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Contents

Monthly Volume 14 Number 7 July 27, 2022

EDITORIAL

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

Clinical and Translational Research

World Journal of Hepatology

Monthly Volume 14 Number 7 July 27, 2022

Contents

RETRACTION NOTE

World Journal of Hepatology

Monthly Volume 14 Number 7 July 27, 2022

Contents

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EDITORIAL

Checkpoint inhibitor-induced hepatotoxicity: Role of liver biopsy and management approach

Fernando Bessone, Einar Stefan Bjornsson

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Abstract

Immunological checkpoint inhibitors (ICIs) have revolutionized therapy of many different malignanices. Concomitant immune-mediated adverse effects are common and can affect many organs such as the skin, lungs, gastrointestinal and endocrine organs as well as the liver. Liver injury has been reported in 3%-8% of patients with grade III-IV hepatitis in retrospective studies. The liver injury is characterized by hepatocellular injury resembling autoimmune hepatitis biochemically but not immunologically as patients with ICI induced hepatoxicity rarely have auto-antibodies or IgG elevation. The role for liver biopsy (LB) in patients with suspected liver injury due to ICIs is controversial and it is not clear whether results of a LB will change clinical management. LB can be helpful when there is diagnostic uncertainty and pre-existing liver disease is suspected. Although there are no distinctive histological features, the finding of granulomas and endothelitis may suggest a specific type of hepatitis induced by ICIs. The natural history of hepatotoxicity of ICI therapy is not well known. Recent studies have demonstrated that 33%-50% of patients improve spontaneously with discontinuation of ICIs. In patients with jaundice and/or coagulopathy corticosteroids are used. The high doses of corticosteroids with 1-2 mg/kg/d of methylprednisolone recommended by the oncological societies are controversial. Recently it has shown that initial treatment with 1 mg/kg/d provided similar liver tests improvement which was also associated with a reduced risk of steroid-induced adverse effects in comparison with higher-dose regimens. Secondary immunosuppression mostly with mycophenolate mofetil has been reported to be helpful.

Key Words: Hepatotoxicity; Checkpoint inhibitors; Biologics; hepatitis; Drug-induced liver injury; Liver biopsy

Core Tip: Liver injury associated with immunological checkpoint inhibitors (ICIs) has been reported in 3%-8% of patients with grade III-IV hepatitis in retrospective studies. Although there are no distinctive histological features, the finding of granulomas and endothelitis may suggest a specific type of hepatitis induced by ICIs. Recent studies have demonstrated that 33-50% of patients improve spontaneously with discontinuation of ICIs. The high doses of corticosteroids with 1-2 mg/kg/d of methylprednisolone recommended by the oncological societies are controversial. Patients with ICI induced hepatoxicity without jaundice and/or coagulopathy should be monitored.

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INTRODUCTION

Immunotherapy has revolutionized the treatment of oncological diseases. Within this large type of compounds, immunological checkpoint inhibitors (ICIs) are increasingly used due to their therapeutic efficacy. These agents exert important beneficial effects on tumor regression and patient survival[1].

ICIs are a large family of co-stimulatory immunotherapy drugs with strong effects that modulate the immune response. They regulate the signaling transduction downstream of T-cell receptors *via* protein-kinase-mediated cascades. Major components of these immune checkpoint molecules are cytotoxic T lymphocyte-associated antigen-4 (CTLA4), programmed cell death-ligand-1, and programmed cell death protein-1 (PD1)[2]. ICIs block these proteins and disable their inhibitory effects, thus evoking an immune response leading to both activation and proliferation of T cells, which results in the killing of tumor cells. CTLA4- and PD1-mediated T-cell inhibition is involved in immunological tolerance to self-antigens as well, and the consequent immune-mediated damage can affect virtually all organs and systems, including the liver[3].

The reported incidence of drug-induced liver injury (DILI) associated with ICIs varies between 0%-30% and depends on the severity, grade, type and drug dose[4]. The occurrence of hepatitis associated with these agents is usually high, and ranges from 3-9% and 1%-2% for anti-CTLA4 and anti-PD1 drugs, respectively. Hepatotoxicity occurs more frequently if combined ICIs schemes are used (up to 17% increased risk) compared to monotherapy[5].

Due to the relatively short period since this type of drugs were approved, many aspects regarding the diagnosis and management of adverse effects are unknown. Besides typical clinical and analytical presentation, different histological findings have been associated with ICIs-induced liver damage (*i.e.* ring granuloma, endothelitis and cholangitis)[6-9]. The role of liver biopsy and the main controversies in the management of liver toxicity induced by ICIs will be discussed in this editorial.

ROLE OF LIVER BIOPSY

Although liver biopsy (LB) is not always required to establish the diagnosis of DILI, it can be helpful in in patients with suspected ICIs- induced hepatotoxicity. LB is particularly indicated when there are diagnostic uncertainties despite noninvasive investigations, in patients presenting with atypical features and in those who fail to respond to conventional therapies[8]. It can also be very useful in patients with potential pre-existing liver disease that cannot be confirmed by imaging or serological tests.

Even though these compounds usually do not trigger a classical autoimmune hepatitis, there is a strong suspicion that liver injury is related to an immune-mediated mechanism[9]. In addition, there are clinicopathologic differences supporting the notion that ICIs –induced DILI is a distinct entity from autoimmune hepatitis. Hepatotoxicity due to ICIs are very rarely associated with autoantibodies and/or IgG elevations and the histological changes are different from those seen in classical autoimmune hepatis[9].

However, differentiating drug-induced autoimmune hepatitis (DIAIH) from a classical autoimmune hepatitis (AIH) is a complex issue for pathologists[10,11]. Interestingly, the histologic pattern most frequently associated with ICIs is commonly described as immune-related hepatitis to differentiate it from classical AIH. Whether liver injury caused by ICIs can be considered a DILI or a DIAIH is controversial[12].

The most conspicuous findings linked to DILI-induced by ICIs is the presence of centrilobular necrosis and acute hepatitis with lobular inflammation associated with acidophil bodies[9,12,13]. Lobular hepatitis indistinguishable from autoimmune hepatitis is one of the most reported patterns usually associated with panlobular inflammation that may be limited to zone 3[13]. Ductal damage has also been described[10] (Figure 1). Inflammatory infiltrates in ICI-induced DILI are predominantly composed of both activated CD3+ and CD8+ T lymphocytes[12]. In contrast to this pattern, autoimmune hepatitis shows higher numbers of both CD20+ and CD4+ T lymphocytes. ICIs-induced DILI may be associated with immune-mediated hepatocellular injury and do not appear to be triggered by T-helper lymphocyte activation or increased immunoglobulin production[12].

De Martin *et al*[9] analyzed 16 patients with liver injury associated with these drugs. A typical pattern of granulomatous hepatitis, characterized by the presence of fibrin-ring granulomas in addition to central-vein endothelitis, were found in patients treated with anti-CTLA-4 monoclonal antibodies (mAbs). Histology findings were considered useful for decision-making regarding therapy. Mild portal fibrosis was found in 50% of patients suggesting a possible trend of acute hepatitis towards chronicity. Fibrin-ring granulomas and central-vein endothelitis were also documented in patients treated with a therapeutic schema combining CTLA-4 with anti-PD-mAbs. These authors emphasized that acute hepatitis associated with immunotherapy agents for cancer treatment is not a frequent clinical event, since it is found in no more than 3.5% of treated patients[9].

Interestingly, they highlight the key role of LB stating that it provides to the clinician with valuable information about the severity of liver injury and helps them to select an appropriate treatment, sometimes avoiding the unnecessary indication of corticosteroids[9].

In a study from Barcelona, 28 cases of severe hepatitis-induced by ICIs were compared with classical AIH[14]. Histological parameters differed between the two conditions. Most patients with AIH underwent a liver biopsy in contrast to only two out of 28 cases (7%) of irH (immune-related Hepatitis) linked to a low response to immunosupression. The authors suggested that liver biopsy should be restricted to patients presenting with irH associated with poor or slow response to corticosteroids[14]. This is in line with guidelines from the European Society of Medical Oncology[15]. In addition, a consultation by a hepatologist and consideration of LB in steroid and mycophenolate-refractory cases is also recommended[15].

On the other hand, Peeraphatdit *et al*[8] analyzed 107 cases in a recent systematic review on management recommendations of DILI-induced by ICIs. They found 83 (78%) patients had grade 3-4 of liver injury. The authors stated that establishing causality for liver damage induced by ICIs can be challenging and a LB should be considered only in cases with at least liver injury grade 2.

Few studies have critically analyzed the predictive value of LB. A recent retrospective study analyzing 60 patients with suspected liver injury due to ICIs showed a pattern of lobular inflammation and injury, endothelitis and the presence of granulomas. The histological findings did not predict the need for corticosteroids, therapy duration, or the need for secondary immunosuppression[16]. The authors questioned the value of LB in the management of patients with typical features of ICI-induced liver injury.

Li *et al*[17] retrospectively analyzed a cohort of 213 patients who developed grade 3 or higher grade of hepatitis linked to ICIs therapy. The most common pattern of DILI was panlobular hepatitis. Patients who underwent a LB had a significatly longer median time to normalization of ALT *vs* those who did not undergo LB (42 *vs* 33 d respectively; $P < 0.01$). This study suggested that LB in patients treated with ICIs and developing grade 3 or higher liver injury presented a delay in the initiation of corticosteroid therapy and not associated with a faster resolution of liver inflammation. These authors also stated that LB can provide valuable information in patients who do not improve despite the indication of corticosteroids before another immunosuppressant is prescribed[17].

A new pattern of cholestasis induced by ICIs displaying imaging and laboratory features similar to those observed in primary eslerosing cholangitis has been recently described[18]. This type of secondary sclerosing cholangitis (SSC) has also been reported in patients with other types of DILI[19,20]. SSC induced by ICIs is characterized by diffuse dilatation and thickening of intrahepatic bile ducts[18]. The absence of biliary obstruction was demonstrated in almost 80% of the cases showing bile duct dilatation [21]. A diffuse hypertrophy in the wall of these biliary ducts was documented in most of them.

Cohen *et al*[16] described a predominantly cholangitic pattern in 16 patients, associated with portal-based inflammation. This histological feature was more likely to be linked to bile duct dilatation or narrowing on cholangiography. Although the biliary involvement induced by ICIs has been well documented to date, its long-term clinical consequences are unknown.

In conclusion, the use of LB is still debatable. Clinicians are faced with pros and cons considering that the final decision should be taken individually for each patient (Table 1).

Table 1 Pros and cons on the indication for liver biopsy in clinical practice

Benefits	Limitations
To rule out pre-existing liver diseases	Invasiveness
To confirm diagnosis (*i.e.*; granulomas)	Cost
Differentiate anti-PD1/PD-L1 from anti-CTLA4- induced DILI	Pathognomic histological features are lacking
To establish the severity of liver injury.	Unclear influence on patient management
To discriminate ICIs-induced DILI from typical seronegative classical AIH	Biochemical features might be sufficient
To assess a possible chronicity evolution	

AIH: Autoimmune hepatitis; DILI: Drug-induced liver injury; ICIs: Immunological checkpoint inhibitors; PD1: Programmed cell death protein-1.

Regarding the benefits, we should consider the usefulness of liver biopsy in different setting as follow: To rule out pre-existing diseases such as metastases or NASH. To confirm the diagnosis of liver injury especially if ring granulomas and endothelitis are observed in patients who are on anti-CTLA4. To investigate the presence of distinctive features of liver toxicity induced by anti-PD1/PD L1 and anti-CTLA4 (ring granulomas and endothelitis). To establish the severity of liver injury. To confirm diagnosis when clinical presentation is associated with features of not typical idiopathic AIH. To assess liver histology in patients with a possible trend from acute hepatitis towards chronicity.

Among the limitations, the cost and invasiveness of LB should always be taken into account and whether the results will change management of the patient. Thus, frequently LB does not show pathognomonic histologic findings and, accordingly, some authors propose that is unlikely to influence patient management. Unfortunately, it is unclear if a liver biopsy is helpful in the decision if another Check point inhibitor can be tried if hepatotoxicity has occurred with the first line Check point inhibitor. A proposed algorithm on the role of liver biopsy in the management of DILI-induced by ICIs is shown in Figure 2.

Finally, a magnetic resonance cholangiopancreatography is recommended when secondary sclerosing cholangitis is suspected and to rule out biliary obstruction.

MANAGEMENT

The management of liver injury considered to be due to ICIs and the role of corticosteroids as therapy for this adverse effect is not evidence based. Randomized controlled studies are lacking in this context and recommendations based on expert opinion in the guidelines of the oncological societies[15,22,23]. According to these guidelines, general advice is probably not controversial. If liver injury is mild, or < 3 × ULN in ALT, the therapy with ICIs is not interrupted and liver tests only monitored. If ALT is 3-5 × ULN, ICIs can be temporarily discontinued and if levels of elevated ALT return to baseline within a week, ICI therapy can be resumed and/or oral corticosteroids can be given. Experience from observational studies support only to monitor patients without corticosteroids in these relatively mild cases[9,24,25].

If ALT levels are > 5 × ULN, classified as grade III hepatitis by the oncological societies, which is > 5 × ULN-20 × ULN and bilirubin > 3 × ULN (15, 21-22), the patients should be monitored and patients given corticosteroids if there is no improvement in liver tests. If the levels of ALT are > 10 × ULN (grade IV hepatitis) and/or if the ALT > 5 × ULN is accompanied by rise in serum bilirubin, ICI therapy should be permanently interrupted[15,22,23]. However, in observational studies a relatively large proportion of patients of these patients have shown spontaneous improvement in liver tests without the use of corticosteroids[9,24,25]. In a study from France, 37% with > grade III hepatitis improved spontaneously and 50% in the study by Gauci *et al*[24] and in a recent study from Texas, 33% of patients were found not to require corticosteroids[25]. There is though no doubt that liver injury can be severe and have severe consequences. In a study from Barcelona, among 28 patients with severe hepatitis (> grade III), two patients developed acute liver failure (ALF) and one of these died from ALF[14]. Two other well characterized patients have been reported who died from hepatotoxicity[24,25]. All of these patients were treated with high dose of methylprednisolon 2mg/kg combined with mycophenolate mofetil[14,25,26]. Thus, it seems that not all cases with hepatotoxicity due to ICIs are steroid responsive. A study analyzing data from World Health Organization pharmacovigilance database (Vigilyze) also reported mortality due to hepatotoxicity[27]. Among adverse effects associated with fatality 22% were due to hepatotoxicity but this is perhaps not completely reliable data as it seems that a formal causality assessment has not been undertaken[27]. However, mortality from hepatotoxicity can occur and it is understandable that mortality from adverse effects in a patient who is in remission from the malignancy is a nightmare for the oncologist. Thus, it is understandable that they want to do everything in their

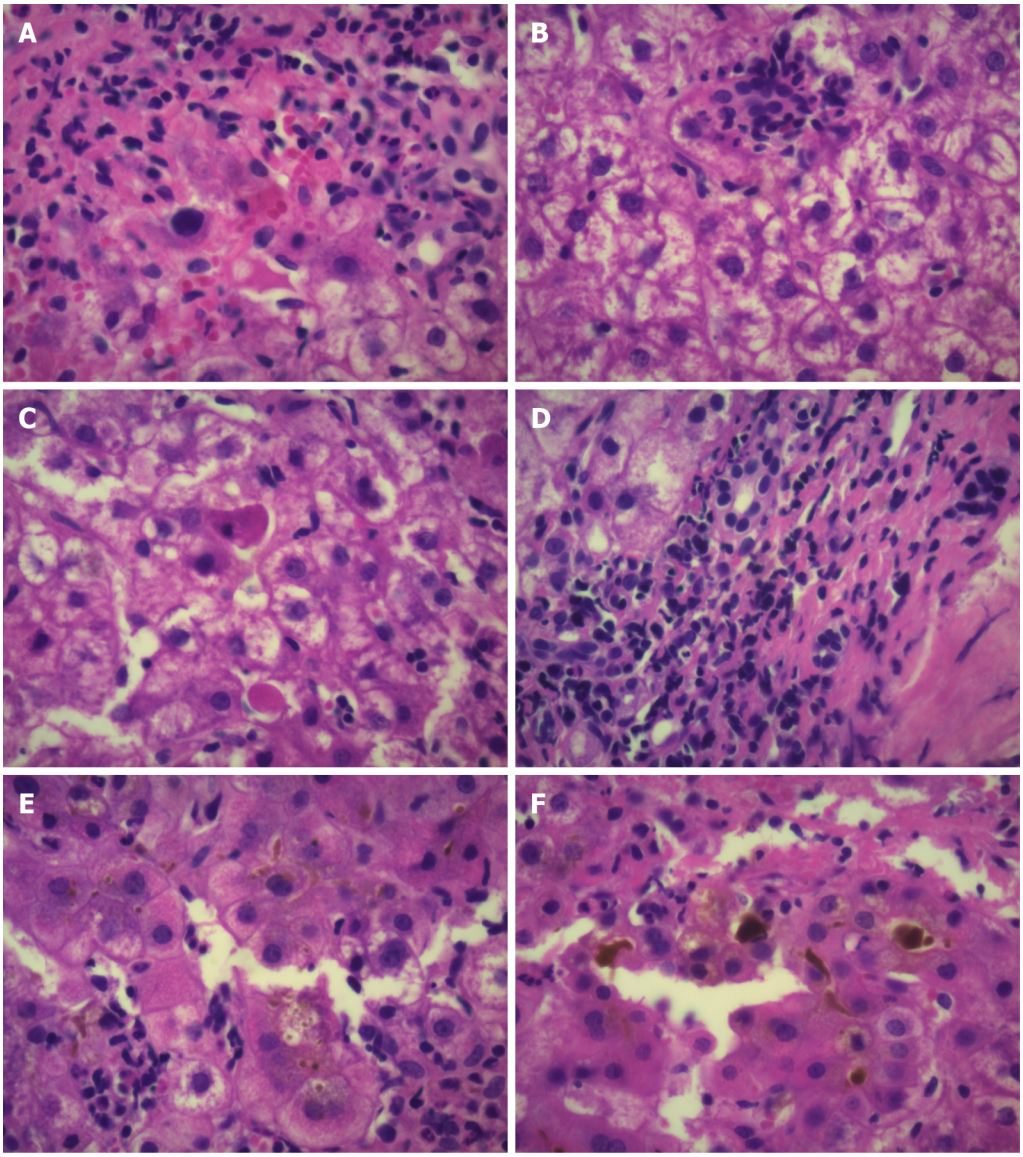

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Figure 1 Pathological changes of drug-induced liver injury -induced by atezolizumab used for treatment of hepatocellular carcinoma (H&E 40×). A: Portal inflammation and interfase hepatitis; B: Focal lobular necrosis; C: Frequent lobular acidophilic bodies; D: Ductal damage and migration of inflammatory cells into ductal epithelium; E: Hepatocyte rosettes as a result of liver regeneration; F: Hepatocanalicular cholestasis and biliary plugs.

power to reverse the hepatotoxicity.

High doses of corticosteroids are recommended by the oncological societies: 1 mg/kg/d for grade III hepatitis and even 2 mg/kg/d for grade IV hepatitis[15,22,23]. As pointed out earlier, these doses are not evidence based. Although high doses of corticosteroids have been used observational studies these have not always been helpful. In a study from the UK only 50% with hepatotoxicity due to ICIs responded to corticosteroids[28]. Two Japanese studies have similarly shown responsiveness between 33 and 50%[29,30]. In a recent study from France, important experience was reported on the clinical management of patients with liver injury due to ICIs[31]. In more than 300 patients with advanced melanoma, 21 had hepatotoxicity and 13/21 (62%) were treated with steroids, whereas 8 were not. Time to resolution of liver tests and survival was not statistically between the groups[31]. The authors suggested that patients with prothrombin levels > 50% and bilirubin < 50 mmol/L should be monitored and not treated with corticosteroids but ICI therapy discontinued until < 5 × ULN, whereas those with prothrombin level < 50% and bilirubin > 50, should be treated with corticosteroids 0.5-1 mg/kg/d. Riveiro-Barciela *et al*[14] found only 2-3 mo of corticosteroid therapy necessary. In a recent study, it was demonstrated that initial treatment with 1 mg/kg/d provided similar liver tests improvement as doses > 1.5 mg/kg/d, which was also associated with a reduced risk of steroid-induced adverse effects in comparison with higher-dose regimens[32].

In patients with worsening jaundice despite high doses of corticosteroids mycophenolate mofetil is most often used because it is probably better tolerated than tacrolimus due to potential nephrotoxicity.

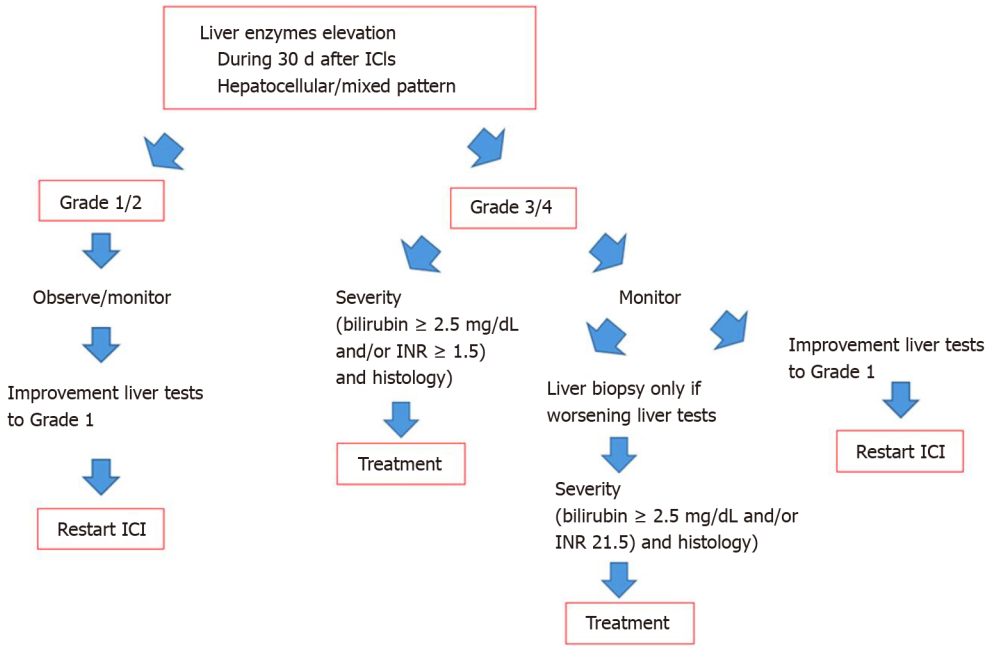

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Figure 2 Proposal algorithm on the role of liver biopsy in immunological checkpoint inhibitors –induced drug-induced liver injury. ICIs: Immunological checkpoint inhibitors.

However, the use of secondary immunosuppression in patients is not evidence based and relies on small cases series and case reports. There is no data to guide us in patients with pre-existing liver disease.

CONCLUSION

The role for LB in the setting of DILI induced by ICIs is controversial and not yet defined. LB can be helpful when there is diagnostic uncertainty and pre-existing liver disease is suspected. Although there are no distinctive histological features, the finding of granulomas and endothelitis may suggest a specific type of hepatitis induced by ICIs. Recent data suggest that liver histology did not predict the need for corticosteroids, therapy duration, or the need for secondary immunosuppression. Patients with ICI induced hepatoxicity without jaundice and/or coagulopathy should be monitored as a large proportion of patients will recover spontaneously with discontinuation of the ICIs. Patients who develop worsening of liver tests with jaundice and/or coagulopathy despite discontinuation of ICIs should be treated with corticosteroids. Recent data suggests that 1mg/kg/d of methylprednisolon are as efficacious as higher doses but it is not clear if doses of 40-60 mg of prednisolon are less efficacious. Secondary immunosuppression mostly with mycophenolate mofetil has been reported to be helpful.

FOOTNOTES

Author contributions: Bessone F and Bjornsson ES contributed to this paper; Bessone F designed the overall concept and outline of the manuscript; Bjornsson ES contributed to the discussion and design of the manuscript; They both contributed to the writing, and editing the manuscript and review of literature.

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REVIEW

Gut microbiota contribution to hepatocellular carcinoma manifestation in non-alcoholic steatohepatitis

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Abstract

Recently, the gut microbiota has been recognized as an obvious active player in addition to liver steatosis/steatohepatitis in the pathophysiological mechanisms of the development of hepatocellular carcinoma (HCC), even in the absence of cirrhosis. Evidence from clinical and experimental studies shows the association of specific changes in the gut microbiome and the direct contribution to maintaining liver inflammation and/or cancerogenesis in nonalcoholic fatty liver disease-induced HCC. The composition of the gut microbiota differs significantly in obese and lean individuals, especially in the abundance of pro-inflammatory lipopolysaccharide-producing phyla, and, after establishing steatohepatitis, it undergoes minor changes during the progression of the disease toward advanced fibrosis. Experimental studies proved that the microbiota of obese subjects can induce steatohepatitis in normally fed mice. On the contrary, the transplantation of healthy microbiota to obese mice relieves steatosis. However, further studies are needed to confirm these findings and the mechanisms involved. In this review, we have evaluated well-documented clinical and experimental research on the role of the gut microbiota in the manifestation and promotion of HCC in

nonalcoholic steatohepatitis (NASH). Furthermore, a literature review of microbiota alterations and consequences of dysbiosis for the promotion of NASH-induced HCC was performed, and the advantages and limitations of the microbiota as an early marker of the diagnosis of HCC were discussed.

Key Words: Gut microbiota; Hepatocellular carcinoma; Non-alcoholic steatohepatitis; Non-alcoholic fatty liver disease; Microbiome

Core Tip: Although the incidence of life-threatening cases of hepatocellular carcinoma (HCC) induced by nonalcoholic steatohepatitis (NASH) has recently increased due to the dramatic increase in steatohepatitis, the pathophysiological mechanisms of the manifestation of HCC nodules have not yet been fully elucidated. There is a lack of tools to diagnose HCC at an early stage, especially considering that HCC can occur in patients with NASH even in the absence of cirrhosis. In this review, we have evaluated the current state of research on the role of the gut microbiota in promoting NASH-induced HCC and the use of the microbiota for the early diagnosis of HCC.

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INTRODUCTION

In the different regions of the world, non-alcoholic fatty liver disease (NAFLD) affects 4%-55% of the population[1,2]. Subjects with NAFLD are constantly at risk of developing chronic liver inflammation leading to nonalcoholic steatohepatitis (NASH) and eventually progressing from liver fibrosis to cirrhosis. The latter has a higher risk of hepatocellular carcinoma (HCC) manifestation[3]. Although the risk of NAFLD progression to cirrhosis is less likely than in viral hepatitis (approximately 10% of NASH [4], and less than 1% of patients with NAFLD developed HCC within 8 years after initial diagnosis[5,6]), NASH alone can cause HCC even in the absence of cirrhosis, and this raises concerns[7-9]. Furthermore, it is estimated that HCC cases related to NASH may increase by up to 56% in the next 10 years[10].

In some cases, prolonged inflammation of the liver caused by steatosis appears to be a sufficient circumstance to cause the rise of the so-called compensatory proliferation of hepatocytes, which triggers the formation of HCC nodules[5], but the precise pathophysiological mechanism is still far from complete elucidation. To some extent, NAFLD/NASH mice models are helpful. However, translating animal studies into a human context is always difficult because only reliable mechanistic information comes from these studies[11].

In addition to liver steatosis / steatohepatitis, the gut microbiota has recently been recognized as an obvious active player in NAFLD-induced HCC. Experimental and clinical studies demonstrate a stimulating role of the intestinal microbiota in maintaining liver inflammation and an alteration of the microbiome composition toward a more pro-inflammatory state with the progression of liver disease from NAFLD to NASH at different stages of fibrosis and HCC[12,13]. It seems like this is a mutually supportive process. This has been confirmed by a study of germ-free mice transplanted with stool from genetically obese patients. Soon after the guts of these mice were colonized by the microbiota of obese subjects, a steatosis manifested in their livers despite a balanced diet[14]. On the contrary, fecal microbiota transplantation from healthy mice alleviated steatohepatitis in mice fed a high-fat diet[15].

The liver is closely related to the intestinal tract and serves as a vital metabolic center for digestion, detoxification, and clearance of microbial products[16]. Research on the gut-liver axis has greatly contributed to understanding the basic pathophysiology of liver diseases, including NAFLD of different severity and malignancy of the liver parenchyma[17,18].

In this review, we conducted a survey of the current state of research on the contribution of the gut microbiota to the manifestation and progression of HCC in patients with NASH.

LITERATURE SEARCH AND ANALYSIS OF CLINICAL AND EXPERIMENTAL STUDIES SELECTED

An electronic search of the literature on the microbiota in NASH-induced HCC was performed. Articles available in the PubMed, Medline, Cochrane, and Web of Science databases were reviewed up to November 12, 2021. The search terms used were "nonalcoholic fatty liver disease AND hepatocellular carcinoma AND microbiome", "nonalcoholic fatty liver disease AND hepatocellular carcinoma AND microbiota", "nonalcoholic steatohepatitis AND hepatocellular carcinoma AND microbiota", "nonalcoholic steatohepatitis AND hepatocellular carcinoma AND microbiome", "nonalcoholic steatohepatitis AND liver cancer AND microbiota" and "nonalcoholic fatty liver disease AND liver cancer AND microbiota". No time restrictions were used for publications. A total of 1,073 articles and abstracts met the initial search criteria.

The titles, abstracts, and full papers were reviewed to identify full-text articles focusing on alterations in the gut microbiota in NASH/NAFLD - HCC compared to healthy controls, as well as animal model studies discussing changes in the gut microbiota in NASH/NAFLD - induced HCC (Supplementary Figure 1).

Inclusion criteria were: Well-documented full-text articles written in English, presence of the following study groups – NAFLD/NASH with/without cirrhosis, NAFLD/NASH-HCC with/without cirrhosis, control group of healthy subjects.

Exclusion criteria after abstract and full text reviews were: articles written in other languages than English, no presence of NAFLD/NASH - HCC, no evaluation of the NASH/NAFLD - HCC microbiota, no control group.

Following a comprehensive review of the current literature, we identified only six publications focusing on the gut microbiota in NASH/NAFLD induced HCC that were fully consistent with the inclusion criteria[12,13,19-22]. Three selected articles were clinical studies, in which the microbiota composition of 86 patients with HCC induced by NAFLD was analyzed among others with NAFLD of different severity (Table 1)[13,19,20]. The other three publications included animal model studies in which mice with NAFLD and HCC microbiota were analyzed (Table 2)[12,21,22]. The circumstantial analysis of the selected studies is presented below.

Human studies

All three identified clinical studies on NASH-induced HCC were cross-sectional. Two of them compared cirrhotic NAFLD with or without HCC with healthy controls[19,20], and one compared patients with NASH together, NASH-HCC with or without cirrhosis, and healthy controls[13]. In total, 168 patients with NAFLD and 70 controls were enrolled. The HCC had 72(55%) of 131 cirrhotic patients and 14(37.8%) of 37 without cirrhosis.

The α-diversity and bacterial richness were analyzed. Behary *et al*[19] confirmed dysbiosis in the NAFLD-HCC and NAFLD-cirrhosis groups compared to healthy controls. Patients in these following groups had reduced α-diversity (a measure of microbiome diversity applicable to a single sample) and the Chao-1 richness index. However, no other differences were observed in other alpha-diversity measures (Shannon's diversity index, Evenness index). A study by Sydor *et al*[13] showed that the rarity index increased in patients with NASH-HCC with cirrhosis compared to the control group. In the third study by Ponziani *et al*[20], α-diversity was reduced in the NAFLD-HCC group compared to healthy controls. However, diversity changes were not specified when comparing NAFLD-HCC with cirrhosis and NAFLD-HCC without cirrhosis.

There is a consistent amount of evidence that the gut-liver axis plays an important role in the progression of liver diseases[17,18]. In a study by Komiyama *et al*[23], the most common phyla of the gut microbiota (*Bacteroidetes, Firmicutes*, and *Proteobacteria*) were also dominant in HCC, suggesting that an increased abundance of these phyla is also found in subjects with HCC induced by NAFLD.

Ponziani *et al*[20] demonstrated an increased quantity of *Bacteroides* and *Lactobacillus* in cirrhotic patients with or without HCC. Furthermore, with deficiency of *Bifidobacterium* and *Blautia,* HCC patients had an even higher abundance of *Bacteroides* and R*uminococccaceae, Enterococcus, Phascolarctobacterium,* and *Oscillospira* than the NAFLD-non-HCC with cirrhosis patient group. A study by Behary *et al*[19] also showed a significant enrichment of *Bacteroides xylanisolvens* and *Ruminococcus gnavus* in both the NAFLD-HCC and NAFLD-cirrhosis groups compared to healthy controls. *Bacteroides caecimuris* and *Veillonella parvula* were specifically enriched in the NAFLD-HCC group compared to the control and NAFLD-cirrhosis groups[19]. However, Sydor *et al*[13] demonstrated a reduction in the abundance of *Bacteroidetes* along with Gram-positive *Actinobacteria* and *Bifidobacterium* and an increased abundance of *Proteobacteria* and *Lactobacillus* in patients with NASH-HCC.

In a previous study, the *Bacteroides* genera were also enriched in HCC *vs* patients with cirrhosis, suggesting that the enrichment of *Bacteroides* in the gut microbiota may be associated with the diagnosis of liver cancer[24].

Animal studies

We identified 3 animal studies (mice) investigating changes in the gut microbiome in NAFLD-induced

Table 1 Clinical studies investigating gut microbiota composition in patients with nonalcoholic fatty liver disease - induced hepatocellular carcinoma

Ref.	Participants (groups)	Exclusion criteria	Main findings	Other metabolites investigated
Behary *et al*[19]	Patients with NAFLD-HCC-cirrhosis *n* = 32; Patients with NAFLD-cirrhosis *n* = 28; Control group (non-NAFLD) *n* = 30.	Unspecified	Subjects with NAFLD-HCC and NAFLD-cirrhosis had reduced α-diversity indices compared to non-NAFLD controls; NAFLD-HCC was characterized by expansion of *Proteobacteria* compared to a non-NAFLD group; Expansion of *Enterobacteriaceae* in NAFLD-HCC compared to NAFLD-cirrhosis and controls; NAFLD-HCC was characterized by a reduction in *Oscillospiraceae* and *Erysipelotrichaceae* compared to non-NAFLD; NAFLD-cirrhosis was characterized by an expansion of *Eubacteriaceae* compared to both NAFLD-HCC and controls; *Bacteroides caecimuris* and *Veillonella parvula*, were both significantly enriched in NAFLD-HCC, compared to NAFLD cirrhosis and controls	Pyruvate carboxylase (pycA), responsible for the production of oxaloacetate from pyruvate, was overexpressed in NAFLD-HCC compared to NAFLD-cirrhosis and non-NAFLD control; Genes related to acetate synthesis (phosphate acetyltransferase) and butyrate/acetyl phosphate synthesis (phosphate butyryltransferase) were both overexpressed in NAFLD-HCC compared to NAFLD cirrhosis and non-NAFLD controls; The feces of NAFLD-HCC subjects were enriched in acetate, butyrate and formate compared to NAFLD-cirrhosis and controls; Fecal SCFA was NAFLD-HCC specific
Sydor *et al*[13]	Patients with NASH-non-HCC without cirrhosis *n* = 23; Patients with NASH-non-HCC with cirrhosis *n* = 11; Patients with NASH-HCC without cirrhosis *n* = 14; Patients with NASH-HCC with cirrhosis *n* = 19; Control group *n* = 20.	Unspecified	B*acteroidetes* and, to a lesser extent, *Actinobacteria* were gradually decreased in abundance from controls to NASH-non-HCC to NASH-HCC; The abundance of *Proteobacteria* was significantly increased in NASH-HCC with cirrhosis; The abundances of *Bacteroides* and *Bifidobacterium* were decreased in NASH-non-HCC and NASH-HCC compared with controls; *Lactobacillus* showed a progressive increase in abundance from controls to NASH-HCC with cirrhosis; Abundance of *Clostridium* and *Escherichia/Shigella* remained unchanged; *Lactobacillus*-related ranks showed a progressive increase in abundance from controls to NASH-HCC with cirrhosis	Significant increase of BA associated with disease severity between healthy, NASH-non- HCC, and NASH-HCC; Individual and conjugated serum BA were associated with the abundance of *Lactobacillus*
Ponziani *et al*[20]	Patients with NAFLD-HCC with cirrhosis *n* = 21; Patients with NAFLD-non-HCC with cirrhosis *n* = 20; Control group *n* = 20.	Patients with CVH, AH, cholestatic disorders such as PBC or PSC, and inherited liver disorders leading to cirrhosis such as hemochromatosis, Wilson's disease, and alpha-1 antitrypsin deficiency; Patients who were taking drugs such as antibiotics, probiotics, prebiotics, PPIs, and laxatives during the last 6 mo; affected by diseases potentially influencing the gut microbiota composition; Patients with a history of cancer.	α-diversity was less diverse in patients with cirrhosis compared to controls; Cirrhosis patients showed enriched *Proteobacteria*, *Bacteroidetes* and *Cyanobacteria* compared to healthy controls; The gut microbiota of the HCC group was enriched with *Bacteroides*, *Ruminococcaceae*, *Enterococcus*, *Phascolarctobacterium*, and *Oscillospira* compared to patients with cirrhosis but without HCC and controls; Reduced abundance of *Verrucomicrobiaceae*, *Bifidobacteriaceae*, *Akkermansia*, *Bifidobacterium*, *Dialister*, *Collinsella*, and *Adlercreutzia* were seen in NAFLD-HCC compared with NAFLD-non-HCC.	Intestinal permeability was increased in all patients with liver cirrhosis, who had higher levels of plasma ZO1 and LPS compared to controls

AH: Autoimmune hepatitis; BA: Bile acids; CVH: Chronic viral hepatitis; HCC: Hepatocellular carcinoma; LPS: Lipopolysaccharides; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; PBC: Primary biliary cholangitis; PPI: Proton pump inhibitors; PSC: Primary sclerosing cholangitis; SCFA: Short-chain fatty acid.

HCC, summarized in Table 2[12,21,22]. To induce HCC, mice were fed a high-fat diet (high-fat/high-cholesterol (HFHC) and high-fat/low-cholesterol (HFLC). In one study, additional intraperitoneal injections of CCl4 were administered once a week to induce HCC[21].

Animal studies demonstrated the same results regarding α-diversity in the gut microbiome in HCC induced by NAFLD. In all studies, α-diversity was reduced in HCC mice compared to the control group. A study by Zhang *et al*[22] also showed that mice fed the HFHC diet had lower bacterial diversity than mice fed the HFLC diet. HFHC-fed mice also had a higher association with the development of HCC.

Increased LPS across the intestinal barrier in mice with NAFLD-induced HCC

Some studies in humans observed increased serum lipopolysaccharide (LPS) levels in HCC patients[25,26]. It indicated an increase in permeability of the intestinal epithelial barrier[23].

Thus, it was no surprise that higher serum LPS levels were observed in three reviewed animal studies [12,21,22]. Mice fed a high-fat streptozocin diet (STZ) and developed HCC had a higher abundance of *Bacteroides* and *Desulfovibrio* in their gut microbiome[12]. Since most *Bacteroides* and *Desulfovibrio* species

Table 2 Animal models investigating gut microbiota composition in nonalcoholic fatty liver disease induced hepatocellular carcinoma

Ref.	Experimental animal	Participants (groups)	Main findings
Xie *et al*[12]	Mice	Mice with STZ-HFD induced NASH-HCC; Control group	STZ-HFD group exhibited lower α-diversity than controls; The most abundant species in both control group and STZ-HFD group were primarily from the *Bacteroides* genus; The most decreased in abundance in the STZ-HFD group were *Parasutterella spp., Bacteroides acidofaciens, Odoribacter spp., Barnesiella spp., Moryella spp., Paraprevotella spp., Lactobacillus intestinalis, and Akkermansia spp*; *Atopobium spp., Bacteroides acidifaciens, Bacteroides spp., Bacteroides uniformis, Bacteroides vulgatus, Clostridium cocleatum, Clostridium xylanolyticum*, and *Desulfovibrio spp.* were significantly positively correlated with LPS in plasma, liver and feces; As most *Bacteroides* and *Desulfovibrio* were LPS-producers, LPS concentration was significantly increased in the STZ-HFD group.
Carter *et al* [21]	Mice	Western diet only (high fat and fructose diet, no CCl4 injection); CCl4 only (CCl4 injection intraperitoneal once a week and normal diet); NASH-HCC (Western diet and CCl4 injection intraperitoneally once a week); Control group (normal diet, no CCl4 injection);	NASH mice display impaired intestinal barrier function, leading to increased leakage of bacterial byproducts such as LPS into the circulation; NASH mice had reduced alpha diversity; Expansion of *Erysipelotrichales* was only observed in NASH mice
Zhang *et al* [22]	Mice	HFHC-fed mice (NAFLD-HCC group); HFHC-fed mice; Normal diet-fed mice (control group).	The microbiota composition changed during NAFLD-HCC formation: *Mucispirillum, Desulfovibrio, Anaerotuncus* were sequentially increased; Gut bacterial metabolites alteration like TCA and IPA were increased in NAFLD-HCC mice; Lower bacterial diversity and increased bacterial richness were observed in HFHC-fed mice with HCC than HFLC diet-fed mice with only steatosis; LPS concentration was elevated in HFHC-fed mice compared to HFLC-fed mice.

HCC: Hepatocellular carcinoma; HFHC: High-fat/high-cholesterol; HFLC: High-fat/low-cholesterol; IPA: Indole-3-propionic acid; LPS: Lipopolysaccharides; NAFLD: Non-alcoholic fatty liver disease; STZ-HFD: Streptozocin-high-fat diet; TCA: Trichloroacetic acid.

are producers of LPS, higher LPS concentrations were found in HCC mice' blood. In a study by Carter *et al*[21], NASH-induced HCC mice had increased gut permeability, which also resulted in elevated serum LPS.

Recent studies showed that circulating LPS was significantly elevated in patients with colorectal cancer compared to healthy controls. Furthermore, the authors concluded that serum LPS can cause chronic inflammation and activate the coagulation system, leading to cancerogenesis[27]. New studies show that elevated levels of circulating LPS may be highly associated with many chronic liver diseases, including liver fibrosis and HCC[28,29].

NASH-INDUCED HCC PATHOGENESIS ASSOCIATIONS WITH GUT MICROBIOTA

The accumulation of lipid droplets alone does not cause liver damage or inflammation. Hepatosteatosis (a.k.a. "bland steatosis") requires a necro-inflammatory mechanism characterized by ballooning hepatocytes, liver injury, and fibrosis[5]. The inflammation of the liver could be triggered by provocative factors, such as oxidative stress, stress of the endoplasmic reticulum, and/or the presence of infectious or commensal organisms[30]. This so-called two-hit hypothesis was first formulated by Day and James[31].

The specific mechanism that links the gut microbiota with the progression of NAFLD is still unclear. However, bacterial overgrowth, translocation of microorganisms, increased endotoxin absorption, and enterohepatic secondary bile acids may be possible explanations[32].

Leaky gut

Patients with exacerbated liver function have increased intestinal permeability and impaired mucosa due to the alternation of the tight epithelial junction[25,33]. This leads to the leakage of chemicals derived from the microbiota into the bloodstream of the portal vein. The more severe and long-lasting the liver disease, the higher the levels of different potentially pro-inflammatory and pro-oncogenic microbial products that might be detected in the blood of patients[25]. It should be noted that this state is often worse in the NASH population due to a high-fat/high-carbohydrate diet that maintains the pro-inflammatory alteration of the intestinal microbiota[34]. Improvement in liver function tests following dietary correction in clinical trials in patients with NASH / obesity is evidence of reduced parenchymal inflammation[35]. Mice experiments also confirmed the importance of diet for the healthy shape of the gut microbiota[15].

Bacterial overgrowth

There is a link between bacteria overgrowth and NAFLD/NASH. Approximately 50%-80% of patients with NAFLD/NASH have small intestine bacterial overgrowth (SIBO)[7]. SIBO, together with alteration of the intestinal microbial community, has been detected in NAFLD-induced chronic liver inflammation conditions of different stages[16].

In several clinical studies, an abundance of the *Veillonella* genus was found in the duodenum and colon of cirrhotic patients, along with the reduction of the genus *Akkermansia* and *Prevotella*[16,36]. Loomba *et al*[37] observed an increased quantity of *Bacteroides vulgatus* and *Escherichia coli* (*E. coli*) in patients with advanced NAFLD-induced fibrosis. *E. coli* was also predominant in patients with SIBO-affected NAFLD[38].

More studies are needed to show the prevalence of SIBO in patients with NASH-induced HCC.

Dysbiosis

Dysbiosis of the gut microbiota has been associated with a higher risk of certain cancers and has been shown to affect the body's reaction to various cancer treatments[39,40]. Furthermore, a reduction in the diversity of the intestinal microbiome has been reported in inflammatory bowel diseases, colorectal cancer, and gastric cancer[41-43]. The diversity of the gut microbiota is now considered an important environmental characteristic of NAFLD, since it can impact host metabolic processes, such as the extraction of energy from food. Through mechanisms such as altered hunger signaling, enhanced energy extraction from the diet, and altered regulation of gene expression involved in de novo lipogenesis or oxidation, the gut microbiota has the ability to increase intrahepatic fat[44].

It should be noted that researchers observed a larger difference in the abundance of bacteria at the levels of phylum, family, and genus levels between healthy and obese subjects, while relatively fewer differences were observed between obese and the NASH microbiome[45]. The only abundance of *Proteobacteria*, *Enterobacteria*, and *Escherichia* differed between obese and NASH[46]. Ezzaidi *et al*[32] found that patients with NASH have a lower abundance of *Faecalibacterium* and *Anaerosporobacter,* but a higher abundance of *Parabacteroides* and *Allisonella*. They also noted that the reduction in *Firmicutes* and the increase in *Bacteroidetes* were associated with an improvement in steatosis. However, *Bacteriodetes* are known as LPS-producing bacteria, which is why they are pro-inflammatory[32].

An elevated abundance of *Bacteroides vulgatus* and *E. coli* has been discovered in NAFLD patients with advanced fibrosis[37]. Fecal *Bacteroides* and *Ruminococcus* were independently related to NASH and fibrosis (stage 2 or above), while *Prevotella* decreased under the same circumstances[36].

The role of the microbiome in NAFLD-HCC is mainly unknown. The clinical studies summarized in Table 1 of this review agree on the decrease in the diversity of bacteria in patients with NASH-HCC, but demonstrate a discrepancy in the abundance of various representatives of the gut microbiota. Only changes at the phyla level toward LPS producers have been confirmed in all studies.

The gut microbiota produces a wide range of bioactive chemicals, including those from food substances [LPS, short-chain fatty acids (SCFA), deoxycholic acid (DCA)], resulting in a complex transgenomic metabolism between the microbiota and the host that significantly affects physiological and pathological states[47]. Through the gut-liver axis, intestinal microbial dysbiosis is linked to hepatic inflammation and HCC[32].

Dysbiosis of the intestinal microbiota appears to be a novel component that promotes the development of NALFD-induced HCC. The manifestation of HCC has been associated with increased *Bacteroides* and *Ruminococcaceae,* but lower *Bifidobacterium* in patients with NAFLD[20]**.**

The increase in *Bacteroides* and *Ruminococcaceae* in the HCC population is associated with higher levels of calprotectin and systemic inflammation[16,19,20,48,49]. In general, researchers agree that the gut bacteria of obese subjects promote HCC. However, the patterns of bacterial abundance were not consistent between studies. For example, some studies claimed an increase in *Bacteroidetes* in advanced NASH[19,20,37], , while other studies showed that patients with NASH possessed a lower abundance of *Bacterioidetes*[13].

MECHANISMS OF MICROBIOTA CONTRIBUTION TO PERSISTENT LIVER INFLAMMATION AND HEPATOCARCINOGENESIS

Since liver disease may be accompanied by SIBO and altered gut permeability, a correlation of the increased level of bacterial products in the portal blood can be expected with the severity of the disease. Due to the altered intestinal barrier, bacterial products derived from gut microbes (microbial-associated molecular patterns (MAMPs): LPS, peptidoglycan, and bacterial unmethylated cytosine–phosphate–guanine dinucleotides (CpG) DNA, DCA, and lipoteichoic acid (LTA), ethanol, acetone, butanoic acid, and many other molecules) can enter the liver and activate toll-like receptors (TLRs) in Kupffer cells, liver stellate cells, and hepatocytes, leading to an inflammatory response that promotes NASH[7,16,32]. In humans, TLR-2, TLR-4, and TLR-9 are known to be involved in the pathogenesis of NASH[50].

According to recent experimental and clinical studies, the intestinal microbiome can contribute to all histological components of NAFLD: liver steatosis, inflammation, and fibrosis[48]. As HCC in patients with NASH can occur in the absence of cirrhosis[8,9,51,52], chronic inflammation of the liver is the most important circumstance for its manifestation[53].

Several studies of NASH-induced HCC reported the correlation of *Bacteroides* and *Ruminococcaceae* expansion with systemic inflammation[19,20,48,49]. It is well known that after pro-inflammatory stimulation by nutrients metabolites or/and bacterial molecules that enter the liver, Kupffer cells, liver stellate cells, and infiltrating macrophages produce a variety of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL) -6, and IL-8, to establish the immune response. Increased levels of these cytokines have been detected in patients with NASH[54,55].

These cytokines contribute to the development of NASH and HCC by activating nuclear factor kappa-B (NF-κB) and STAT3 in initiated hepatocytes[30]. However, it is not yet clear how pro-inflammatory events trigger the development of HCC and how malignant hepatocytes escape the immune attack. Evidence from the experimental study elucidated a suppressive impact of immunoglobulin A+ plasma cells on cytotoxic T lymphocytes by expression of programmed death ligand 1 (PD-L1) that leads to the exhaustion of CD8 + T lymphocytes[56]. PD-L1 inhibitors appeared to be highly effective for HCC treatment[57]. The inflammatory cytokine profile and TNF-α activated NF-κB signaling, as well as the exhaustion of CD8+ T lymphocytes, are characteristic of HCC of non-NASH etiology[5].

LPS producing bacteria can induce liver inflammation and promote carcinogenesis

LPSs are active components of bacterial endotoxins released by Gram-negative bacteria after their death. LPS-specific TLR-4s are expressed by monocytes, mast cells, B cells, and the intestinal epithelium[1]. After release from the wall of the bacteria cell, LPS forms a complex with the lipopolysaccharide binding protein, CD14, and TRL4 and enters circulating blood due to increased intestinal permeability[58].

Hepatocytes, Kupffer cells, and liver stellate cells also express LPS-specific TLR-4. After activation of TRL-4 by LPS in Kupffer cells, an intracellular inflammatory cascade is triggered, inducing the production of pro-inflammatory cytokines (TNF-α, IL-6)[59,60].

TLR-4 activation also leads to overexpression of hepatomitogen epiregulin, which promotes mitosis of hepatocytes and, therefore, hepatocarcinogenesis. At the same time, LPS-activated liver stellate cells gain a pro-inflammatory state and start to secrete collagen, inducing liver fibrogenesis and vascular endothelial growth factor, which participates in hepatocarcinogenesis by promoting neoangiogenesis [47,61].

Furthermore, caspase-3 cleavage, responsible for cell apoptosis, appears in hepatocytes through the NF-κB-mediated mechanism[47]. All of the mentioned events lead to the survival of malicious hepatocytes and the formation of HCC nodules. In patients with liver cancer, the activated LPS-TLR-4 pathway is associated with increased invasiveness of tumor cells induced by NF-κB-mediated epithelial-mesenchymal transition and, consequently, metastasis and poor prognosis[62,63].

Other pro-inflammatory and pro-oncogenic impacts of the microbiota in NASH-induced HCC

Alongside TLR-4, Kupffer and hepatic stellate cells possess TLRs with specificity to other MAMPs. TLR-2 can be activated by components of Gram-positive bacterial cell walls, such as peptidoglycan and lipoteichoic acid. Through mitogen-activated protein kinases (MAPKs) induced by MyD88/MAL and NF-κB-mediated transcriptional programs, they promote liver tumorigenesis[16,64]. TLR-2, activated by lipoteichoic acid, along with secondary bile acid deoxycholate, promotes DNA damage, cell senescence, and apoptosis, and incites obesity-associated tumorigenesis through a pro-inflammatory and immunosuppressive pro-tumorigenic environment involving prostaglandin E2[65,66]. NASH progression and NASH-induced HCC have been prevented in an experimental model by treating mice with sequestrant bile acids[67].

TLR-9 is an intracellular receptor that detects bacterial and viral DNA. It recognizes DNA containing unmethylated CpG motifs, which are common in bacteria[64,68]. The TLR-9 signaling pathway induces IL-1b production by Kupffer cells, leading to steatosis, inflammation, and fibrosis. IL-1b promotes lipid accumulation and cell death in hepatocytes[69,70].

Modifying bile acid metabolism and other small metabolites contribute to the development of HCC induced by NASH

Metabolites produced by the gut microbiota have received much attention in the scientific community, and they are helping us to understand the metabolic changes that contribute to the development of NAFLD and NAFLD-HCC. Liposomes (SCFA), glucose, amino acids, and bile acids are now being investigated to improve our understanding of the pathophysiology of NAFLD-HCC[32,71].

Bile acids and their metabolites play an important role in the regulation of hepatic glucose, cholesterol, and triglyceride balance, and their changes can cause NAFLD by affecting lipid and energy metabolism[7]. In addition, bile acids can directly affect the intestinal microbiome by altering bacterial membranes[72].

The colon microbiota, particularly Gram-positive bacteria belonging to *Clostridium* clusters, convert primary bile acids, which were not resorbed in the small intestine, into secondary bile acids,

deoxycholate and lithocholate, which are then transported back to the liver with portal blood[73]. Dysbiosis promotes the increase of levels of such secondary bile acids in the liver. Consequently, a senescence hepatic stellate cell phenotype appears, which is characterized by the overproduction of various pro-inflammatory and tumorigenic factors that promote the development of HCC[7,16]. Sydor *et al*[13] have determined the direct correlation of blood levels of conjugated bile acids with the severity of NAFLD, although independent of the occurrence of HCC. Enterohepatic DCA also promotes the development of HCC in mice[74].

On the other hand, liver inflammation has been shown to cause intrahepatic retention of bile acids, directly promoting the development of HCC[67].

By activating TGR5 (Takeda G protein receptor 5), secondary bile acids may participate in the regulation of insulin sensitivity[16,75]. Activation of FXR (Farnesoid X receptor) by the gut microbiota may also influence bile acid metabolism during the onset and progression of hepatic steatosis[16,76].

Other small bacterial metabolites generated by the gut microbiota are also attractive objects to study metabolic alterations that may play a role in the progression of NAFLD and NAFLD-HCC[32,77].

Branched chain amino acids (leucine, isoleucine, valine, and phenylalanine) and bile acids (glycocholic acid, taurocholic acid, glycochenodeoxycholate) were found to be strongly associated with progression of steatosis to NASH, NASH-cirrhosis, and HCC[78], while glutathione was inversely associated[79].

SCFAs (formate, acetate, propionate, and butyrate) can enter the portal vein and promote lipid build-up and glucogenesis in the liver and possibly promote inflammation and oncogenesis[19,80]. The feces of patients with NAFLD-induced HCC were enriched in those SCFs[19]. Although other researchers propagate the anti-inflammatory effects of aromatic amino acid metabolites, especially butyrate[81,82].

Intestinal bacteria can convert dietary choline to trimethylamine (TMA), which is then further metabolized in the liver to trimethylamine-N-oxide (TMAO). Contrary to the useful choline metabolite, phosphatidylcholine, TMAO promotes the accumulation of triglycerides leading to hepatic steatosis and, thus, contributes to inflammation[7].

The difference between bland and NASH steatosis is the accumulation of free non-sterified cholesterol in the latter[5]. Free cholesterol and its oxidized derivatives are cytotoxic and can cause liver damage[5,83].

NAFLD patients had higher serum alcohol concentrations than healthy controls and obese subjects, indicating the possible impact of ethanol-producing bacteria on the pathogenesis of NASH[7].

How the aforementioned bacterial metabolites contribute to the manifestation of HCC in subjects with NASH must be elucidated.

Modifying antitumor immunity

The multilayer immune components of the colon wall, together with the genetic diversity of the colon microbiota, create an ideal environment for intestinal microbe-human immunological interactions[84]**.** The gut microbiota and its metabolites alter host gene pathways implicated in immunological and metabolic diseases[85].

In addition to promoting inflammation, the gut microbiota can possibly affect antitumor immunity. *A. muciniphila* and *Ruminococcaceae spp*. were found to be enriched in the gut of HCC patients who respond to anti-PD-1 immune checkpoint inhibitor compared to nonresponders[86]. The gut microbiota of patients with unresectable HCC differs: Those with progressive HCC were characterized by the abundance of fecal *Prevotella*, while those with a good response to immune checkpoint inhibitors were distinguished in the amount of *Veillonella, Lachnospiraceae, Lachnoclostridium, Lactobacillales, Streptococcaceae,* and *Ruminococcaceae*[87].

In several clinical studies of using an anti-CTLA-4 treatment for cancers of other etiology, the promoting effect for response to treatment by several species of the gut microbiota was also reported. However, the possible mechanism of such an impact is not very clear[84]. Furthermore, molecules born of the microbiota, including genomic material, the so-called bacterial signature, have been found in the liver parenchyma and the HCC nodules themselves[16]. These molecules could certainly play an active role in modulating the immune response in favor of more severe inflammation and hepatocarcinogenesis. A direct association of intrahepatic *Gamma-proteobacteria* abundance with liver disease progression from non-NAFLD to NAFLD and NASH of different severity was reported[88]. And finally, bile acids themselves possess immunomodulatory properties. Therefore, their modulation by the gut microbiota directly impacts host immunity.

LIMITATIONS AND FUTURE PERSPECTIVES

Most healthy individuals demonstrate relative stability of their gut microbiota with the transient effect of diet and the slightly longer effect of antibiotics[89-91]. For example, shared housing promotes the preservation of the same microbiota profiles[92]. On the contrary, discrepancies in the data on the composition of the gut microbiota are observed in clinical studies, including those of NASH-induced HCC. Due to the small number of subjects enrolled, the absence of control groups, different sample

collection techniques, and distinctive sequencing methods, the results of clinical studies are difficult to compare, and there are always doubts about their reproducibility.

Estimated differences between the composition of the gut microbiota of a healthy population, NAFLD, NASH, and those with NASH-induced HCC, even at the phyla level, can be considered as evidence of the participation of the microbiota in the pathogenesis of HCC, especially with a shift towards LPS-producing phyla. However, the collected data is not sufficient to draw reasonable conclusions so far.

Moreover, even in the generally pro-inflammatory LPS-producing phyla, there is a huge difference between the properties of bacteria depending on the species. Furthermore, bacterial strains belonging to the same species can also vary greatly in properties. Since affordable measures, such as a balanced diet and aerobic exercises, gradually shift the microbiota toward a healthy shape, it can be presumed that substantial changes are likely to occur at the species/strain level. Possibly, the research of some representative of the gut microbiota at the species/strain level in subjects with NASH-induced HCC in comparison with those without HCC will provide us with more definitive hepatocarcinogenesis provokers in the NASH population, or at least a noninvasive marker of early HCC will be confirmed. One such candidate – *Veillonella parvula* – has already been discovered. However, it is too early to draw conclusions about whether it was an incidental finding or a reliable HCC marker[93].

The microbiota as a potential noninvasive marker for the diagnosis of HCC, especially in the early stages, is intensively studied and might be promising since researchers determine some peculiarities distinguishing the microbiota composition in cirrhotic patients with HCC patients[48,49]. A more attentive study of comparing the gut microbiota of non-cirrhotic NAFLD-HCC patients with cirrhotic ones may prove useful in clarifying the most provocative representatives of liver oncogenicity. HCC of different stages can also be characterized using a dysbiosis index[49]. Although the cohorts of patients in such studies are too small to expect reproducibility of the results.

Experimental studies of the gut microbiota are characterized by another limiting aspect, different methodological approaches. These problems were perfectly elucidated in the Ponziani *et al*[94] review. However, the authors state that despite existing limitations, research on the impact of the gut microbiota on liver diseases has diagnostic, preventive and therapeutic potential, especially in patients with early stage HCC[94].

The therapeutic potential of the microbiota is currently intensively studied. In multiple clinical trials, fecal microbiota transplantation is applied with the expectation of reducing the progression of various etiology liver diseases, including NAFLD of different stages and NASH-induced HCC. Unfortunately, the published results are not promising so far[95]. More clinical trials are needed to better understand the efficacy of intestinal microbiota transplantation in NASH liver and HCC. Prebiotic and probiotic therapy appears to be more promising for the prevention and/or treatment of HCC, although it is necessary to determine its long-lasting effect[96,97].

The other members of the gut microbiome community, including fungi, viruses, and bacteriophages, are also worthy of consideration by researchers as possible participants in the pathogenesis of liver diseases, including NASH and HCC. They can also potentially contribute to the relief of liver disease. For example, Duan *et al*[98] presented experimental research on the beneficial effect on reducing liver disease of bacteriophages targeting *Enterococcus faecalis* that produces toxin cytolysin. Due to more affordable and powerful sequencing technologies, in addition to bacterial components, enteric fungal and viral species will certainly become objects of future research not only in connection with NASH-induced HCC, but also in elucidating the pathophysiological mechanisms of liver diseases of other etiologies[32]. Furthermore, a healthy lifestyle is an affordable approach that can be an effective measure in modulating the microbiota to a healthier shape, reducing obesity, and prophylaxis of NASH and NASH-induced HCC[2,99].

CONCLUSION

Current research claims that in the long run, steatohepatitis and the gut microbiota establish mutually maintaining pathological circuit that trigger liver inflammation. This can result in the manifestation of HCC and the growth of malignant nodules, even in the absence of obvious cirrhosis. However, a definite picture of that circuit treads remains blurred.

FOOTNOTES

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REVIEW

Hepatogenous diabetes: Knowledge, evidence, and skepticism

Ramesh Kumar, Diego García-Compeán, Tanmoy Maji

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Abstract

The diabetogenic potential of liver cirrhosis (LC) has been known for a long time, and the name "hepatogenous diabetes" (HD) was coined in 1906 to define the condition. Diabetes mellitus (DM) that develops as a consequence of LC is referred to as HD. In patients with LC, the prevalence rates of HD have been reported to vary from 21% to 57%. The pathophysiological basis of HD seems to involve insulin resistance (IR) and pancreatic β-cell dysfunction. The neurohormonal changes, endotoxemia, and chronic inflammation of LC initially create IR; however, the toxic effects eventually lead to β-cell dysfunction, which marks the transition from impaired glucose tolerance to HD. In addition, a number of factors, including sarcopenia, sarcopenic obesity, gut dysbiosis, and hyperammonemia, have recently been linked to impaired glucose metabolism in LC. DM is associated with complications and poor outcomes in patients with LC, although the individual impact of each type 2 DM and HD is unknown due to a lack of categorization of diabetes in most published research. In fact, there is much skepticism within scientific organizations over the recognition of HD as a separate disease and a consequence of LC. Currently, T2DM and HD are being treated in a similar manner although no standardized guidelines are available. The different pathophysiological basis of HD may have an impact on treatment options. This review article discusses the existence of HD as a distinct entity with high prevalence rates, a strong pathophysiological basis, clinical and therapeutic implications, as well as widespread skepticism and knowledge gaps.

Key Words: Cirrhosis; Diabetes; Hepatogenous diabetes; Glucose intolerance; Insulin resistance; Metabolism

Core Tip: Hepatogenous diabetes appears to be the most prevalent form of diabetes in patients with liver cirrhosis. It is linked to the pathophysiological alterations and severity of cirrhosis. However, it is still an underappreciated problem and is not recognized as a distinct entity by scientific organizations. This article discusses the current state of knowledge about hepatogenous diabetes, including evidence of its existence and clinical implications.

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INTRODUCTION

The maintenance of glucose homeostasis necessitates a coordinated response of insulin secretion, hepatic and peripheral glucose uptake, and suppression of hepatic glucose synthesis. The same is achieved *via* a complex control process involving several tissues and inter-organ crosstalk, including the liver, pancreas, muscles, and adipose tissues, as well as a number of circulating factors[1]. The liver plays a key role in glucose homeostasis by regulating multiple glucose metabolism pathways such as glycolysis, glycogenolysis, gluconeogenesis, and glycogenesis[2-4]. Therefore, hepatic dysfunction is likely to have an impact on glucose metabolism. In fact, the association between liver cirrhosis (LC) and diabetes mellitus (DM) has been known for a long time[5,6]. The prevalence of diabetes in patients with LC ranges from 20% to 70%, which is significantly higher than the 6.28% prevalence of type 2 DM (T2DM) in the general population[7,8]. The wide range of reported prevalence rates appears to be due to heterogeneity in the studied population, stage of liver disease, and evaluation method(s).

In 1906, Naunyn first coined the term "hepatogenous diabetes" (HD) to describe DM caused by LC [9]. In the subsequent years, the association between DM and LC was studied more thoroughly, with hyperinsulinemia, insulin resistance (IR), and pancreatic-β-cell dysfunctions being commonly reported [10-14]. Although cirrhosis due alcohol, non-alcoholic fatty liver disease (NAFLD), hepatitis C viruses (HCV), and hemochromatosis has been deemed a diabetogenic condition, multiple studies have shown that diabetogenic potential of cirrhosis cuts across etiologies. Emerging evidences suggest that in patients with LC, a complex interplay between the liver, pancreas, skeletal muscles, gut, and adipose tissues is involved in the pathogenesis of impaired glucose tolerance (IGT) and HD[7,9,15,16]. However, despite plethora of evidence, HD is still not regarded as a distinct disease or a recognized complication of LC[7,17,18]. Such skepticism among scientific bodies appears to be paradoxical. The global acceptance of this term is important in order to spur more research in this area.

DEFINITION AND CHARACTERISTICS OF HD

DM that develops as a result of LC is referred to as HD[19]. For HD to be diagnosed, DM must have occurred after the onset of cirrhosis. In practice, however, distinguishing HD from T2DM can be challenging, especially in early cirrhosis, because both DM and cirrhosis have a long, indolent, and clinically silent course, making it difficult to determine which condition appeared first. Furthermore, the association between diabetes and LC is bidirectional, as patients with T2DM can develop NAFLD which may progress to cirrhosis[20]. Certain etiological agents of LC such as ethanol, NAFLD, HCV, and hemochromatosis, have a direct diabetogenic effect which can lead to DM even before onset of cirrhosis, posing a classification dilemma. Therefore, there is an unmet need to develop a consensus-based criteria for defining HD in LC patients in order to ensure consistency in future clinical research.

There are a number of soft indicators that can help distinguish HD from T2DM[7,17-19]. Unlike T2DM, HD can occur in patients with LC who don't have metabolic risk factors including a high body mass index, hyperlipidemia, or a family history of diabetes. HD patients frequently have normal fasting blood glucose (FBG) and glycated hemoglobin (HbA1c) but abnormal oral glucose tolerance tests (OGTTs), whereas T2DM patients often have high FBG. In addition, the degree of hyperinsulinemia and IR is substantially higher in HD than in T2DM patients (Table 1).

PREVALENCE OF HD IN LC

The prevalence of all types of DM (T2DM + HD) in patients with LC has been reported to range from 20% to 70%[7]. Overall, the prevalence of DM varies depending on the etiology of LC. In a recent

Table 1 Characteristics of diabetes in patients with cirrhosis that favour a diagnosis of hepatogenous diabetes over type-2 diabetes mellitus

Following characteristics favour a diagnosis of HD
Occurrence after the onset of liver cirrhosis
Low prevalence of metabolic risk factors[1] or a family history of DM
Normal fasting glycemia but abnormal oral glucose tolerance test
Low prevalence of microvascular complications, such as diabetic retinopathy
Associated with higher levels of hyperinsulinemia, insulin resistance, and an increased risk of hypoglycemia due to high glycemic variability
Higher association with the severity of liver cirrhosis and liver related complications
Remission after a liver transplantation

[1]Obesity, hyperlipidemia.
HD: Hepatogenous diabetes; T2DM: Type-2 diabetes mellitus.

systematic review of 58 studies (n = 9705), the overall prevalence of DM in adult patients with LC was 31%. Patients with NAFLD-cirrhosis had the highest prevalence of diabetes (56%), followed by cryptogenic cirrhosis (51%), while patients with HCV and HBV cirrhosis had 32.2% and 22.2%, respectively[21]. Due to multiple shared risk factors, the prevalence of DM is higher in metabolic cirrhosis than in viral cirrhosis. However, because HD in its true sense refers to diabetes induced by liver dysfunction *per se*, the etiology of cirrhosis may have little bearing on its occurrence. Many studies, however, have not reported the differential prevalence of T2DM and HD. In studies where prevalence of HD was specifically looked at using OGTT, the rates ranged from 21% to 57% (Table 2). Wang *et al*[22] and Ramachandran *et al*[23] reported HD prevalence rates of 15.9% and 29.2%, respectively, based on clinical history alone, *i.e.*, onset of DM after diagnosis of LC. The relatively lower prevalence rates of HD in their studies signify the relevance of performing an OGTT. To detect DM in LC patients, an OGTT is required because FBG and HbA1c levels may be erroneously low[24,25]. LC patients who have normal FBG and HbA1c values but an abnormal OGTT are likely to have HD. Because of the pathophysiological differences as well as clinical and therapeutic ramifications, HD must be distinguished from T2DM.

The severity of liver disease appears to influence the prevalence of DM in LC[26,27]. In a prospective study on compensated LC patients with a normal glucose tolerance (n = 100) at baseline, a diabetic response to OGTT was noted in 4.4% and 21.2% after a 1-year and 4-year follow-up, respectively. The incidence of DM was even higher (35.3% at 2 years) among patients whose Child-Pugh class worsened during follow-up. Notably, the incidence of diabetes was unaffected by gender, etiology, or a family history of diabetes, suggesting that diabetes was likely to be hepatogenous[26]. In another study, DM was present in 20.5%, 56%, and 61% in Child Pugh class A, B, and C, respectively[27]. The presence of HD was significantly related to a higher model for end stage liver disease (MELD) scores (> 15), large varices, and hepatocellular carcinoma (HCC) in a study[28]. HD was significantly associated with a high Child-Pugh's scores [odds ratio (OR) = 1.43] and hepatic venous pressure gradients (HVPG) (OR = 1.15) in a study by Jeon *et al*[29]. García-Compean *et al*[19] found that renal impairment and family history of DM were only two factors significantly differed between T2DM and HD[30]. Holstein *et al*[31] reported a very high prevalence of HD (57%) in a study cohort in which 56% of LC patients belonged to Child-Pugh class B or C. Thus, the available evidence suggests that the severity of LC, rather than the etiology, influences the development of HD[26-29]. In summary, HD seems to constitute a significant proportion of DM in patients with LC. The worsening diabetogenic potential of LC in parallel with the severity of liver disease suggests a detrimental impact of liver failure on glucose metabolism.

PATHOPHYSIOLOGY OF HD

The pathophysiology of HD is complex and poorly understood. This appears to be caused by two major factors: IR and pancreatic β-cell dysfunction (Figure 1). The development of IR is triggered by neurohormonal changes, endotoxemia, and chronic inflammation of LC. The toxic effects eventually reach the pancreatic islets, causing β-cell dysfunction, which leads to the development of HD. Recently, roles of hepatokines, adipokines, gut dysbiosis, hyperammonemia, sarcopenia and myosteatosis have emerged in the pathogenesis of metabolic disturbances in LC, including IR and glucose intolerance (Figure 2). Thus, the identification of mechanisms that connect multi-organ dysfunction might unravel a novel understanding of HD pathophysiology.

Table 2 Reported prevalence rates of hepatogenous diabetes in patients with liver cirrhosis

Ref.	Patients (*n*)	Diagnostic method	HD, *n* (%)	IGT, *n* (%)
Holstein *et al*[31]	35	OGTT	20 (57)	13 (37)
Tietge *et al*[114]	100	OGTT	35 (35)[1]	38 (38)
Nishida *et al*[25]	46	OGTT	21 (38)[1]	13 (23)
García-Compeán *et al*[30]	130	OGTT	28 (21.5)	36 (38.5)
Jeon *et al*[29]	195	OGTT	108 (55.4)	169 (86.7)
Ramachandran *et al*[23]	202	Clinical history[2]	59 (29.2)	NS
Wang *et al*[22]	207	Clinical history[2]	33 (15.97)	NS
Vasepalli *et al*[28]	121	OGTT	52 (42.9)	58 (47.9)

[1]Likely hepatogenous diabetes (diabetes diagnosed after oral glucose tolerance test in non-diabetic cirrhosis).
[2]Onset of diabetes after diagnosis of cirrhosis.
HD: Hepatogenous diabetes; LC: Liver cirrhosis; IGT: Impaired glucose tolerance; OGTT: Oral glucose tolerance test; NS: Not stated.

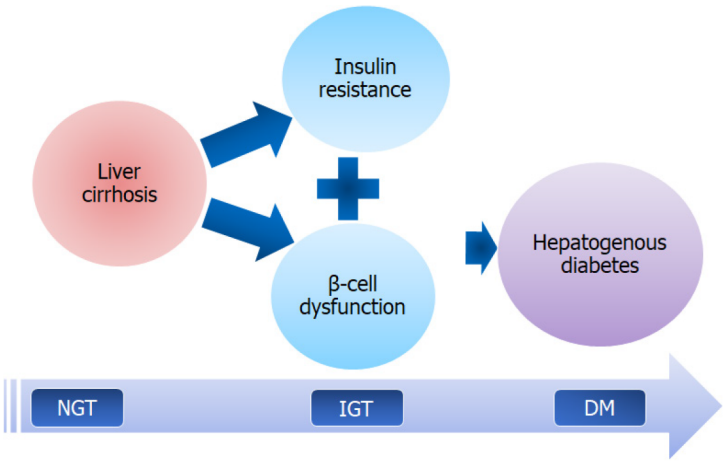

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Figure 1 Key events leading to development of hepatogenous diabetes in patients with liver cirrhosis. Insulin resistance, followed by β-cell dysfunction, occurs as a result of liver cirrhosis, culminating in a progressive worsening of glucose tolerance and the onset of hepatogenous diabetes. Both of these occurrences are linked to the pathophysiological changes in the body caused by liver cirrhosis. NGT: Normal glucose tolerance; IGT: Impaired glucose tolerance; DM: Diabetes mellitus.

Hyperinsulinemia and IR

Several studies have confirmed hyperinsulinemia and IR in LC patients[10-12,32]. Hyperinsulinemia appears to be caused primarily by two abnormalities: decreased hepatic extraction and portosystemic shunting of insulin. Hyperglucagonemia, due to insufficient hepatic metabolism, may also contribute to hyperinsulinemia[33]. An augmented insulin secretion due to pancreatic islet hypertrophy may contribute to hyperinsulinemia before the development of significant β-cell dysfunction[34]. Persistent hyperinsulinemia leads to IR as insulin receptors are downregulated over target cell membranes[35]. Insulin sensitivity has been reported to be normalized when hyperinsulinemia is reduced[36]. Many studies have found a link between clinically significant portal hypertension and elevated IR[37,38], which could be due to the existence of a portosystemic shunt. In LC patients, hyperinsulinemia deteriorates after the placement of a trans jugular intrahepatic portosystemic shunt (TIPS)[39]. In contrast, balloon-occluded retrograde transvenous obliteration (BRTO) of portosystemic shunts has been shown to ameliorate hyperinsulinemia in portal hypertensive patients[40].

Clamp studies of whole-body glucose utilization have shown that IR in patients with LC is due to reduction in nonoxidative glucose disposal, which includes glucose conversion to glycogen or fat, as well as anaerobic glycolysis[41,42]. Since extrahepatic glucose metabolism accounts for majority (approximately 85%) of total body glucose metabolism under glucose clamp condition, reduction in nonoxidative glucose disposal leads to significant IR at peripheral tissues[43,44]. Many other studies have consistently demonstrated that diminished insulin-dependent glucose transport into skeletal muscle and a reduction in glycogen synthesis are mainly responsible for the reduction in peripheral

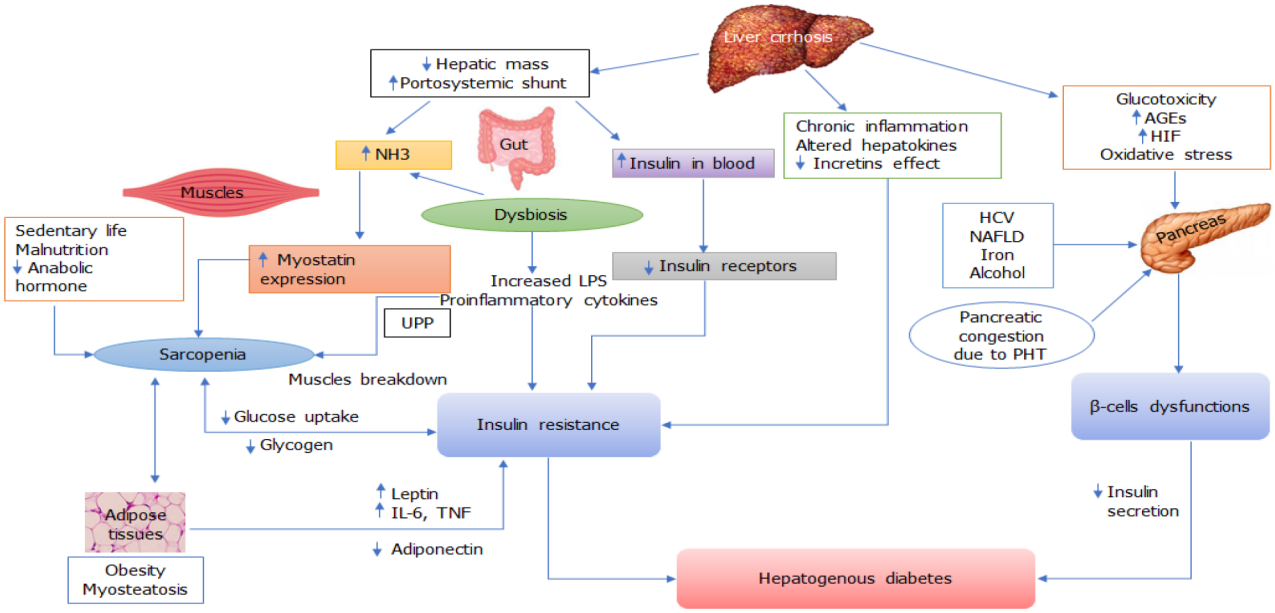

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Figure 2 Complex pathophysiological mechanisms likely to play important roles in the development of hepatogenous diabetes. Neurohormonal alterations, endotoxemia, and chronic inflammation induced by cirrhosis and portal hypertension all contribute to the development of insulin resistance and β-cell dysfunction. These changes may be further modulated by other concomitant abnormalities, such as gut dysbiosis, hyperammonemia, sarcopenia, adiposity, and myosteatosis. NH_3: Ammonia; AGE: Advanced glycation end products; HIF: Hypoxia inducible factor; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; LPS: Lipopolysaccharides; IL-6: Interleukin-6; TNF: Tumour necrosis factor; UPP: Ubiquitin proteasome pathways.

glucose turnover in patients with LC[45,46]. On the other hand, there appears to be no significant hepatic IR in LC[47]. Thus, skeletal muscle is the primary site of IR in patients with LC.

Pancreatic dysfunction

Despite reports of pancreatic islet cell proliferation in patients with LC, insulin-positive islet area was found to be considerably reduced[34,48,49]. In comparison to the control and T2DM groups, Sakata *et al* [49] found that patients with LC have lower insulin expression and higher expression of the pancreatic transcription factor PDX-1 in their islets. Studies on animal models of LC and portal hypertension have also found a decreased insulin secretion from the pancreatic islets despite hyperinsulinemia[13,14]. In a recent study, pancreas in LC patients showed congestive changes on dynamic contrast enhanced ultrasound and histopathology. In addition, decreased insulin secretion was found to be associated with pancreatic congestive changes. Despite the islets' expansion, the fraction of insulin-positive region per islet decreased, and this was negatively correlated with thickness of pancreatic vein due to portal hypertension[14]. These data indicate that even when glucose tolerance is impaired, pancreatic hyposecretion can occur in LC patients. The inability of the pancreatic β-cell to compensate for worsening IR appears signal the switch from IGT to HD. An improved β-cell function is also required for the regression of diabetes after liver transplantation (LT)[50].

Chronic hyperglycemia can produce toxic damage to the pancreatic islets, resulting in β-cells' dysfunction[51-53]. The accumulation of advanced glycation end products (AGEs), which are normally eliminated by the liver, accelerates this process by causing oxidative stress in β-cells. The systemic low-grade hypoxia generated by advanced LC contributes to the further deterioration of β-cells function[54]. Increased expression of hypoxia-inducible factors (HIF, mainly HIF-1α) has been reported in many liver diseases, including NAFLD and alcoholic liver disease[55]. HIF-1α is known to regulate cellular glucose uptake, glycolytic enzyme activity, and insulin sensitivity[56,57]. Apart from directly affecting glucose metabolism, activation of HIF-1 in patients with LC can elicit an inflammatory response in β-cells, contributing to the development of overt DM[58].

In patients with LC, a number of disease-specific mechanisms of β-cell dysfunction may be operating. Chronic alcohol use and hemochromatosis produce glucokinase downregulation and increased oxidative stress, resulting in increased β-cells apoptosis and decreased glucose-induced insulin production, respectively[59,60]. The combination of chronic hyperglycemia and high free fatty acid levels in NAFLD causes glucolipotoxicity, leading to pancreatic β-cells injury[61]. Chronic HCV infection, on the other hand, causes pancreatic islets injury by a combination of autoimmune-mediated and direct cytopathic processes[62-64].

Reduced incretins effect

Incretins serve an important function in maintaining glucose homeostasis. Enteroendocrine cells produce two naturally occurring incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide, which regulate glycemic control by boosting insulin secretion and lowering glucagon secretion during postprandial period. Dipeptidyl peptidase 4 (DPP-4) is a membrane-associated peptidase that has a wide range of organ distribution and exhibits pleiotropic effects through its peptidase activity. DPP4 inactivates GLP-1, which leads to the development of IGT and DM[65]. Cirrhotic patients had higher serum DPP-4 activity and hepatic DPP-4 expression, which reduces incretin effects[66]. Thus, decreased incretin effects could play a role in the development of HD.

Gut dysbiosis

The gut microbiota is involved in the host immunity, metabolism, and intestinal endocrine function[67]. Patients with LC frequently have changes in the composition and function of the gut microbiota with associated damage to the gut barrier, bacterial translocation, and systemic inflammation[68,69]. The translocation of gut-derived endotoxins (notably lipopolysaccharide, LPS), which activates of toll-like receptors, is involved in the pathogenesis of IR[70]. Metabolic endotoxemia mediated by LPS/CD14 system dysregulates inflammatory tone, leading to diabetes and adiposity[70]. Dysbiosis of the gut has been associated with obesity, metabolic diseases, and DM[71]. Gut dysbiosis also contributes to hyperammonemia in LC patients which has some role in the development of peripheral IR[72]. The human gut microbiota produces a variety of compounds, including branched-chain amino acids, whose circulation levels are linked to the risk of IR and DM[73]. The gut microbiome of CLD patients with sarcopenia was found to be pro-diabetogenic in a recent study, with a high abundance of gram-negative bacteria containing LPS on the one hand and a low Firmicutes/Bacteroidetes ratio on the other[74]. Therefore, gut dysbiosis could play an important role in the pathogenesis of HD in advanced LC.

Hyperammonemia

Hyperammonemia is a frequent abnormality in LC due to impaired hepatic detoxification to urea and bypass *via* portosystemic shunt. Hyperammonemia has been associated with IR for a long time[75]. Hyperammonemia in LC is linked to enhanced myostatin expression in the skeletal muscle[76]. Because myostatin is a negative regulator of muscle protein synthesis, a greater serum ammonia level can promote a rise in myostatin in skeletal muscle, leading to sarcopenia progression and its adverse consequences[77,78]. Hyperammonemia is also associated with myosteatosis which affects glucose transport and glycogen synthesis by downregulating muscle insulin receptors[79,80]. Thus, myostatin appears to have a role in cirrhosis-induced peripheral IR, as it causes sarcopenia and myosteatosis.

Sarcopenia

Skeletal muscle is responsible for the majority of postprandial glucose consumption, making it an essential insulin target organ for glucose uptake and utilization[81]. As a result, skeletal muscle loss might result in substantial IR[81,82]. Sarcopenia, or the loss of skeletal muscle mass, quality, and strength, is common in LC patients and is linked to IR and DM[83,84]. Sarcopenia in LC is caused by an imbalance in muscle protein turnover, which is influenced by a number of metabolic variables such as hyperammonemia, amino acid deficiency, hormone imbalance, gut dysbiosis, IR, and chronic inflammation[81-84]. Sarcopenia and DM appear to have a bidirectional link. On the one hand, sarcopenia is common among DM patients; on the other hand, sarcopenia has been linked to an increased risk of DM [85]. Sarcopenia is frequently accompanied by myosteatosis, mitochondrial dysfunction, macrophage infiltration, and inflammatory cytokine release, all of which contribute to IR as well as lower glucose uptake and utilization[83,84]. Previous studies have shown that skeletal muscles secrete a variety of cytokines, such as IL-6 and irisin, that regulate insulin sensitivity and promote metabolism[86,87]. Thus, impairment of muscle secretory function due to sarcopenia may contribute to the development of DM in LC patients. Sarcopenic obesity, which affects up to 35% of patients waiting for a liver transplant, has a higher influence on metabolic profile than either condition alone[88].

Hepatokines and adipokines

Hepatokines and adipokines, proteins that regulate systemic metabolism and energy homeostasis, are secreted by the liver and adipose tissues, respectively[89-91]. Crosstalk between hepatokines, adipokines, and myokines influences inflammation and fat metabolism in adipose and skeletal muscle, which can contribute to IR[90,92,93]. Additionally, some hepatokines influence insulin secretion by the pancreas, which can independently affect peripheral tissue glucose uptake and metabolism. Hepatokines are known to contribute to the pathogenesis of metabolic syndrome, NAFLD, and 2DM[90, 92]. Furthermore, several hepatokines control pancreatic insulin secretion, which can affect glucose uptake and metabolism in peripheral tissues independently. Many hepatokines, including fetuin A, fetuin B, retinol-binding protein 4, and selenoprotein P, have been linked to the induction of metabolic dysfunction[93]. Therefore, their significance in metabolic abnormalities of LC is worth investigating. Resistin is an adipokine that reduces insulin sensitivity in adipocytes, skeletal muscles, and hepatocytes. Serum resistin level has been found to be significantly elevated in patients with LC, which may

contribute to IR[94].

CLINICAL IMPACT OF DM ON LC

The presence of DM (HD+T2DM) in patients with LC is associated with numerous complications and poor outcomes (Table 3). Because most studies have not stratified DM into HD and T2DM, the individual impact of HD cannot be ascertained[16,18,95,96]. However, because HD is a direct complication of liver cirrhosis, it is likely to have a greater negative impact on prognosis of liver cirrhosis than T2DM.

Impact on complications of LC

Many complications of LC, including hepatic encephalopathy (HE), variceal hemorrhage (VH), sepsis, and hepatocellular carcinoma (HCC) have been associated with DM. DM has been associated with an increased incidence and severity of HE in patients with LC. In a study, the proportion of patients with severe HE was found to be higher in diabetic than in nondiabetic patients (60% *vs* 20%, $P = 0.007$)[97]. Jepsen *et al*[98] reported that diabetic LC patients had a higher incidence of first-time overt HE in a year (26% *vs* 15.8%) as well as greater risk of HE progression > grade 2 (64% *vs* 42%), compared to non-diabetic LC. DM is an independent predictor of HE after TIPS in LC patients[99,100]. Possible mechanisms by which DM can promote HE include induction of intestinal glutaminase, intestinal bacterial overgrowth, hyperammonemia, sepsis, and development of a chronic inflammatory state[101-103].

Chronic hyperglycemia may induce splanchnic hyperemia in LC patients leading to an increased portal pressure and risk of VH[22,29,104]. In a prospective study ($n = 194$), HD (55.4%) was significantly associated with increased portal pressure and risk of VH[29]. Yang *et al*[104] also reported DM as an independent predictor of VH in LC patients (OR = 2.99). Wang *et al*[22] have reported an increased risk of rebleeding following endoscopic variceal ligation in HD patients (44% *vs* 13.9% in 6 mo). DM also increases the mortality risk following upper gastrointestinal bleeding in LC patients (OR = 5.7)[105].

DM increases the risk of bacterial infections in patients with LC[106]. In a study on hospitalized LC patients ($n = 178$), the prevalence of bacterial infections was higher among diabetics than non-diabetic (85% *vs* 48%, $P < 0.0001$)[107]. DM increases the risk of spontaneous bacterial peritonitis in LC (HR = 1.51)[108]. Furthermore, uncontrolled DM in LC has greater risk of bacterial infection, suggesting that glycemic control could be a modifiable target[108,109]. DM is also a risk factor for HCC in LC patients. In a cohort study, DM increased the risk of HCC in patients with non-HCV cirrhosis (HR = 2.1) but not in HCV-cirrhosis, who already have a very high risk of developing HCC[110]. Takahashi *et al*[111] have reported that 2-hours post-glucose-challenge hyperglycemia was significant factor for HCC development in HCV-RNA–positive patients (HR = 6.9).

Impact on outcome and LT

The survival rate in patients with LC is significantly reduced in presence of DM[25,106,112,113]. Bianchi *et al*[112] reported that 5-year survival of LC patients with or without DM was 41% and 56%, respectively, $P = 0.005$). In a prospective study on 100 compensated LC patients, 5-year cumulated survival rates were lower (31.7% *vs* 71.6%) in those with abnormal OGTT normal OGTT ($P = 0.02$)[113]. In an another prospective study, the cumulative 5-year survival was 94.7% in LC with normal glucose tolerance compared to 56.6% in those with DM on OGTT[25]. In a recent French study, DM had a greater impact on survival in early stages of LC patients (MELD score < 10), suggesting that the severity of liver disease can mask the deleterious effect of DM[106]. In a longitudinal study, Holstein *et al*[31] also found that all deaths in HD patients were due to complications related to LC rather than diabetes-related complications. This could be because of advanced liver failure in HD patients which shortens the time for diabetes complications to emerge.

The pre-transplant DM also has adverse impact on outcomes of liver transplantation. Tietge *et al*[114] demonstrated that pre-liver transplant IGT or DM are the major risk factors for post-transplant diabetes. In a meta-analysis of 20 studies ($n = 4580$), impaired glucose metabolism was among the risk factors for new onset DM after LT[115]. Post-LT DM is associated with increased risk of mortality and multiple morbid outcomessuch as cardiovascular disease, infection, biliary complications, renal impairment, and graft rejection[116-118].

SKEPTICISM ABOUT HD

Ever since Naunyn first coined the term "hepatogenous diabetes" in 1906, there has been a lot of research on this subject, especially in the1970s and 1980s, but the momentum faded little bit and the term HD began to lose its recognition and appeal. To date, the most scientific bodies, such as the American Diabetes Association (ADA) and the American Association for the Study of Liver Disease

Table 3 Studies depicting clinical impact of diabetes mellitus/hepatogenous diabetes in patients with liver cirrhosis

Ref.	Design	*n*	Main outcomes/remarks
Bianchi *et al*[112]	Retro-prospective	354	5 yr survival: 41% with DM and 56% without DM (P = 0.005)
Holstein *et al*[31]	Prospective cohort	52	51% of HD patients died within median of 5.7 yr after diagnosis of DM. Remark: No data on non-diabetic control
Moreau *et al*[136]	Prospective cohort	75	Survival in patients with and without DM: 18% and 58%, respectively
Sigal *et al*[97]	Cross-sectional	65	Incidence and severity of HE was higher in diabetics and DM was an independent risk factor for HE (P = 0.0008). Remark: study involved only HCV cirrhosis
Nishida *et al*[25]	Prospective cohort	56	5 yr survival was 94%, 68% and 56%, with NGT, IGT and DM, respectively
Tietge *et al*[114]	Case-control study	100	Pre-transplant IGT or DM was risk factor for post-LT DM. Remark: Only 31 patients were prospectively evaluated
Jeon *et al*[29]	Prospective cohort	195	HD correlated significantly with HVPG and VH. Post-prandial hyperglycemia correlation with risk of VH in 6 mo
García-Compeán *et al*[113]	Prospective cohort	100	5 yr cumulated survival was lower in IGT patients than NGT (31.7% *vs* 71.6%, P = 0.02)
Elkrief *et al*[106]	Retrospective cohort	348	DM was independently associated with ascites, infections, HE, HCC and mortality. Remarks: Only HCV cirrhosis studied
Yang *et al*[104]	Prospective cohort	146	DM was among independent predictors of VH (OR = 4.90)
Jepsen *et al*[98]	Database analysis	863	Diabetic patients had a higher episode of first-time overt HE and HE progression beyond grade 2 than non-diabetics. Remarks: Original trials used vaptan which could be a confounder
Khafaga *et al*[137]	Prospective case-control	60	Proportion of VH (46.4% *vs* 10%), HE (36% *vs* 10%) and mortality (16.6% *vs* 6.7%) was higher among diabetics compared to non-diabetic LC
Qi *et al*[105]	Retrospective	145	In-hospital mortality was 20.6% in diabetics and 4.3% in nondiabetics (P = 0.003)
Hoehn *et al*[116]	Retrospective	12442	Diabetic recipients had longer hospitalization (10 *vs* 9 d) and higher peri-transplant mortality (5% *vs* 4%)
Yang *et al*[110]	Retrospective cohort	739	DM increased the risk of HCC in non-HCV cirrhosis (HR = 2.1)
Routhu *et al*[100]	Retrospective cohort	895	DM was an independent predictor of HE
Ramachandran *et al*[23]	Prospective cohort	222	HD patients had higher incidence of gall stones (27% *vs* 13%) and urinary infection (28% *vs* 7%), compared to those without DM
Tergast *et al*[108]	Prospective	475	DM patients had an increased risk for SBP (HR = 1.51), especially when HbA1c values ≥ 6.4%
Wang *et al*[22]	Retrospective	207	Rebleeding rate following variceal endotherapy was higher (approximately 5 times) in diabetics, including HD, than non-diabetics at 1, 3, and 6 mo
Rosenblatt *et al*[109]	Retrospective (National database)	906559	Uncontrolled DM was associated with an increased risk of bacterial infection (OR = 1.33) and death (OR = 1.62)
Labenz *et al*[138]	Prospective cohort s	240	DM was independently associated with covert HE. The risk of HE and overt HE was more pronounced when HbA1c ≥ 6.5%

DM: Diabetes mellitus; HCC: Hepatocellular carcinoma; HCV: Hepatis C virus; HD: Hepatogenous diabetes; HE: Hepatic encephalopathy; HR: Hazard ratio; IGT: Impaired glucose tolerance; NGT: Normal glucose tolerance; OR: Odds ratio; VH: Variceal hemorrhage.

(AASLD), do not recognize HD as a distinct entity. As a result, HD is underestimated by most medical fraternity belonging to gastroenterology and endocrinology departments. There are no consensus-based diagnostic criteria or therapeutic guidelines for HD. Such skepticism does not appear to be justified. A strong link between LC and diabetes, several evidences of impaired glucose metabolism in LC patients, and a number of characteristics that distinguish HD from T2DM, all point to HD being a separate disease (Table 1). In addition, a number of factors have recently been identified as playing a role in the pathogenesis of impaired glucose metabolism in LC, including sarcopenia, sarcopenic obesity, gut dysbiosis, hyperammonemia, and hepato-adipokines. We believe that the time has arrived for scientific bodies to acknowledge HD as a distinct entity. This will pave the road and create doors for a large number of researchers to work on this topic in greater depth.

THERAPEUTIC CONSIDERATION

There are no standardized guidelines for managing diabetes in LC patients. Currently, T2DM and HD

are being treated in a similar manner[7,20,50,96]. In general, insulin is recommended for LC at all stages, while many oral hypoglycemic agents (OHA) are being used in LC up to Child-Pugh class B. A number of pathophysiological changes caused by LC, such as changes in the hepatic blood flow, fluid balance, hypoalbuminemia, and gut dysbiosis, might impact the bioavailability, distribution, and metabolism of antidiabetic medicines, posing a risk to patients. As a result, the majority of current OHA are considered unsafe for LC in Child-Pugh class-C, the stage which has the highest frequency of HD. Because the pathophysiology of T2DM and HD differs, the therapeutic approach should differ accordingly. However, because the pathophysiology of T2DM and HD differs, the therapeutic approach may need to be adjusted. Several pathophysiological changes produced by cirrhosis, such as degree of hepatic dysfunction, large portosystemic shunt, sarcopenia, gut dysbiosis, and hyperammonemia, all of which have an indirect impact on HD, could influence treatment choices, including drug selection (Table 4).

General measures

A moderate caloric restriction may be recommended for HD patients, particularly those who are overweight or obese. However, because of sarcopenia and sarcopenic obesity, it is important to maintain a sufficient protein intake to avoid muscle loss. Physical exercise may aid in the preservation and restoration of muscle function and mass while also improving IR. Physical exercise has also been shown to improve the HVPG and nutritional status in LC patients[119,120]. Because HD is a direct complication of LC and is associated with severity of cirrhosis, improving hepatic dysfunction and portal hypertension should be one of the important goals of HD treatment. Etiology-specific therapy (for HCV, hepatitis B, autoimmune hepatitis, *etc.*) and non-selective β-blocker to control portal hypertension may play a role in preventing, delaying, or attuning HD in LC patients. In a recent prospective study of 96 acute-on-chronic liver failure patients, 51 (53.1%) of whom had new-onset diabetes, most likely HD, the glycemic indices improved in one-third of patients following improvement of their liver function without taking anti-hyperglycemic medication[121].

OHA

Among the OHAs that can be considered for HD patients are metformin, glucagon-like peptide-1 (GLP-1) agonists, dipeptidyl peptidase 4 (DPP-4) inhibitors, thiazolidinediones (TZD), alpha-glucosidase inhibitors (AGI) and sodium glucose co-transporter-2 (SGLT2) inhibitors[[7,20,50,96]. Glycemic targets for HD patients should be set based on postprandial glucose levels rather than HbA1c or FBG. Metformin can be an important therapeutic agent for HD, because it is free of hepatic metabolism, plasma protein binding, and hypoglycemia risk, as well as having other benefits like cardio protection and a lower risk of HCC and HE[122-124]. However, metformin should be avoided if there is concurrent renal impairment with an eGFR of less than 45 mL/min per 1.73 m^2 due to the significant risk of lactic acidosis[50]. Upregulation of DPP-4 expression in LC patients contributes to the development IR[66]. Therefore, incretin-based antidiabetic agents, like GLP-1 receptor agonists (Liraglutide) and DPP-4 inhibitors can be an important agent for HD. They are generally safe in LC patients, increase muscle mass, and pose little risk of hypoglycemia or weight gain[125,126]. Recently, a group of investigators from Taiwan have raised safety concerns about use of metformin and DPP4 inhibitors in LC[127,128]. From analysis of Taiwan's National Health Insurance Research Database, investigators found that metformin use (> 1000 mg/d) in patients with compensated LC patients was associated with higher risks of mortality and decompensation[127]. Similarly, DPP-4 inhibitor was found to be associated with higher risks of hepatic decompensation and failure in another study[128]. These results should be viewed with caution, as the findings need to be validated in prospective studies. In a recent study, sulfonylureas (SU) was found to be associated with lower risks of all-cause mortality and major cardiovascular events in LC patients with diabetes[129]. However, SU should be better avoided in HD patients because of a high risk of hypoglycemia. HD patients are already at high risk of hypoglycemia due to poor glycogen storage and reduced gluconeogenesis capacity. Due to hypoglycemia, stringent glycemic control should not be attempted in HD patients.

In obese HD patients, metformin, SGLT2i, and GLP-1 agonists can be preferred because they tend to promote weight loss. When sarcopenia is severe, metformin, GLP-1 agonist (Liraglutide), and DPP-4 inhibitors are preferable[123]. SUs and SGLT2 inhibitors may increase the risk of sarcopenia[130,131]. Metformin or AGI, both of which have a positive effect on blood ammonia levels and the risk of HE, should be considered in hyperammonemic HD patients. Metformin effect is mediated partially by inhibition of glutaminase activity in enterocytes, while AGI (acarbose) stimulates the gut peristalsis and proliferation of the saccarolytic bacteria[132,133]. If there is a large portosystemic shunt in such patients, shunt occlusion using BRTO may be considered. Alteration of gut dysbiosis using probiotics is another option that requires investigation.

Insulin

Insulin therapy is considered to be the safest and most effective for patients with LC, and it is currently the sole option available for LC patients of Child-Pugh class C. However, there are many concerns about the use of insulin in HD patients who have a higher degree of hyperinsulinemia and IR than LC patients with T2DM. The insulin requirements in such patients might vary greatly, making it difficult to

Table 4 Factors that might influence selection of antidiabetic medication for hepatogenous diabetes

Condition	Antidiabetic drug with pros and cons	Preferences
Obesity	Metformin, SGLT2i, and GLP-1 agonists promote weight loss; DPP-4 inhibitors are weight neutral; Sulfonylureas, Pioglitazone, and Insulin promote weight gain	Should be preferred; May be considered; Consider alternative
Sarcopenia	Metformin and TZD appears to have favorable effect on muscles mass; SGLT2 inhibitors, SUs (especially glibenclamide and glinides) may increase the risk of sarcopenia	Should be preferred; Consider alternative
Hyperammonemia/Recurrent HE	Metformin and AGIs cause reduction of blood ammonia levels and risk of HE	May be preferred
Renal impairment	Insulin and linagliptin appear to be safe; SGLT-2 inhibitors may be considered with dose modification. It has added diuretic advantage; Metformin increases the risk of lactic acidosis	Should be preferred; May be considered; Should be avoided
Hypoglycemia	Insulin in SU have high risk of hypoglycaemia; Metformin, PZD, DPP4i and SGLT2 inhibitors have low risk of hypoglycaemia	Should be avoided; May be considered
LC with dysplastic liver lesion/high serum AFP	Metformin decreases the risk of HCC; DPP4 inhibitors and pioglitazone inhibit HCC development in experimental model; Insulin increases risk of HCC	Should be preferred; May be consider; Should be avoided

TZD: Thiazolidinedione; SU: Sulfonylurea; GLP-1: Glucagon-like peptide-1; DPP-4: Dipeptidyl peptidase 4; AGI: Alpha-glucosidase inhibitors; SGLT2: Sodium glucose co-transporter-2; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; AFP: Alfa-fetoprotein.

maintain glycemic control without increasing the risk of hypoglycemia. Insulin use has also been associated with HCC in LC patients[133]. Hence, it should be avoided in patients who are at high risk of developing HCC, such as those with dysplastic liver nodules and elevated serum alpha fetoprotein levels. In a recent study, insulin use in LC patients with diabetes was found to be associated with increased risks of hypoglycemia, cardiovascular events, liver-related complications, and mortality compared to insulin nonusers[134]. Given these considerations, insulin cannot be regarded an optimal anti-diabetic treatment for LC patients, and the search for a better alternative should be prioritized.

LT

Finally, HD should be reversible after LT because it is caused by LC. There have been reports of HD reversibility with LT, however this does not occur in all patients[135]. In one study, DM regressed in 63.9% of patients after LT, while DM never regressed in 36% of patients after two years of follow-up. The reversibility of HD appears to be determined by the level of pre-LT pancreatic ß-cell injury and its improvement after LT. Grancini *et al*[50] found that improved β-cell function plays a major role in favoring diabetes regression following LT, in the presence of a sustained improvement of IR. With progression of LC, progressive accumulation of toxic materials (AGEs, HIF, *etc*.) may lead to severe non-repairable ß-cells injury, making the chances of HD reversibility less likely. The diabetogenic potential of immunosuppressive therapies could also be one of the reasons behind non-reversibility of diabetes following LT.

CONCLUSION

In conclusion, the evidence suggests that patients with LC can have two forms of diabetes: T2DM and HD, with HD appearing to be the predominant type. HD is a direct complication of LC since it is strongly linked to the pathophysiological alterations and severity of LC. However, HD is still an underappreciated problem that isn't even recognized as a separate entity by scientific organizations. To maintain consistency in clinical research, future directions will first require recognition of HD as a distinct entity, followed by the creation of a consensus definition for HD. Understanding the complex pathophysiology of LC leading to HD, including changes in the liver-multiorgan cross-talk, will also be critical for providing evidence-based management recommendations.

FOOTNOTES

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REVIEW

Small extracellular vesicles and liver diseases: From diagnosis to therapy

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Abstract

Extracellular vesicles (EVs), especially small EVs (sEVs) derived from liver cells, have been the focus of much attention in the normal physiology and pathogenesis of various diseases affecting the liver. sEVs are approximately 100 nm in size, enclosed within lipid bilayers, and are very stable. The lipids, proteins, and nucleic acids, including miRNAs, contained within these vesicles are known to play important roles in intercellular communication. This mini-review summarizes the application of sEVs. First, liver diseases and the related diagnostic markers are described, and the current active status of miRNA research in diagnosis of hepatocellular carcinoma (HCC) is reported. Second, the biodistribution and pharmacokinetics of sEVs are described, and the liver is highlighted as the organ with the highest accumulation of sEVs. Third, the relationship between sEVs and the pathogenesis of liver disorders is described with emphesis on the current active status of miRNA research in HCC recurrence and survival. Finally, the possibility of future therapy using sEVs from mesenchymal stem (stromal) cells for cirrhosis and other diseases is described.

Key Words: Small extracellular vesicles; Liver; cirrhosis; Hepatocellular carcinoma; Mesenchymal stem cells

Core Tip: Extracellular vesicles (EVs), especially small EVs (sEVs) derived from liver cells, have been the focus of much attention in the normal physiology and pathogenesis of various diseases affecting the liver. sEVs are approximately 100 nm in size, enclosed within lipid bilayers, and are very stable. The proteins and nucleic acids, including miRNAs, contained within these vesicles are known to play important roles in intercellular communication. This mini-review summarizes the application of sEVs in the diagnosis of liver diseases, along with their distribution post administration, their role in pathogenesis, and their potential therapeutic effects in hepatic disorders.

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INTRODUCTION

The study of extracellular vesicles (EVs) is an active area of research. Recent evidence has established that these EVs are released not only by human cells, but also by plant, bacterial, and yeast cells[1]. EVs are sub-organellar entities that act as "cargo" carriers that transmit information between cells thus exerting a variety of effects on biological activities[1]. Since the vesicles released are unique to the cells that release them, they have been widely studied in diseases of various organs and systems, including diseases of the liver in the context of diagnosis, pathogenesis, and therapeutic applications[2-5]. In particular, small extracellular vesicles (sEVs; referred to as exosomes), with a particle size of approximately 100 nm, have garnered much attention in recent years[6-11].

Secretory vesicles were first described in the 1980s, and they have been referred to by a number of different names based on their size and cellular origin such as exosomes, ectosomes, microvesicles, shedding vesicles, apoptotic bodies, oncosomes, and prostasomes. The International Society for Extracellular Vesicles recommends the usage of extracellular vesicles as a general term for these entities. Small EVs (sEVs), or exosomes, are formed from early endosomes that are generated by endocytosis and subsequently mature into late endosomes[12,13].

The late endosomes expand to form intraluminal membrane vesicles, also referred to as multivesicular bodies (MVBs), which then fuse with the plasma membrane and are released into the extracellular space. Secreted vesicles with a diameter of 30–200 nm are called sEVs or exosomes, and they are known to encapsulate a content of proteins, mRNAs, and miRNAs within a membrane composed of cholesterol, sphingomyelin, ceramide, and lipid rafts[13]. Although these vesicles vary between cells, there are common markers that characterize most exosomes including membrane transport and fusion proteins (GTPases, annexins, flotillin, *etc.*), heat shock proteins (HSP60, HSP70, HSP90, *etc.*), tetraspanins (CD9, CD63, CD81, *etc.*), MVB formation and transport proteins (TSG101, ALIX, Annexins, *etc.*), and cytoskeletal proteins (actin, tubulin, *etc.*)[11-13]. sEVs can be have important applications in the diagnosis and treamtent of various diseases and malignancies, and their study can also contribute to the ellucidation of the pathogenesis of these disease. For example, the stable inclusion of drugs within the lipid bilayers of sEVs creates novel therapeutic drug delivery systems that can be implied in the treatment of different diseases[13] (Figure 1).

Investigations on EVs are rapidly moving beyond basic research to clinical trials, and the global market of the diagnostic and treatment strategies that use sEVs, although still in its infancy, is expected to progress rapidly. This paper reviews the role of sEVs in the context of diagnosis, pathogenesis, and treatment of liver diseases.

sEVs AND DIAGNOSIS OF LIVER DISEASES

Although different types of EVs were studied in the context of liver disorders, in this report we focus mainly on the role sEVs in the pathogenesis, diagnosis, and treatment of liver diseases. sEVs in particular have been analyzed in various chronic liver diseases such as non-alcoholic steatohepatitis (NASH), alcoholic liver disease (ALD), viral hepatitis caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), cirrhosis, acute liver disease, and hepatocellular carcinoma (HCC); and in various specimens including blood, urine, bile, and ascitic fluid[5]. There are various techniques that were employed in the collection ofsEVs including ultracentrifugation, size exclusion chromatography, and methods utilizing precipitation kits and bead kits[14]. Details of sEV collection have been described in the minimal information for studies of extracellular vesicles guidelines 2018 (MISEV2018)[13]. After their collection, sEVs have been evaluated by western blotting, ELISA, flow cytometry, and nano

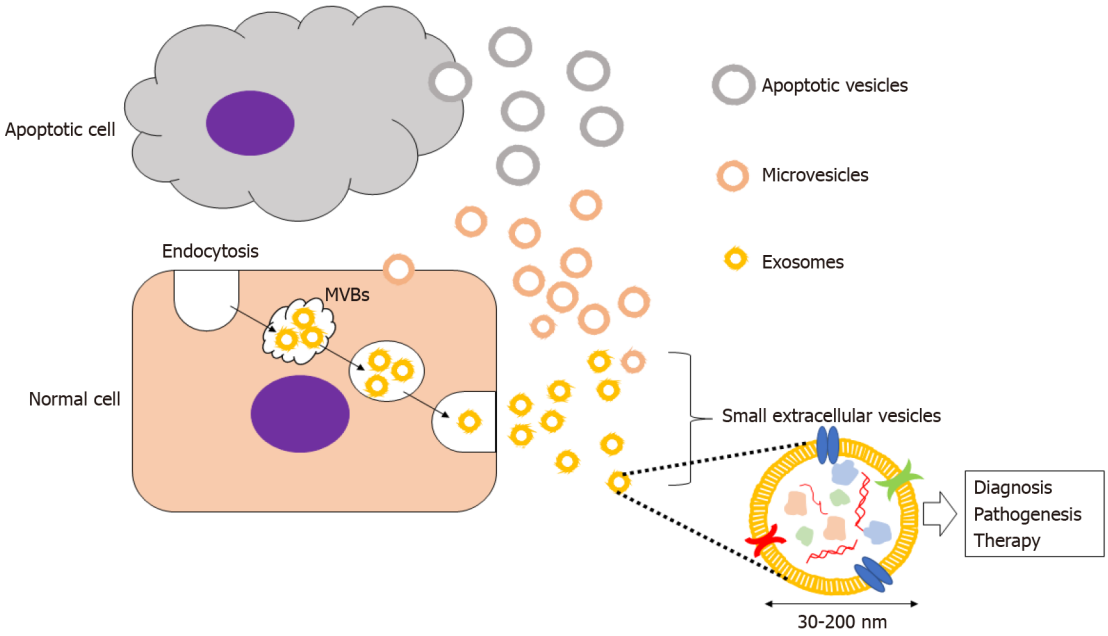

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Figure 1 Extracellular vesicles and liver diseases. Extracellular vesicles (EVs) include apoptotic vesicles, microvesicles, and exosomes. Small EVs (sEVs), or exosomes, are formed from early endosomes that are generated by endocytosis and subsequently mature into late endosomes. The late endosomes expand to form intraluminal membrane vesicles, also referred to as multivesicular bodies, which fuse with the plasma membrane and are released into extracellular space. These sEVs, or exosomes, are analyzed for diagnosis, pathogenesis, and therapy of various diseases including liver diseases.

tracking analysis to study the expression of common sEVs proteins, such as tetraspanins, and to identify markers including lipids, proteins, and nucleic acids, such as miRNAs and lincRNAs. Although the aforementioned studies are in the pre-clinical stage, they are expected to yield specific markers that can aid the processes of early and definitive diagnosis, treatment, and follow-up of liver diseases, in addition to helping in the ellucidation of the pathophysiology governing many of these liver disorders. Table 1[15-71] summarizes the various liver diseases and their related sEVs diagnostic markers. The bulk of the studies reported on miRNAs as sEVs diagnostic biomarkers of liver diseases which may be due to the ease of evaluating them using qRT-PCR. Markers of HCC have been the most frequently analyzed, and diseases such as NASH and ALD have received the most attention in recent years. Extracting sEVs produced by target cells and using them as markers of disease can contribute greatly to the field of diagnosis and treatment of liver disorders. However, we believe that there are some limitations and challenges to be acknowledged and addressed in the future, such as the efficient collection of target sEVs, recognition of target molecules (*e.g.*, protein miRNA), cost, and high reproducibility.

BIODISTRIBUTION AND PHARMACOKINETICS OF sEVs

Numerous studies have demonstrated the importance of the liver in the biodistribution and pharmacokinetics of sEVs. This has been accomplished by employing techniques such as lipophilic fluorescent and luminescent, radio-labeling, and magnetic resonance imaging. Studies have conclusively shown that post systemic administration of sEVs, these vesicles are cleared from the bloodstream within a few minutes of their half-life *via* phagocytosis by macrophages and neutrophils[72]. While they disappear from the blood, they have been reported to persist longer within organs, with the largest accumulation occurring in the liver. This accumulation peaks in the liver and kidneys approximately 1 h post administration, which is earlier than that in the lungs where maximal accumulation is achieved 2–12 h post administration. It has been shown that high concentrations of sEVs can be maintained in the liver for about 12-24 h, although there have been contradictory reports about this[72,73]. Some studies suggest that the macrophages primarily take up scaffold in the liver, while others report that hepatocytes and other cells also do the same. The abundant expression of scavenger receptors in macrophages is thought to play a crucial role in this process[73]. Additionally, phosphatidyl serine (PS) has been found to easily accumulate in the liver unlike the phosphatidylcholine-rich lipids[73]. Hoshino *et al*[74] have demonstrated the importance of integrins by showing that integrin αvβ5 in sEVs is essential for its accumulation in macrophages. However, results from these studies must be interpreted with caution since most of them employed the technique of labeling lipid bilayers, which may have resulted in the

Table 1 Diagnostic small extracellular vesicles markers in relation to liver diseases

Diseases	Types of molecules	Markers	Refs.
HCC	Protein	Aminopeptidase N, Galectin-3-binding protein, SMAD, ANGPT2, 14-3-3 ζ, β-catenin, P120-catenin, EPCAM	[15–21]
	RNA	miR-21, miR-21, -96, miR-122, miR-18a, -221, -222, -224, miR-10b-5p, -215-5p, miR-101, -106b, 12, -195, miR-519d, -595, -939, miR-19b,-92, miR-125, miR-9-3, miR-122, 148a, -1246, miR-122, miR-93, miR 144-3p, -21-5p, miR210, miR-638, miR-665, miR-774, miR-1262, miR-320d, miR-23a/b, miR-45-1a, miR-224, miR-21, -10b, miR-122, -125b, -145, -192, -194, 29a, 17-5p, -106a, miR-26a, -29c, -21, lncRNA Jpx, lncRNA FAL1, lncRNA-RP11-513I15.6, mRNA RAB11A, miR-1262, lncRNA HEIH, lncRNA LINC00161, lnc RNA HULC, AFP mRNA	[22–50]
HBV	miRNA	miR-21-5p	[33]
HCV	RNA	HCV-RNA, miR-122-5p, -222-3p, -146-5p, -150-5p, -30c-5p, -378a-3p, -20a-5p,	[51–53]
NAFLD/NASH	Protein	ITGβ1, CD68	[54,55]
	RNA	miR-192-5p	[56]
	Lipid	ceramides and sphingosin 1-phosphate	[57]
ALD	Protein	ASGR2 and CYP2E1, CD163, 206, ASGPR, CD40L, CK18, Glutathione synthetase	[55,58–62]
	RNA	miR-122, -155, miR-Let-7f, 29a, -340, miR-122, let7f, -21, -29a, -146a, miR-192-5p, miR-192, -30a	[56,63–66]
	Lipid	Sphingosin 1-phosphate	[67]
Cirrhosis	Protein	CD163, 206, PDGFRβ, urinary maltase and glucoamylase (for AKI during cirrhosis)	[59,68–70]
	RNA	miR-19a, -19b, -92, 17a, -20a	[27]
ALI	Protein	Apolipoprotein A-1, Argininosuccinate synthase-1	[62]
	RNA	Gnb21 mRNA,	[71]

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; ALD: Alcoholic liver disease; ALI: Acute liver injury; RNA: Ribonucleic acid; SMAD: Suppressor of Mothers against Decapentaplegic; ANGPT: Angiopoietin; EPCAM: Epithelial cell adhesion molecule; lnc: Long non-coding; FAL1: Focally amplified lncRNA on chromosome 1; HEIH: High expression in hepatocellular carcinoma; HULC: Highly up-regulated in liver cancer; LINC: Long intergenic noncoding; AFP: α-fetoprotein; ITG: Integrin; CD: Cluster of differentiation; ASGR: Asialoglycoprotein receptor; CYP: Cytochrome P450; ASGPR: Asialoglycoprotein receptor; CK: Cytokeratin; PDGFR: Platelet-derived growth factor; AKI: Acute kidney injury; Gnb: Guanine nucleotide binding protein.

visualization of cells that ingested phospholipids rather than the sEVs. Given their miniscule size, sEVs by themselves have never been directly visualized in isolation. Furthermore, the majority of these reports have made observations under conditions of normal physiology, so it is possible that the biodistribution and pharmacokinetics of sEVs in pathological conditions may be significantly different.

sEVs AND LIVER PATHOGENESIS

Many reports described the implication of sEVs in various aspects of the pathogenisis of liver diseases. These entities are highly stable *in vivo* and play an important role in the communication between both neighboring and distant cells. Table 2[75-99] summarizes the different sEV markers that have been linked to certain processes of liver pathogenesis.

sEVs exert their effect on inter-cellular communication between neighboring cells *via* the peri-sinusoidal space. For instance, it has been reported that sEVs secreted by HCV-infected hepatocytes exert an effect on hepatic stellate cells (HSCs) driving hepatic fibrosis[100]. Additionally, as shown in Table 2, sEVs produced by hepatocellular carcinoma (HCC) have a profound effect on the surrounding environment. This effect is mediated by the modulation of the immune system by sEVs that have an inhibitory effect on macrophages, monocytes, NK cells, B cells, and T cells[101]. These vesicles can also promote HSC and the transformation of fibroblasts to cancer-associated fibroblasts (CAFs), promote migration of hepatocellular carcinoma cells in the vicinity, act on vascular endothelial cells to promote angiogenesis, and induce drug resistance in surrounding cancer cells[101]. Additionally, sEVs released from hepatocytes are believed to function as drivers of inflammation and state formation in inflammatory cells such as macrophages in NASH[102].

Table 2 Small extracellular vesicles markers in relation to liver pathogenesis

Pathophysiology	Types of molecules	Markers	Refs.
NASH inflammation and fibrosis	miRNA	miR-122, -192	[75]
NASH fibrosis	miRNA	miR-122	[76]
ALD outcome	protein	ASGR2 and CYP2E1	[58]
HBV fibrosis	miRNA	miR-150, -192, -200b, -91a	[77]
HCV treatment response	miRNA	miR-122, -199a, miR-122, miR-122-5p, -222-3p, -146-5p, -150-5p, -30c-5p, -378a-3p, -20a-5p	[53,78,79]
HCV fibrosis	protein	CD81	[80]
	miRNA	Let-7s, miR-122, -150, -192, 200b, 92a, miR-19a	[77,81]
HCC recurrence	protein	SMAD3, CASC9	[16,82]
	RNA	miR-718, miR-125, miR-21, miR-103, miR1247-3p, miR-92b, miR-21 and lncRNA-ATB, miR-21, -10b, miR-215-5p, miR-155, mRNA RAB11A, miR-211-3p, -6826-3p, -1236-3p, 4448	[25,26,36,42,83–90]
HCC survival	RNA	miR-125, miR-21, miR-103, miR-22a-3p, miR-335, miR-25-5p, miR-320a-PBX3, miR-718, miR-210, miR-122, miR-93, miR-21, -96, -122, miR-1247-3p, miR-638, miR-665, miR-21 and lncRNA-ATB, miR-30d, -140, miR-106a, miR-224, miR-320d, long non-coding RNA (ENSG00000258332.1 and LINC00635), hnRNPH1, circPTGR1, circRNA-100, -338, circ DB	[18,23,31,32,34,36,38,41,43,82–86,88,91–99]

NASH: Nonalcoholic steatohepatitis; ALD: Alcoholic liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; RNA: Ribonucleic acid; ASGR: Asialoglycoprotein receptor; CYP: Cytochrome P450; CD: Cluster of differentiation; SMAD: Suppressor of Mothers against Decapentaplegic; CASC: Cancer Susceptibility; PBX: Pre-B cell leukemia homeobox; lnc: long non-coding; ATB: Activated By TGF-Beta; ENSG: Ensembl Gene ID; LINC: Long intergenic noncoding; RNPH: Ribonucleoprotein H; PTGR: Prostaglandin Reductase; DB: Deubiquitination.

It has been also reported that these vesicles drive the pathogenesis of disease through an effect on distant cells. This is exemplified by the crosstalk between the sEVs produced by adipocytes and those produced by hepatocellular carcinoma cells, which contributes to cell proliferation, angiogenesis, invasion, epithelial-mesenchymal transition, and the creation of a favorable environment for metastasis [103]. Recent reports have also demonstrated that microbiota-derived EVs in the intestine affect other organs and tissues in the body, including the liver, heart, brain, kidney, lung, and adipose tissue[104]. Though there have been some studies describing the effects of sEVs on various cells in the body, it is still unclear how these sEVs that are produced by specific cells selectively reach their target cells. Consequently, further analysis from a broader perspective is essential to describe the specificities and dynamics of this interaction between sEVs and their target cells.

sEVs AND THE TREATMENT OF LIVER DISEASES

To date, there have been no reports or ongoing trials on the application of sEVs in the treatment of liver diseases. This scarcity might be attributed to the fact that there are still many unknowns regarding the effects of sEVs on liver disease, but further mechanistic analysis in the future may lead to the development of new therapies. However, the potential of sEVs as anti-fibrotic and anti-cancer therapeutic agents needs to be explored. In the case of anti-fibrotic therapy, sEVs may be the most convenient therapeutic agents that target macrophages on account of their massive accumulation in these cells within the liver[5,105]. Furthermore, Mesenchymal stem (stromal) cells (MSCs) have been investigated for their anti-fibrotic properties due to their ability to suppress fibrogenesis by reducing the inflammatory responses of inflammatory cells and by inducing fibrolysis *via* their effect on macrophages and matrix metalloproteinases[106].

Basic research has recently revealed that sEVs secreted by MSCs transmit information to macrophages. Additionally, the potency of these sEVs has been enhanced by pre-conditioning the MSCs with IFN-γ to augment their therapeutic effects in a mouse model of liver cirrhosis[105]. In view of this, it might be possible to create and evaluate a therapeutic strategy that employs sEVs obtained from pre-conditioned or modified MSCs to transmit information to macrophages and exert anti-fibrotic effects suppressing fibrogenesis. Although such attempts have been made, the production of sEVs from preconditioned/modified MSCs has not yet been successful due to regulatory concerns, lack of appropriate quality control, and difficulties associated with mass purification.

Warnecke *et al*[107] reported a first-in-human case study that utilized MSC-EVs derived from umbilical cord tissues to reduce inflammation during cochlear implantation. Briefly, the authors obtained 1.03 × 10^{11} particles/mL of EVs with a diameter range of 110–130 nm, as measured by nanoparticle tracking analysis, *via* a combination of tangential flow filtration (TFF) and diafiltration techniques post culture. This showed that MSCs-derived exosomes may find successful application in clinical use. Furthermore, improvements in the methods that can reduce the quantity of exosomes needed for efficient treatment will further expedite their use in clinical scenarios[107].

The development of cancer therapies that employ sEVs are also theoretically possible, such as those that aim to suppress sEVs derived from cancer cells. Mendt *et al*[108] have developed a therapeutic strategy for pancreatic cancer using sEVs that is currently under clinical trials. Their study involved the optimization of iExosomes to enable them to deliver higher concentrations of sEVs to pancreatic cancers *via* a two-pronged strategy. This includes the selection of CD47 that protects exosomes from phagocytosis by macrophages and engineering exosomes to carry siRNA or shRNA specifically targeted against the oncogenic KRASG12D, the key driver of pancreatic cancer. The study also reported the feasibility of large-scale production of clinical grade iExosomes by a bioreactor-based methodology[108, 109]. Therefore, sEVs have a promising potential in anti-fibrotic and anti-cancer therapy, and their applications may be expanded by developing techniques that efficiently load therapy enhancing substances, aid their incorporation into target cells, and improve high-throughput collection methodologies.

CONCLUSION

In summary, small extracellular vesicles (sEVs) have a promising potential in the diagnosis and treatment of liver diseases. The challenge in the therapeutic uses of sEVs is that it is not easy to harvest large amout of sEVs for human systemic therapy. However, the collection of sEVs can be greatly enhanced by pre-conditioning or modifying the source cells, thereby greatly expanding their possible applications. Furthermore, the potential for using EVs in therapy may be enhanced by utilizing larger EVs in addition to sEVs. These vesicles can potentially be harvested from cell sources other than mesenchymal stem cells (MSCs), such as induced pluripotent stem cells (iPS cells). In addition, with using these larger EVs there are fewer risk of embolization especially to the lungs after the administration. Consequently, in spite of issues with future regulatory trends and establishment of manufacturing processes, sEVs remain a promising therapeutic option for liver ailments.

FOOTNOTES

Author contributions: Tsuchiya A collected and analyzed the data and wrote the manuscript; Natsui K, Ishii Y, Koseki Y, Takeda N, Tomiyoshi K, Yamazaki F, and Yoshida Y collected the data; Terai S supervised the manuscript; and All authors reviewed the manuscript.

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REVIEW

Hepatocellular carcinoma and microbiota: Implications for clinical management and treatment

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Abstract

Gut microbiota plays an essential role in host homeostasis. It is involved in several physiological processes such as nutrients digestion and absorption, maintenance of intestinal epithelial barrier integrity and immune system self-tolerance. Especially the gut microbiota is assumed to play a crucial role in many gastro-intestinal, pancreatic and liver disorders. Its role in hepatic carcinogenesis is also gaining increasing interest, especially regarding the development of therapeutic strategies. Different studies are highlighting a link between some bacterial strains and liver disease, including hepatocellular carcinoma (HCC). Indeed, HCC represents an interesting field of research in this perspective, due to the gut-liver axis, to the implication of microbiota in the immune system and to the increasing number of immunotherapy agents investigated in this tumour. Thus, the assessment of the role of microbiota in influencing clinical outcome for patients treated with these drugs is becoming of increasing importance. Our review aims to give an overview on the relationship between microbiota and HCC development/progression and treatment. We focus on potential implications on the available treatment strategies and those under study in the various stages of disease. We highlight the pathogenic mechanisms and investigate the underlying molecular pathways involved. Moreover, we investigate the potential prognostic and/or predictive role of microbiota for target therapies, immune checkpoint inhibitors and loco-regional treatment. Finally, given the limitation of current treatments, we analyze the gut microbiota-mediated therapies and its potential options for HCC treatment focusing on fecal microbiota transplantation.

Key Words: Hepatocellular carcinoma; Gut microbiota; Gut–liver axis; Fecal microbiota transplantation; Carcinogenesis; Immune checkpoint inhibitors

Core Tip: The gut-liver axis plays an important role in the pathogenesis of liver diseases, including hepatocellular carcinoma (HCC). Growing evidence has supported the role of the gut microbiota in the development of HCC and as a prognostic and predictive factor. Thus, manipulation of the gut microbiota might represent a novel way to treat or prevent HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive malignancy and almost exclusively develops in patients with chronic liver disease and cirrhosis. While viral hepatitis, especially hepatitis B virus (HBV) infection and hepatitis C virus (HCV) infection represent one ofe the most important cause of cirrhosis and HCC in low-income countries and Asia, alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) are the main cause for developing cirrhosis and HCC in high income countries. The pathogenesis of HCC is multi factorial, driven by a circle of liver injury, inflammation, and regeneration that typically spans decades. Next to predisposing factors, as already mentioned, increasing evidence points towards a key role of the bacterial microbiome and bacterial metabolites in the development of chronic liver disease (CLD).

The human gut is one of the most complex structures in the body and is colonized by trillions of microorganisms including bacteria, fungi, viruses and protists. Among them, bacteria are the main inhabitants[1]. In recent years there has been increasing attention to the possible relationship between the gut microbiota and the process of carcinogenesis. Increasing data have suggested that the gut microbiota is related to a variety of cancers, particularly of the gastrointestinal tract, and consequently it has been hypothesized that this link may lead to the development of targeted therapies against the gut microbiome[2,3].

Among the various types of cancer, hepatocellular carcinoma is also included. As is known, the liver does not contain a microbiome, but is closely connected to the gut *via* the portal venous system, constituting the intestine – microbiota – liver axis[4].

The balance of the intestinal microbiota is essential for a physiological and correct functioning of the metabolism and immunity and, even more importantly, of the intestinal barrier[5]. In fact, homeostasis between the host and the microbiota is maintained precisely by the multilayered intestinal barrier. A disruption in this balance can lead to a malfunction of the intestinal barrier, resulting in chronic inflammation and dysbiosis. Although the mechanisms by which the microbiota is related to cancer are not yet fully understood, the above two are key factors in the carcinogenesis process[6] and several studies have observed significant alterations in the composition of gut microbiota in patients with chronic liver disease especially with a reduction in beneficial bacteria and an increase in pathogenic bacteria[7]. For this reason, numerous studies have investigated the potential use of therapies targeting the microbiota, such as prebiotics, probiotics and fecal microbiota transplantation (FMT). In particular, their potential role in the treatment of different liver diseases (such as hepatic encephalopathy, steatohepatitis and cirrhosis) and in different types of cancer (such as gastrointestinal cancers, breast cancer and melanoma) has been studied[8].

Indeed, in recent years, researchers have focused their attention on the possible prognostic and predictive role of gut microbiota composition, in particular in the response to therapy with immune checkpoint inhibitors (ICIs). It seems that a variation in the composition of the gut microbiota can influence the efficacy of treatments, in particular of immunotherapy, and the presence of specific gut microbes increases this efficacy[9].

IMPACT OF MICROBIOTA IN HCC DEVELOPMENT

Microbiome role in the development and growth of neoplastic lesions has assumed ever greater interest in recent studies. Human microbiota, defined as the population of microorganisms that colonize the body, has in fact been shown to play a crucial role both in physiological and pathological mechanisms. In addition to bacteria, the gut microbiota also contains eukaryotes as fungi, and some types of viruses.

Bacterial gut microbiota promotes disease development and progression not only locally, such as inflammatory bowel disease (IBD)[10,11], but also in distant locations such as the brain, heart, hematopoietic system and liver[12-16].

Given its direct anatomical connection through the portal vein, the liver is closely connected to the intestine. This is called "intestine-microbiota-liver axis"[6,17]. In fact, the liver receives blood rich in nutrients absorbed by the intestine, but it is also the first "filter" organ for the intestinal microbiota, of the MAMP (microbe-associated molecular pattern), toxins and bacterial metabolites. These products can subsequently trigger inflammatory responses *via* pattern recognition receptors.

Damage to the intestinal barrier, associated with alterations of the intestinal microbiota in CLD contribute to the onset of chronic inflammation. The inflammation, in turn, progresses, leading to tissue reworking with fibrosis. The processes of rehashing on an inflammatory basis increase the risk of developing HCC as the last step of the entire pathological process[7,18-20].

Bile acids represent another factor that acts in this complex system. In fact, these have the function of regulating the intestinal epithelial barrier, the proliferation of epithelial cells of the mucosa *via* the farnesoid X-activated receptor - dependent and the epidermal growth factor receptor - dependent pathways and controlling the growth and adhesion of intestinal bacteria[21,22].

At the hepatic level, bacterial products activate toll-like receptors (TLRs), in particular TLR-4, which, in turn, activates the NF-kB pathway, which determines the constitutive initiation of a mitogenic signal that is associated with an inhibition of programmed cell death. Chronic damage exposes the liver to a prolonged action of several TLR ligands and other bacterial substances, which represent inflammatory mediators that promote the development of chronic liver disease as well as laying the foundations for the subsequent development of hepatocellular cancer[20] (Figure 1).

Most cases of HCC develop on a basis of fibrosis and cirrhosis, which is the most important risk factor for the development of liver cancer. However, the presence of underlying liver diseases of various etiologies may contribute to the increased specific risk for developing HCC on a cirrhotic basis[23] (Table 1).

NAFLD

Although the percentage of NAFLD patients who develop HCC is small, the high incidence carries a high risk of developing hepatocellular carcinoma. Several studies in animal models have shown that the microbiome of obese patients has the ability to extract more nutrients, and in mice deprived of bacterial flora there was a reduction in body weight despite an increased caloric intake[24,25].

In dysbiotic mice fed a high-fat diet, choline is converted to methylamine, which involves a reduction in circulating plasma levels of phosphatidilcholine. Subsequently, low phosphatidylcholine levels lead to impaired secretion of VLDL, reducing hepatic lipid export, inducing hepatic steatosis[26,27].

The contribution of gut microbiota to non alcoholic steatosis hepatitis, a progressive form of NAFLD, is not as well documented as its role in earlier disease stages. An high-fat diet (HFD) increases intestinal permeability in mice with a noticeable increase in LPS serum levels[28,29].

ALD

About half of the cases of cirrhosis are caused by alcohol consumption. Indeed, serum LPS levels are increased in patients who have made chronic use of alcohol. Ethanol and its metabolite acetaldehyde have the ability to interrupt the intercellular junctions in the intestine, allowing bacteria and their products to pass into the bloodstream[30].

The microbiota-TLR4 axis plays an important role in this process. In fact, in several studies conducted on animal models, TLR4 deprived mice, subjected to intestinal disinfection, have shown a reduction in inflammation and oxidative stress[31-33].

Liver fibrosis

Fibrosis represents a risk factor for the development of HCC. Literature data highlight an important contribution of the microbiota-TLR4 axis to liver fibrosis[34].

Studies in knockout mice have shown a key role for TLR4 and other mediators in the TLR4 signaling pathway, such as CD14 and lipopolysaccharide binding protein, in experimental models of hepatic fibrosis.

Conversely, other studies have shown a protective role of bacterial flora against the development of liver inflammation and fibrosis[35,36].

Evidence supports the role of the commensal microbiota as a hepatoprotective, although an alteration in its internal balance can lead to the prevalence of harmful species that can cause liver damage.

Viral Hepatitis

There is currently little data on the role of the microbiota in chronic viral hepatitis at the moment. Current data suggests that dysbiosis in patients with viral hepatitis cirrhosis is similar to that in patients with cirrhosis from other causes[37]. Further studies will be needed to investigate the close interconnections between microbiome composition and tumor development and growth.

Table 1 Human studies involving gut microbial composition in various hepatocellular carcinoma-related etiologies

		Microbiota balance	
Ponziani *et al* [38]	Cirrhosis + HCC	↑ *Bacteroides*; ↑ *Ruminococcus*; ↑ *Enterobacteriaceae*	↓ *Bifidobacterium*; ↓ *Akkermansia*
Ren *et al*[41]	HBV cirrhosis + HCC	↑ *Actinobacteria*	↓ *Verrucomicrobia*
Liu *et al*[97]	NBNC cirrhosis + HCC	↑ *Escherichia*; ↑ *Enterococcus*	↓ *Faecalibacterium*;↓ *Ruminococcus*;↓ *Rumino-clostridium*
Huang *et al*[39]	HBV cirrhosis + HCC	↑ *Bacteroides*; ↑ *Lachnospiracea incertae sedis*; ↑ *Clostridium XIVa*	

↑: Increased; ↓: Decreased; NBNC: Non-hepatitis B virus non- hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

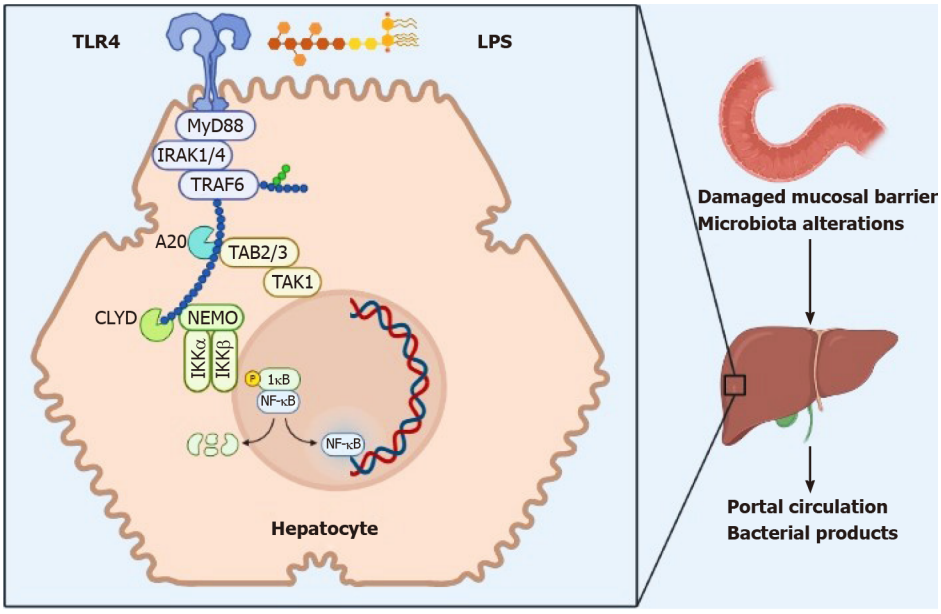

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Figure 1 Interaction between Bacterial Lipopolysaccharides and Toll-like receptor 4 signaling pathway in hepatocellular carcinoma development. LPS: Lipopolysaccharides; MyD88: Myeloid differentiation primary response protein 88; IRAK1/4: IL-1 receptor associated kinase 1/4; TRAF6: TNF receptor associated factor 6; TAK1: TGF-activated kinase 1; TAB2/3: TAK1-binding protein 2/3; NEMO: NF-kappa-B essential modulator; IKB: Inhibitor of nuclear factor kappa-B; NF-κB: Nuclear factor kappa B.

PREDICTIVE AND PROGNOSTIC ROLE OF MICROBIOTA

Given the important role of the gut microbiota in liver carcinogenesis, growing attention towards using microbiome patterns as a predictive, prognostic or diagnostic biomarker of HCC is observed. The gut microbiome dysbiosis has been evaluated in NAFLD- and HBV/HCV-related HCC patients to find new clinical features and outcomes biomarkers in this setting[38,39]. In 2019, Ponziani *et al*[38] found that cirrhotic patients with NAFLD and HCC lack protective bacteria and have an enhanced intestinal inflammation with an increased level of IL8, IL13, CCL3, CCL4, CCL5, and faecal calprotectin concentration. HCC was associated with increased abundance of Bacteroidetes together with a reduction of Verrucomicrobiaceae, Bifidobacteriaceae, Akkermansia, Bifidobacterium.

In a more recent study, HCC tumour burden was associated with the presence of specific gut microbes, distinguished by the enrichment of Bacteroides, Lachnospiracea incertae sedis, and Clostridium XIVa. Patients with these three genera mounted a weaker host liver anti-tumour inflammatory response[39]. Primary bile acids increased CXCL16 expression, which regulates NK cell accumulation, whereas secondary bile acids showed the opposite effect. Feeding secondary bile acids or colonization of bile acid-metabolizing bacteria reversed both NK cell accumulation and inhibition of liver tumour growth in mice with altered gut commensal bacteria. Removing gram-positive bacteria by antibiotic treatment with vancomycin, which contains the bacteria mediating primary-to-secondary bile acid conversion, induced hepatic NK cell accumulation and decreased liver tumour growth in mice. These data suggest that Clostridium XIVa influences bile acid-controlled NK cell accumulation. In normal liver tissue from human HCC patients, primary bile acid cheno-deoxycholic acid levels

correlated with CXCL16 expression, whereas an inverse correlation was observed with secondary bile acid glycolithocholate[40]. Higher bile acid levels (≥ 16 μmol/L) indicated worse clinical outcomes among HBV-related HCC patients with enrichment of Bacteroides, Lachnospiracea incertae sedis, and Clostridium XIVa. These results show that correlation between gut microbiota and serum bile acids in tumour immune microenvironment could potentially influence tumour burden and clinical outcomes in HBV-related HCC[40].

In 2019, Ren *et al*[41] evaluated the potential role of microbiome as a non-invasive biomarker for HCC. The authors collected 486 faecal samples from East, Central, and Northwest China. Using 16S rRNA Miseq sequencing, 3 groups were identified: early HCC, cirrhosis, and healthy controls. Actinobacteria, 13 genera including Gemmiger and Parabacteroides were increased in HCC compared with cirrhosis. Additionally, butyrate-producing genera was decreased, and lipopolysaccharide-producing was increased in HCC in comparison to healthy controls. Interestingly, 30 microbial markers were identified through a fivefold cross-validation on a random forest model between 75 early HCC and 105 non-HCC samples. This was the first study characterizing the gut microbiome in early HCC as a non-invasive tool to diagnosed early stage of HCC.

In literature, three studies analysed the potential predictive and prognostic role of gut microbiota in HCC.

Zheng *et al*[42] analyzed the characteristics and changes in the gut microbiota during treatment with anti-PD-1 immunotherapy drugs in eight patients with HCC. Responders (R) had, during the entire treatment, a higher richness of taxa and a greater number of genes than the no-responder (NR). Before the start of treatment, Bacteroidetes was the most abundant phylum, followed by Firmicutes and Proteobacteria in both R and NR[41]. As treatment progressed, the microbial composition at the phylum level in R remained relatively stable, while proteobacteria often increased in NR, with a prevalence of Escherichia coli. Furthermore, the methanogenesis pathway was found to be correlated with R. obeum and Lactobacillus species, and furthermore the generated methane would appear to improve oxidative stress damage and suppress host inflammatory response. Other pathways with potential benefits include sulfate reduction and carbon fixing functions that were correlated with R. obeum, carotenoid biosynthesis correlated with B. cellulosilyticus and A. colihominis, and unsaturated fatty acid metabolism associated with C. comes[42].

Li *et al*[9] collected microbiome samples from 65 patients with metastatic HCC being treated with ICI therapy. The analyzes showed that patients with a high presence of Faecalibacterium had a significantly prolonged PFS compared to those with low presence (P = 0.006). In contrast, patients with a high presence of Bacteroidales had a reduced PFS compared with those with low presence (P = 0.002).

Chung *et al*[43] studied the effects of the gut microbiota in eight adult patients with HCC treated with nivolumab (anti PD-1). Reported data showed that responder patients had a significantly lower Firmicutes/Batteroidetes ratio (10% *vs* 66.7%, P <0.05) and a higher Prevotella/Batteroides ratio (22.99 *vs* 2.312, P = 0.024) than non-responders. These results indicate that the F/B ratio and P/B ratio could serve as predictive markers of non-response to nivolumab therapy. Akkermansia species was also found in two responders, indicating that this could also be a useful prognostic marker of response to nivolumab therapy in patients with advanced HCC.

In the last decade, several research efforts have been made to identify potential prognostic and predictive biomarkers of response with target therapy[44-46]. In this perspective, interest in interaction between the gut microbiome and HCC targeted therapy is increasing, even if no data are available up to date. It has been shown that among anthocyanins, delphinidin possesses strong antitumor activity through various mechanisms such as downregulation of matrix metalloproteinase (MMP)[47], inhibition of angiogenesis and tumour cell migration[48], growth suppression of ERα-positive cancer both *in vitro* and *in vivo*[49] and apoptosis promotion[50,51].

These mechanisms are, in turn, the target of targeted therapies. Therefore, a possible implementation of the targeted therapies activity can be profoundly influenced by the composition of the intestinal microbiome. Furthermore, particular microbes could become a future therapeutic target to potentially improve the effectiveness of cancer treatment. However, further studies are needed to better understand the predictive and prognostic role of the gut microbiome in HCC and to investigate the potential benefits of microbiome modulation.

THE ROLE OF GUT MICROBIOTA IN PATIENTS WITH HEPATOCELLULAR CARCINOMA TREATED WITH IMMUNE CHECKPOINT INHIBITORS

Gut microbiota dysfunction is known to lead to dysfunction of local, locoregional, and systemic immune systems, causing the dissolution of epithelial barriers, and as a consequence, transfers biofilm microbes and their components into mesenteric lymph nodes and peripheral circulation. Furthermore, dysbiosis may induce a neutrophils gathering into the intestinal epithelium that modifies the profiles of inflammatory cytokine and chemokine, stimulates the T helper 17 and effector T-cells, resulting in a negative feedback control of the microbiota[52]. Aging, antibiotics, xenobiotics, smoking, hormones, and diet can be responsible for dysbiosis which is a risk factors for cancer onset and, on the other hand, influences

the therapeutic outcomes, as for chemotherapy or immunotherapy, interfering directly or indirectly with therapeutic mechanisms[53].

ICIs are promising anticancer agents and according to several recent studies, the gut micro-biome may play a critical role in regulating immunotherapy responses. Thus, the microbiome can influence the host response to ICIs (PD-1/ PD-L1 blockade, or CTLA-4 inhibition)[52,53] (Table 2).

CD152, well known as CTLA-4, a T-cell surface receptor activated by two ligands (CD80 or CD86) expressed on antigen-presenting cells, induces an inhibitory signal in the early activated T-cell[54,55]. The other immune checkpoint repressing T-cell response is PD-1 (programmed death cell 1) sided is on the surface of activated T-cells, whereas its ligand PD-L1 is expressed on tumor cells surfaces and antigen-presenting cells (macrophages and dendritic cells). Their bond induces T-cell inactivation[56].

Various studies have recently examinated the role of gut microbiota in melanoma patients treated with ICIs showing a strict correlation between gut microbiota and response to immunotherapy and how differences in microbiome composition could influence treatment efficacy[56-60].

Notably, Akkermansia muciniphila was associated with a better response to ICIs. In metastatic melanoma patients with a benefit from ICIs treatment, the so-called responders, several bacteria phyla were detected in abundance: Faecalibacterium, Ruminococcaceae, and Clostridiales. Nevertheless, non-responder patients had a higher amount of Bacteroidales.

According to several studies, both preclinical and clinical, A. muciniphila, Alistipes indistinc-tus, Bacteroides, B. cepacia, D. formicigenerans, Parabacteroides merdae/distasonis, C. aerofa-ciens, Eubacterium spp., Veillonella parvula, Klebsiella pneumoni-ae, Bifidobacterium spp., Lactobacillus spp., Streptococcus parasanguinis, Blautia spp., E. hi-rae, E. faecium, H. filiformis, Faecalibacterium prausnitzii, Gemmiger formicilis, and Ruminococ-caceae family seems to improve the effectiveness of ICIs facilitating antitumor immunity.

Gut microbiota enhanced with B. thetaiotaomicron, Roseburia intestinalis, Anaerotruncus colihominis, Blautia obeum, and some combination of antibiotics were associated with a compromised ICIs' efficacy[53].

Changes in the expression of cytokines and immune cells due to alterations in the gut microbiota induced distinct therapeutic responses[61].

As for HCC, ICIs are approved for clinical practice usage[62-64]. Up to now, programmed cell death (PD) ligand-1 (PD-L1) expression, tumor mutation burden, and microenvironmental immune cells have been associated with ICIs effectiveness. A previous study conducted by Shen *et al*[65] investigated the relationship between the gut microbiome, analyzed through 16S rRNA and shotgun whole-genome sequencing on stool samples collected at baseline and after eight weeks treatment, and the effectiveness of immunotherapy in patients with advanced HCC. The study enlisted thirty-six patients (31 males and 5 females). There was no difference in the baseline gut microbiome between responders and non-responders. Also, the composition of gut microbiota showed no difference induced by ICIs. The study failed to demonstrate an association between gut microbiota and ICI efficacy, maybe due to the size of the sample examined, the different treatment, and the host factors influencing and affecting the gut microbiota, along with a non-standardized method to collect and process the stool samples leading to poor reproducibility. Also, dysbiosis is common in patients with chronic liver diseases. Therefore, it may affect the response to ICIs treatment as the study results, too.

On the contrary, a study by Chung *et al*[43] enrolled eight patients with HCC who received nivolumab as second- or third-line treatment after sorafenib. At baseline and after three months of treatment, fecal samples to assess the microbiome were collected. Patients with Child-Pugh A were 87.5%. Various bacterial taxa, like Dialister pneumosintes, Lactobacillus reteri, Enterococcus faecium, Streptococcus Gordonii, Streptococcus mutans, Escherichia coli, Veillonella atypica, Granulicatella sp., and Trchuris trichiura, were detected in non-responders. Citrobacter freundii, Azospiril-lum sp., and Enterococcus durans were part of responders' microbiota. Yet, the bacteria detected didn't match with the species found in previous studies. A skewed Firmicutes/Bacteroidetes ratio seems related to a scarcity of response to immunotherapy. Moreover, a high Prevotella/Bacteroides ratio seems associated with a better response to nivolumab as proved in a study that examined fecal samples in patients with advanced gastrointestinal cancer treated with ICIs[66]. However, the exiguity of the samples examined leads to low statistical power. Dysbiosis appears as an element that plays a critical role in the immunotherapy response in patients affected with hepatocellular carcinoma.

A retrospective study by Li *et al*[9] focused on the composition of gut microbiota in patients with HCC treated with ICIs. In particular, patients were gathered on the basis of the abundance of Bacteroidales in nonresponders and Faecalibacterium in responder patients. As a result, an abundance of Faecalibaterium correlated with an increased PFS, while a great amount of Bacteroidales is as-sociated with low PFS. However, the small size of the sample and the retrospective nature of this study limited the validity of the results observed.

Another study by Zheng *et al*[42] enlisted eight patients with HCC, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and a Child-Pugh Class A receiving camrelizumab, an anti-PD-1 agent, after progression to sorafenib. Stool samples were collected and analyzed at baseline and week 3 and 12, respectively. In the responders patients the species that composed the gut microbiota were: Bacteroidetes, Firmicutes and Proteobacteria. In non-responder Proteobacteria were predominant. Oral intake of Bifidobacterium may promote antitumor growth induced by ICIs. A. Muciniphila and

Table 2 Correlation studies between intestinal microbial composition and response to immune checkpoint inhibitors

	Treatment	Responder	Non responder
Zheng *et al*[42]	Anti-PD1	↑ *Akkermansia muciniphila*; ↑ *Ruminococcaceae*	↑*Proteobacteria*
Li *et al*[9]	Anti-PD1	↑ *Faecalibacterium*	↑ *Bacteroidales*
Chung *et al*[43]	Anti-PD1	*Dialister pneumosintes*; *Escherichia coli*; *Lactobacillus reteri*; *Streptococcus mutans*; *Enterococcus faecium*; *Streptococcus gordonii*; *Veillonella atypica*; *Granulicatella sp.*; *Trchuris trichiura*; ↑ *Firmicutes/Bacteroidetes ratio*; ↓ *Prevotella/Bacteroides ratio*	*Citrobacter freundii*; *Azospirillum sp.Enterococcus durans*; ↑ *Akkermansia species*

↑: Increased; ↓: Decreased.

Ruminococcacaeae were observed in the intestinal microbiota and have a role in immunomodulatory functions. Oral A. Mucinophila could revive the effectiveness of ICIs.

A clinical trial was recently opened at the National Cancer Institute that aims to combine vancomycin-based antibiotic treatment with immune checkpoint inhibitors (NCT03785210). This study may attempt to answer the question of whether the combination of immunotherapy with microbiota selection could have a beneficial effect in HCC patients[67].

Summing up, evidence with low statistical power is available up to now. Further studies are warranted for the time being. Also, a standardized method to collect and analyze fecal samples may help obtain reproducibility.

HCC AND MICROBIOTA TRANSPLANT

Curative options for advanced HCC are very limited, and driving the need to develop new therapeutics. Greater understanding about bacterial function, its impact on the host, its contribution to the loss of barrier function and the gut–liver immune system will lay the foundations for novel therapeutic approaches to the treatment of chronic liver disease that will attenuate progression to cirrhosis and HCC. To improve their efficacy, these therapies should focus on preventing the progression from chronic liver disease to cirrhosis and from compensated to decompensated cirrhosis. Bacteriotherapy could restore microbiome composition, reduce intestinal permeability (and thus reduce endotoxemia) and attenuate the chronic inflammatory environment in the liver; in this way, there is the potential that progression of disease and tumour development could be delayed or halted. Ideally, these therapeutic approaches would be more effective if they targeted earlier stages of disease and aimed to reduce chronic inflammation and cirrhosis, rather than directly reduce tumour mass[68].

Potential routes to target intestinal microbiota community include diet, probiotics, prebiotics, antibiotics[69-73] and FMT.

FMT aims to replenish the gut with a "physiological" microbiome taken from the stool of healthy subjects. Fecal donors are carefully selected[74], with exclusion criteria such as a low or high body mass index (new-onset obesity has been reported in a transplant recipient of fecal microbiota isolated from an overweight donor[75]), high risk behavior for infectious diseases, gastro-intestinal disease, recent microbiota-altering treatment (antibiotics, immunosuppressive medication, antineoplastic agents), presence of specific medical issues such as auto-immune, atopic or neurologic disorders, cancer, or chronic pain syndrome. The method for microbiota isolation is simple: fecal matter is collected from selected donors, suspended (usually in saline solution) and mixed in a blender; the resulting liquefied stool is filtered through a strainer to remove fibers, and thus ready for transplant[8]. Currently, FMT has been approved as a clinical method for treating recurrent Clostridium difficile infection by 2013 guidelines[76] and its clinical effectiveness has reached approximately 90%[77]. Fecal microbiota can be delivered *via* endoscopy (*e.g.*, colonoscopy or nasojejunal), enema or colonic transendoscopic enteral tubing[78-80]. Oral capsules has been developed showing efficacy comparable to delivery by colonoscopy regardless of whether fresh, frozen or lyophilised stools were used[81-83]. However, frequency of doses and optimal overall duration is still unclear as study parameters were not directly comparable across different studies.

There are several clinical studies regarding the use of probiotics as a novel and effective approach to treat or prevent chronic liver disease and HCC. Probiotic VSL#3, a combination of Bifidobacteria, Lactobacilli and Streptococcus thermophilus, could short inpatient time for patients with liver cirrhosis and hepatic encephalopathy[84]. A randomized controlled multicenter study investigates the role of probiotics in patients with alcoholic hepatitis (AH)[85]. The 117 patients were prospectively randomized to receive the 7 d of cultured Lactobacillus subtilis/Streptococcus faecium (1500 mg/d) or placebo (probiotics 60 and placebo 57). In the probiotics group albumin and TNFα showed significant difference. In addition, 7 d of oral supplementation with cultured L. subtilis/S. faecium was associated with restoration of bowel flora and improvement of microbial lipopolysaccharide in patients with AH.

Another study in mice aimed to evaluate the role of FMT in reducing HFD-induced steatohepatitis in mice. The analysis was conducted by examining the microbiota structure of the rodents, the butyrate present in the caecal content, the intrahepatic lipids and the liver pathological conditions 8 weeks after FMT. The results documented a reduction in the degree of steatohepatitis after FMT, as indicated by the finding of a decrease in intrahepatic lipids, NAS score and intrahepatic pro-inflammatory cytokines (INF-γ and IL-17), with an increase in Foxp3, IL -4 and IL-22 [86]. Another study investigate the role of FMT in patients with untreated sever alcoholic hepatitis (SAH). Philips *et al*[87] discovered that Indices of liver disease severity improved significantly within the first week after FMT compared to HC and even survival was better in patients treated with FMT (87.5% *vs* 33.3%, P = 0.018). Philips *et al*[88] reported a case of a young male patient with corticosteroid nonresponsive severe alcoholic hepatitis in 2017. FMT led to rapid amelioration of appetite and hyperbilirubinemia. Notably, FMT was performed in 18 patients with persistent positive HBeAg[89]. FMT was effective for these patients *via* inducing HBeAg clearance, suggesting that regulating intestinal microbiota might be beneficial to chronic hepatitis B treatment. A Phase I clinical trial demonstrated that FMT restored antibiotic-induced microbial dysbiosis in patients with advanced liver cirrhosis[90]. Even more, the effect of FMT on hepatic encephalopathy has been confirmed in both animal models and human beings. FMT alleviated cognitive function and prevented hepatic necrosis in animal models, thereby triggering improvement of hepatic encephalopathy[91]. Kao *et al*[92] reported a significant improvement in serum ammonia and quality of life in a patient with hepatic encephalopathy after performing FMT. Bajaj *et al*[93] conducted a randomized clinical trial, which suggested that FMT has the potential to improve cognition and reduce hospitalizations in hepatic encephalopathy patients.

Recently, Baruch *et al*[94] reported the first-in-human clinical trials where they discovered how treatment with FMT was associated with favorable changes in immune cell infiltrates and gene expression profiles in both the gut lamina propria and the tumor microenvironment. These early findings have implications for modulating the gut microbiota in cancer treatment.

These results encourage further studies on the possible beneficial impact of gut microbiota "resetting" by FMT in HCC.

LIMITATIONS AND FUTURE PERSPECTIVES

Despite the abundance of studies in the literature, the understanding of the mechanisms of the alteration of the gut microbiota in HCC remains incomplete and inadequate. Most clinical studies have limitations due to the fact that they are single center studies with small population samples, which compromises the applicability of the results. Furthermore, it is often complex to analyze the various etiologies of the hepatic disease, the stage of cirrhosis, the diet, the use of antibiotics for other causes, the consumption of alcohol; all these elements represent confounding factors that can determine important variations in the intestinal microbiota. Therefore these factors should be considered in the design of future studies, which would involve multiple centers, on a large scale.

The characteristics of dysbiosis change among patients with hepatocellular carcinoma depending on the different etiologies. Despite this, some studies have shown that the different etiology of HCC is not related to a condition of intestinal microbial dysbiosis[95], while other studies are conflicting. Chen *et al* [96] evaluated the differences in the gut microbiota in consideration of the etiology of liver disease. they found that the discriminant between HBV-related cirrhosis and primary biliary cirrhosis was indicated by two operative taxonomic units (OTU), OTU-23 (Neisseria) and OTU-36 (Gemella). Furthermore, the level of bacterial diversity and composition varied differently between patients with non-HBV and non-HCV HCC (NBNC-HCC) and patients with HBV-HCC[97], and between patients with HBV-HCC and NAFLD-HCC[41,98]. Therefore, these data suggest that in designing future studies, the underlying etiology of liver disease should also be taken into account for identifying patterns of dysbiosis in HCC.

Currently, although several studies on the association between intestinal microbiota and HCC are emerging, data analyzing the causal relationship are still very limited[99]. From a methodological point of view, traditional sequencing technology and 16S rRNA sequencing (the most established genetic marker used for bacterial identification and classification), does not take into account rare eukaryotic cells and the absence of which could lead to loss of important information. Furthermore, it is necessary to consider that the use of low biomass samples, such as blood, can lead to contamination in the microbiome analysis[100].

Finally, despite extensive preclinical evidence, clinical trials focusing on the prevention and treatment of HCC by modulating the microbiota are still lacking. The greatest difficulties are found, in this sense, in the applicability of in vitro or in vivo studies to the human context.

To optimize the therapeutic response it could be useful to host protective intestinal bacteria depending on the type of treatment proposed. To confirm this, despite the current lack of clinical studies, the data obtained up to now with animal models have given interesting results in increasing the efficacy of treatments by modulating the bacterial flora. Some studies suggest that to increase the efficacy of the treatments it is necessary to keep intact the commensal microbiota which would mediate the cellular functions of the myeloid-derived cells present in the tumor microenvironment[101]. The

results obtained from the implantation of fecal material of patients with melanoma in germ-free mice, led to an improvement in tumor control, increased the response of T lymphocytes and obtained a greater efficacy of treatment with the anti programmed cell death protein 1 (PD-1)[59]. Among these, Bifidobacterium spp. has been identified. as a component of the microbiota that improved the efficacy of treatment with anti programmed death-ligand 1 (PD-L1)[58], while several species of Bacteroides appear to have an implication in the antitumor effect of antigen-blocking anti-cytotoxic T cells (CTLA) -4[57].

As regards hepatocarcinoma, a recent study has documented that the intestinal microbiota can increase the effectiveness of treatment with anti PD-1, increasing the sensitivity to immunotherapy[42, 102]. Given the important results obtained from immunotherapy treatment in patients with advanced HCC[103], it is important to explore the data relating to the microbiota and the bacterial species interested in contributing to the beneficial effects of the immunotherapy. Furthermore, in patients with NAFLD-HCC, given the immunosuppressive phenotype exerted in peripheral blood mononuclear cells from a bacterial extract of these patients, the possible modulation of the microbiota could help in overcoming any resistance to immunotherapy in HCC[98].

CONCLUSION

In recent years, enormous progress has been made in the characterization of the gut microbiota and its association with different etiologies and severity of cancer diseases; various hypotheses have also been advanced in chronic liver disease and in the development of HCC and studies have been launched to characterize it. Furthermore, the work in rodent models and the greater understanding of the etiology of bacterial pathogens affecting liver disease has established the contribution of the gut microbiome in the progression of liver disease and its potential role as predictive and prognostic biomarkers.

Considering that patients with HCC and other CLDs are prone to alterations in the intestinal microbiota, it is tempting to assume that dysbiosis affects the efficacy of anticancer treatments including ICI in some categories of patients and that consequently the modulation of components of the microbiota can be managed to increase the activity of available treatments. Despite the growing interest on the part of researchers on the subject, for now it remains to be clarified whether the recent data obtained through animal models on the interaction of the immune response and the microbiota in some types of tumors, can also be applied to patients with hepatocarcinoma. Therefore, the orientation of research towards the intestinal microbiota, in particular the use of probiotics or the transplantation technique of the fecal microbiota, will be able to better direct towards new paradigms and personalized treatments with the aim of improving the effectiveness of the treatments available for HCC.

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MINIREVIEWS

Challenge of managing hepatitis B virus and hepatitis C virus infections in resource-limited settings

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Abstract

The global burden of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and coinfection represents a major public health concern, particularly in resource-limited settings. Elimination of HCV by 2030 has become foreseeable, with effective direct-acting antiviral oral therapies and the availability of affordable generics in low-and-middle-income countries (LMICs). However, access to oral nucleos(t)ide therapy for HBV remains critical and is limited outside the existing global HIV program platforms despite affordable prices. Prevention of mother-to-child transmission of HBV through scaling up of birth dose implementation in LMICs is essential to achieve the 2030 elimination goal. Most individuals living with HBV and/or HCV in resource-limited settings are unaware of their infection, and with improved access to medications, the most significant barrier remains access to affordable diagnostics and preventive strategies. The coronavirus disease 2019 pandemic interrupted hepatitis elimination programs, albeit offered opportunities for improved diagnostic capacities and raised political awareness of the critical need for strengthening health care services and universal health coverage. This review underpins the HBV and HCV management challenges in resource-limited settings, highlighting the current status and suggested future elimination strategies in some of these countries. Global efforts should continue to improve awareness and political commitment. Financial resources should be secured to access and implement comprehensive strategies for diagnosis and linkage to care in resource-constrained settings to fulfill the 2030 elimination goal.

Key Words: Hepatitis B virus/hepatitis C virus; Chronic hepatitis; Resource-limited settings; HBV and HCV elimination

Core Tip: This minireview presents the data and challenges associated with hepatitis B virus (HBV) and hepatitis C virus (HCV)/coinfection in resource-limited settings. It also underlines the key gaps and strategies for elimination of HBV and HCV infections in the low and middle-income countries. Global efforts should continue to improve awareness and political commitment. Equally important is securing financial resources for access and implementation of comprehensive strategies for diagnosis and linkage to care in resource-constrained settings to fulfill the 2030 elimination goal.

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INTRODUCTION

Despite the coronavirus disease 2019 (COVID-19) pandemic, viral hepatitis B and C still claim thousands of lives daily. Both viruses are responsible for 96% of all hepatitis-related mortality worldwide because of the chronicity of these diseases. Globally, approximately 325 million people have viral hepatitis B and C, and most of them are undiagnosed or untreated[1].

Low-income countries (LICs) have a high implication of hepatitis B virus (HBV) infection, with limited availability of detection, prevention, and management[2,3]. The incidence and prevalence of HBV infection are 9.2 and 7.4 times higher, respectively, in LICs than in high-income countries. Anyhow, the proportion of diagnosed subjects decreases from 18% in high-income countries to 0.8% in LICs[2]. Moreover, among the diagnosed individuals the proportion of those accessing therapy, also decreases from 14% in high-income countries to 9% in LICs[2]. Different programs often lead to interventions to prevent viral hepatitis, *e.g.*, immunization, blood transfusion services, and infection control measures. As a result, the proportion of hepatitis C virus (HCV) infected individuals who are diagnosed is higher (46%) in high-income countries than in low- and middle-income countries (LMICs) (6%). Furthermore, annual rates of treatment initiation are higher in high-income countries (8%) than in LMICs (2%)[2]. In 2015, access to direct-acting antivirals (DAAs) was low in LMICs; therefore, the projected cure rates were lower in LMICs than in high-income countries. However, the access patterns are changing rapidly with the availability of affordable generics[2].

Coinfection with HBV and HCV is not uncommon, particularly in LMICs. Although the primary site for HBV and HCV replication is the hepatocyte, their life cycles are totally different. HCV is an RNA virus that replicates in the cytoplasm, while HBV is a DNA virus that replicates in the nucleus. But, both have RNA replicative intermediates and theoretically can interact in coinfected cells, inducing different viral expression and serologic patterns[4]. There is a lack of sufficient data regarding the intracellular interplay between both viruses because of a proper *in vitro* cellular model. Superinfection is the dominant mechanism for developing coinfection, whereas HCV superinfection is more commo[5]. In HBV and HCV coinfection and immune-related regulations, HCV is usually dominant and thus overt, whereas HBV presents either an overt or occult pattern[4,6,7]. However, the possibility that HCV and HBV can alternate their dominance during coinfection cannot be excluded[8]. Coinfection with HCV and HBV can result in the spontaneous viral clearance of either one or both viruses, chronic infection, or development of acute fulminant hepatitis[5]. Chronic coinfection is associated with adverse hepatic outcomes than HBV or HCV monoinfection[9], warranting effective treatment[10]. It is noted that disease progression is faster in HBV/HCV dual infection than in those with monoinfection[5]. Recently, an extensive study was conducted on 8513 chronic HCV patients, of whom 87 were positive for both hepatitis B core antibody (HBcAb) and HBV surface antigen (HBsAg), 1577 were only HBcAb positive, and 6849 were HBcAb negative. The results suggested that prior HBV infection adversely affects liver health despite apparent clearance[11]. The risk of developing hepatocellular carcinoma (HCC) was also reported to be higher in patients with dual chronic HCV/HBV infection than that with mono-infection [12]. It was also shown that dual/triple infection by HIV/HBV/HCV increases the risk of HBV/HCV-associated HCC[13]. Thus, it is important to recognize coinfection, where viral interaction has implications for disease severity, clinical picture, and management strategy[4]. The current success in treating HCV infection highlights the need for proper selection of antiviral regimens for long-term suppression of any concurrent viral coinfection[13].

The current review addresses the current challenges in managing HBV and HCV infections in resource-limited settings and suggested elimination strategies in some of these countries to achieve the 2030 goal.

WHO 2030 ELIMINATION GOAL FOR HBV AND HCV

In May 2016, the World Health Assembly adopted the Global Health Sector Strategy (GHSS) on Viral Hepatitis 2016-2021, targeting the elimination of viral hepatitis as a public health threat by 2030 (reducing new infections by 90% and mortality by 65% and expanding HCV diagnoses from < 20% to 90% and number of eligible individuals getting HCV treatment from < 10% to 80%). The GHSS also aims to decrease hepatitis incidence from 6-10 million cases to 0.9 million cases and decrease annual hepatitis mortality from 1.4 million to 0.5 million by 2030[14]. To achieve the above-mentioned goal, five core intervention areas are documented by the GHSS: (1) HBV vaccination; (2) prevention of mother-to-child transmission of HBV; (3) injection and blood safety; (4) harm reduction; and (5) testing and treatment of HBV and HCV[14]. Different countries have developed their strategies for elimination. By November 2017, 84 countries had developed hepatitis control programs[15]. In addition, 62% of HCV-infected persons live in countries that can buy generic DAAs (LMICs)[16]. According to the Polaris records, 18 countries are working toward elimination, and 12 countries, Australia, Egypt, France, Georgia, Iceland, Italy, Japan, Mongolia, the Netherlands, Spain, Switzerland, and the UK, are on track to meet hepatitis C elimination targets[16]. In contrast, only 20 countries will not meet the 2030 and 2020 targets for HBV prevalence, 12 of which are in sub-Saharan Africa (SSA)[17].

The global hepatitis prevention, testing, treatment, and immunisation programmes were disrupted as a result of the COVID-19 pandemic. Implementing and maintaining successful interventions across the full range of hepatitis-related critical services is key to meeting the 2030 viral hepatitis elimination targets and goal. This spurred the theme "Hepatitis Can't Wait" for World Hepatitis Day 2021, which aims to ensure the longterm viability of viral hepatitis services and to investigate opportunities presented by the COVID-19 pandemic[1].

HIGH PREVALENCE AREAS FOR HBV AND HCV IN RESOURCE-LIMITED SETTINGS

Globally, viral hepatitis B and C are the most common causes of liver cancer, leading to 1.100 million deaths every year[1], comparable to the number of deaths caused by tuberculosis and higher than that caused by HIV and malaria[2]. Viral hepatitis is now ranked as the seventh leading cause of mortality worldwide[18]. Despite the fact that LMICs have implemented universal HBV vaccination as part of their expanded immunisation program, a previous WHO Global Hepatitis Report showed that the number of HBsAg-positive persons was highest in the WHO Western Pacific Region (115 million, prevalence estimated as 6.2%; 95% uncertainty interval (UI) 5.1–7.6) and African Region (60 million, prevalence estimate 6.1%; 95% UI 4.6-8.5), which together accounted for 68% of the global burden. Approximately 2.7 million of the 36.7 million individuals living with HIV are also infected with HBV, with a 7.4% global HBV prevalence in HIV-infected persons[2]. In an Egyptian community-based cross-sectional study of 3600 children aged 9 months to 16 years who were fully vaccinated with HBV vaccine during infancy, seroprotection was detected in 57.2 percent, HBsAg was positive in 0.11 percent, and breakthrough infection was 0.36 percent and 0.39 percent, depending on anti-HBc and DNA detection positivity, respectively[19].

Remarkably, the burden of HCV is increasing over time and is affecting all regions as a mostly "silent pandemic"[20], with significant disparities between and within nations.

The WHO Eastern Mediterranean Region (2.3%), followed by the European Region (1.5%), has the highest reported prevalence of HCV[2]. However, reports from Egypt have suggested a decrease in incidence over time[21]. In most LMICs, the prevalence of HCV infection is 2%[22].

Worldwide, most of chronic hepatitis B patients acquire infection at birth or in early childhood, and perinatal or horizontal transmission dominates in SSA and Asia[23]. HCV infection is most typically linked to unsafe health and injection practices in healthcare institutions with insufficient infection control measures, particularly in resource-limited settings[24].

HBV/HCV coinfection prevalence is about 5%-20% in HBsAg-positive patients and 2%-10% in HCV-positive patients, with rather different geographical distributions[25]. A study conducted in Cameroon between January 2008 and December 2014 on 524 anti-HCV-positive patients found a low coinfection rate with HBV (3.6%)[26], notably, however, the prevalence of HBV/HCV coinfection is not known or underestimated in cases with occult HBV infection[27]. HCV superinfection in patients with chronic HBV has been reported as the most common clinical feature of coinfection in the Asia-Pacific Region [27]. A recent systematic review and meta-analysis from India revealed a prevalence of 1.89% for HBV/HCV coinfection[27]. The prevalence was 0.16% among 3750 patients at a tertiary care hospital in Barabanki, Uttar Pradesh[28]. An estimate of the global prevalence of HBV/HCV coinfection is critical for developing testing and care cascades, particularly in resource-limited settings.

DIAGNOSTIC CHALLENGES AND EXPENSES NEEDED TO ACCESS DIAGNOSTICS IN RESOURCE-LIMITED SETTINGS

The indicator for treatment is the proportion of infected persons diagnosed and thus underwent the treatment protocol. Early detection of chronic HBV/HCV infection is critical for initiating therapy and thus delaying the progression of liver damage.

However, a recent WHO report emphasized the limited access to affordable hepatitis testing, where; only 9% of HBV-infected persons (22 million) and 20% of HCV-infected persons (14 million) have been diagnosed. This report dictates the acceleration of the diagnosis rate of such infections by tracking those already infected and linking them to treatment[2]. In low- and middle-income settings, it is estimated that less than 1% of chronic HBV or HCV patients know their illness, because of lack of awareness, limited facilities or services, limited access to reliable and low-cost HBV and HCV diagnostics, poor hepatitis surveillance programs, and lack of political and financial commitment[3]. Meanwhile, those who have HBV and HCV co-infections are untreated and unaware of their infection[29].

A recent modeling study in 120 countries included a literature review of PubMed and Embase, followed by interviews with experts, to quantify the historical epidemiology of HBV infection found that in 2016, the global prevalence of HBsAg was 3.9% (95% UI 3.4-4.6). Of these infections, 10% were diagnosed; only 5% eligible for treatment received antiviral therapy, and less than 1% of mothers with high viral loads accessed antiviral treatment to decrease mother-to-child transmission[17]. Notably, the cost of diagnostics remains one of the most significant barriers in LMICs. A report released in 2017 showed increased demand for HCV diagnostics in 29 LMICs, representing 80% of absolute HCV viremic burden in LMICs, and in countries with high relative prevalence and active HCV programs. In middle-income countries, laboratory-based immunoassays are mainly used for HCV screening, while for cost and accessibility reasons, most LICs use rapid diagnostic tests (RDTs)[30]. WHO proposes using RDTs, viral load (VL) testing, and DAAs in a streamlined screening and treatment strategy[31].

It was found that 79% of the projected demand for VL assays was driven by four countries (China, Egypt, Pakistan, and India), with 36% of the total demand being driven by Egypt. The prices of HCV VL tests remain high in most countries (from $15–30 per test in the public sector to $60-200 per test in the private sector)[30].

IMPACT OF ANTIVIRAL THERAPY ON HBV/HCV REACTIVATION

Hepatitis B and hepatitis C dual infection have a negative impact on the prognosis of liver disease, but the data is insufficient, and no clear treatment guidelines are known. Because of the interaction between both viruses and the possibility for reactivation of either virus with antiviral therapy directed against only one of them, treatment decision is not a straightforward task[4]. Before implementing antiviral therapy, full serological and virological evaluations are required to verify the activity of each virus and choose the optimal antiviral regimen[8]. The general management approach is to treat the dominant virus as a monoinfection and then monitor for reactivation of the other one. HCV likes to be the priority target to be managed in HBV/HCV coinfected patients with active hepatitis C. Treatment of HCV showed that HBV breakthrough infection and reactivation have been recorded by many researchers[32, 33]. However, for coinfected patients with active hepatitis B or with established cirrhosis, more researches are needed to determine the optimal regimen to manage both viruses simultaneously[8]. Advanced fibrosis was reported to be common in HBV/HCV-coinfected patients (58%) than in HBV monoinfections (32%, $P < 0.0001$), but the frequency was similar to that in HCV-monoinfections (52%, $P = 0.3142$). Decompensated cirrhosis was found to be common in coinfections (11%) than in either HBV or HCV-monoinfections (2%, $P = 0.0002$) and (4%, $P = 0.0275$) respectively[34]. A recent Egyptian study reviewed data extracted from the National Network of Treatment Centers database for HCV viremic patients diagnosed during the national campaign for HCV elimination (October 2018-April 2019). Among 297965 patients who underwent HBsAg testing, 2347 patients (0.8%) were positive. HBsAg +ve patients showed less advanced fibrosis by FIB-4 ($P < 0.01$). Only 14% of HBsAg +ve patients showed liver cirrhosis by ultrasound and two patients had HCC[35].

The DAAs are more effective for HCV clearance than interferon (IFN)-based therapy, exhibiting better tolerability and cure rates > 95%[10,36]. A retrospective study including 40 HBV/HCV dually infected patients to assess their clinical profiles and treatment outcomes showed DAAs are efficient for HCV eradication and recommended screening for HBV and monitoring for reactivation[37]. A systematic review and meta-analysis evaluated the risk of HBV reactivation following treatment for HCV infection with DAAs in patients with active or resolved HBV infection. The study showed that HBV reactivation occurred earlier and was clinically significant in dually infected chronic hepatitis C patients with overt and occult HBV treated with pan-oral DAAs than in those treated with IFN-based therapy. HBV screening was, therefore, recommended for the management of patients during pan-oral DAA therapy[38]. An updated systematic review and meta-analysis documented frequent HBV reactivation in dually infected chronic HBV and HCV patients receiving DAA therapy, albeit a rare encounter among patients with resolved HBV infection. Therefore, use of antiviral prophylaxis might be

warranted in HBsAg positive patients, particularly those with quantifiable HBV DNA[39].

ACCESS TO MEDICINE/PREVENTIVE MEASURES FOR HBV AND HCV IN RESOURCE-LIMITED SETTINGS

Treatment of HBV infections has been possible since 1985 and has progressively improved, first with IFN-based therapy and subsequently with the development of new medicines[2]. In 2015[40], the WHO prepared a recommendation to include nucleos(t)ide analogs with high barriers to resistance (*i.e.*, Tenofovir and Entecavir). Both compounds are easy to be given (one pill a day), highly effective, have few side effects, and induce relatively little resistance, but rarely result in cure[2].

Although HBV treatment is available through the WHO HIV programs in LMICs, access to HBV-monoinfected individuals is quite limited. Nucleos(t)ide analogs that are active against HBV are currently used as part of antiretroviral combinations and are taken by most HIV patients[41]. In addition, Tenofovir is now recommended for use as part of first-line treatment for HIV and to treat chronic HBV infection[2]. Thus, extension of Tenofovir-based treatment for HIV will provide effective treatment for HBV infection for individuals dually infected with HIV and HBV and will prevent transmission of HBV from mother-to-child[42]. However, data are scarce on the actual coverage of Tenofovir-based treatment for patients infected with HIV and HBV[2]. The Polaris Observatory records showed that only 5% of patients eligible for HBV treatment were treated, and most of these patients were from high-income countries[17]. This shows that HBV/HCV mono- and co-infections are underdiagnosed and undertreated in resource-limited settings.

The São Paulo Declaration on Hepatitis from the World Hepatitis Summit in 2017 recommended that LMICs promote fair access to and availability of high-quality, effective, safe diagnostics, vaccines, services, and treatment and make them affordable at the country level[43]. However, it was recognized that the proportion treated with WHO-recommended antivirals of those individuals diagnosed with HBV infection did not more than 8% (1.7 million patients). Among patients diagnosed with chronic HCV infection, 7% began treatment in 2015 (1.1 million persons). As of 2015, a cumulative total of 5.5 million chronic HCV patients had ever received treatment, but most of these treatments were older, less effective IFN-based regimens[2]. The WHO report following the World Hepatitis Summit in Brazil in 2017 emphasized that 3 million people could get treatment for HCV within the last two years, and an additional 2.8 million people started lifelong treatment for HBV infection in 2016[43].

The WHO-recommended treatment of HBV infection is available in a generic form in most LMICs and costs as little as US$30 for a year of treatment. The prices of WHO-recommended DAAs for HCV vary substantially (US$200–45 000 for a curative course), but prices have been dropping, and most LMICs should be able to buy generic medicines at affordable prices[2,43]. Currently, HCV can be treated within 8–12 wk with highly effective DAAs and high cure rates[44,45]. Introducing locally produced pharmaceutical products paves the way for further lowering prices. Prices for a full treatment course have been reported to be as low as US$45 in Egypt, which is considered an LMIC, yet many countries are not accessing these low prices[46]. Since October 2014, treatment in Egypt has been established on DAAs, and as of March 2017, at least a million individuals have obtained treatment in the public sector at the expense of the State (with more being treated in the private sector). Through the 100 Million Healthy Lives Initiative, the country has undertaken an ambitious model elimination program with a treatment scale-up. Egypt is also actively testing the general population (18-59y) to eliminate HCV[2]. A national population-screening program was initiated in October 2018[46]. Nearly 49.6 million individuals were screened, of whom approximately 2.2 million were seropositive for HCV and were referred for evaluation and treatment[47]. Uniquely, Egypt implemented a school screening program testing more than 9 million students above the age of 12 years, linking them to treatment, but COVID-19 disrupted school attendance and the program temporarily. With the support of the WHO, Egypt pledged to provide testing and treatment for one million persons in fourteen African countries that bear a high hepatitis burden[48]. Similarly, a collaborative simplified public health approach was used to support a hepatitis C elimination program in seven countries, including Cambodia, India, Indonesia, Myanmar, Nigeria, Rwanda, and Vietnam (with anti-HCV antibody prevalence ranging from 0.85 percent to 4 percent), with drug and diagnostic costs as low as US$80 per patient (country dependent). By December 2019, over 5900 healthcare personnel had received hepatitis C training, over two million patients had been screened, and over 120000 patients had begun treatment, with cure rates above 90% [49].

Gaining access to preventive strategies in LMICs remains challenging. Safe injections reduce HCV transmission by 70%. Globally, 5% of health-care-related injections remain unsafe, with about 1.75 million new HCV infections occurring worldwide in 2015[2]. Access to safe injection programs and devices is limited and remains a major contributing factor to the continuous transmission of blood-borne infections in resource-limited settings.

Prevention of neonatal and early childhood infection with HBV is also crucial for preventing chronic infection and further complications[18]. Universal infant and birth dose HBV vaccines to reduce mother-to-child transmission remain key strategies for the prevention and control of the HBV epidemic[50]. In

1991, the WHO-recommended inclusion of HBV vaccines into the Expanded Program of Immunization in all countries[51], and in 2009, the WHO-recommended the use of the HBV birth dose vaccine in all countries[52], followed by two or three doses to complete the primary series[2]. In 2015, the worldwide coverage of the three doses of HBV vaccine in infancy reached 84% (90% is the global target), and the coverage was 39% for the initial birth dose vaccination (global target 50% by 2020 and 90% by 2030), with a consequent reduction of HBV among children to 1.3%[2]. The latest WHO reports estimate that the proportion of children under five years of age chronically infected with HBV dropped to just under 1% in 2019, from 5% in the pre-vaccine era between the 1980s and early 2000s[53]. However, access to hepatitis B immunoglobulin and the birth dose of the HBV vaccine remains limited in LICs and needs to be increased to achieve global elimination goals. Globally, it was estimated that only 42% of children have access to the birth dose of the HBV vaccine, where, low coverage of the vaccine in some regions, particularly in SSA, is documented[1]. All immunization programs should use delivery of hepatitis B birth dose vaccine as a performance indicator[22]. In SSA, however, where qualified birth attendants deliver barely 50% of newborns, WHO estimates that birth dose coverage is no more than 10%[54].

Expansion to include health care workers is also recommended[43]. HBsAg prevalence in the WHO African Region remains at 3%[2]. Innovative approaches to confirm timely administration of the HBV vaccine birth dose have been successful in Vietnam, Indonesia, and China[55-57]. A recent modeling study showed that percentage of infants who had received the three-dose HBV vaccination in the first year of life was 87%; that for infants who had received timely birth dose vaccination was 46%, and it was 13% for those who had received HBV immunoglobulin along with the full vaccination regimen[17]. In 2020, the WHO had new guidelines for preventing HBV mother-to-child transmission, demanding universal birth dose vaccination of infants. These guidelines also provide evidence-based advice on using peripartum antiviral prophylaxis, namely Tenofovir, in HBsAg-positive pregnant women to prevent mother-to-child transmission of HBV infection[58].

RECOMMENDATIONS FOR SCREENING AND TESTING

Worldwide, the rate of diagnosis of viral hepatitis is very low, and there remains an enormous burden of undiagnosed infection. It was estimated that 9 out of 10 people living with viral hepatitis are not aware of their status and thus do not benefit from clinical care, treatment, and interventions that lessen further transmission[18]. As a result, good integration with other disease program and approaching case discovery through high-risk subpopulations should be beneficial[1].

The World Hepatitis Alliance (WHA) surveyed in 2018 and outlined the five main causes for misdiagnosis of viral hepatitis: (1) Shortage of public knowledge of the disease; (2) Need of knowledge of the disease among healthcare professionals; (3) lack of easily accessible testing; (4) stigma and discrimination; and (5) out-of-pocket costs to patients. The WHA consequently started an initiative named "Find the Missing Millions" for massive scale-up of screening, diagnosis, and linkage to care to find the millions of undiagnosed people living with viral hepatitis[59]. Key challenges in hepatitis testing currently include a shortage of quality-assured serological and low-cost virological *in vitro* diagnostics, testing limited facilities, deficient data to guide country-specific hepatitis testing approaches, stigmatization of those with or at risk of viral hepatitis, and need of guidelines on hepatitis testing for resource-limited settings[3]. The availability of noninvasive feasible and cost effective serological and imaging modalities to measure hepatic fibrosis would also enable identifying patients with significant or advanced liver fibrosis[60,61]. Current costs for enzyme immunoassays range from 1 to US$9 per test, and those for RDTs range from 0.5 to US$7, while the costs of hepatitis nucleic acid test (NAT) assays currently range from 30 to US$120, which seems unaffordable for a majority of patients in low-resource settings[3,62]. Additionally, FibroScan is not widely used in resource-limited settings because of its high cost, the need for trained personnel, and continuous maintenance[18]. Innovations in testing and sampling approach can increase access to testing and reduce the enormous burden of undiagnosed infection[62]. The 2017 WHO hepatitis testing guidelines for adults, adolescents, and children in LMICs outline the public health approach to strengthen and expand current testing practices for viral hepatitis. The guidelines also address testing approaches (who to test) and strategies (which serological and virological test to use) as well as interventions to promote linkage to prevention and care[3]; these guidelines recommended the use of the HCV core antigen with comparable clinical sensitivity to NAT assays as an affordable alternative[30,62]. The current availability of pan-genotypic HCV treatment using DAA terminates the need for expensive genotyping, unattainable in resource-limited setting[62]. In 2017, Peeling *et al*[61] examined a range of technological testing innovations to provide simplified affordable approaches for testing of HBV and HCV infection and monitoring of the treatment response to improve access to testing *via* alternative sampling methods (use of dried blood spots (DBS), oral fluids, and self-testing). Four systematic reviews and meta-analyses confirmed the high diagnostic accuracy of using DBS specimens for serological testing and NAT assays of HBV and HCV[63,64]. Combined rapid tests for the detection of HIV, HBV, and HCV infection and affordable confirmatory testing for the HBV and HCV viral genomes, such as point-of-care molecular assays, HCV core antigen testing, and multi-disease polyvalent molecular platforms, make use of existing centralized laboratory-

based or decentralized TB and HIV instrumentation for viral hepatitis testing[57]. Resources needed for implementing WHO-recommended hepatitis testing and treatment have been estimated across 67 low-income and middle-income countries, from 2016 to 2030 and it was found that access to affordable medicines in all countries will be key to reach hepatitis elimination and the feasibility of hepatitis elimination will be achieved in the context of universal health coverage[65]. This is confirmed by the last global progress report on accelerating access to hepatitis C diagnostics and treatment despite limited services caused by the current ongoing COVID-19 pandemic[66]. The report also emphasized that many LMIC increased access to testing and treatment by up to 20-fold increase in the number of individuals treated with safe and effective direct-acting antiviral drugs between 2015 and 2018[66]. However, access to HCV testing and treatment has not come to adequate levels of coverage to achieve the global goal of viral hepatitis elimination as a major public health problem by 2030[67]. It is recommended that point-of-care rapid diagnostic and viral load testing be used, as well as existing platforms and capabilities created for other diseases such as HIV and COVID-19.

CHOICE OF TREATMENT AND AVAILABILITY OF GENERICS

The WHO has developed guidelines for managing HBV infection that apply to resource-limited countries, but each should develop its guidelines according to its needs[40]. Only patients with HCV RNA-confirmed infection should start antiviral therapy[21].

Current HBV and HCV treatment rates are very low. According to the Global Hepatitis Report 2017, treatment has reached only a small fraction of diagnosed individuals[2]. In 2015, 8% of individuals diagnosed with HBV infection (1.7 million persons) were on treatment, while 7.4% of individuals diagnosed with HCV infection (1.1 million persons) had started treatment[1,2]. Tenofovir or Entecavir should be part of the treatment for patients with active HBV infection, those coinfected with HIV, and those with cirrhosis[10]. HCV treatments based on IFN/ribavirin are poorly tolerated and are associated with marked side effects, and these treatments have resulted in cure rates between 40% and 65%, depending on different factors. In 2012, Hartl *et al*[68] reported a case study of a 38-year-old Caucasian male coinfected with HCV (genotype 3a), HBsAg, and an antibody to the hepatitis B core antigen, which effectively responded to the pegylated-IFN plus ribavirin treatment regimen for HBV and HCV coinfection. A noticeable advance in HCV therapy followed the introduction of DAAs that directly inhibited the HCV replication cycle, which were better used in combination[2]. The WHO released its first guidelines on HCV treatment in 2014[69] and updated these guidelines in 2016[36] and 2018[70]. The Eastern Mediterranean Region accounted for the largest proportion of individuals that started treatment (12%), which was boosted by the large-scale elimination plans in Egypt[15]. In 2015, the HCV elimination program in Egypt was based on DAAs. Despite the development of generic antivirals that help reduce treatment costs, treatment remains unaffordable in some low-income settings where patients have to pay for their treatment.

CONCLUSION

In order to eliminate viral hepatitis in resource-constrained settings by 2030, a worldwide commitment to tackle this health burden with increased scale-up investment is essential. Political will, financial support, accessible pricing, integration with other current programs, community engagement, and multi-stakeholder collaboration are all critical. Active contribution of the private health sector in the management agenda together with an adequate strategic plan are required to solve problems regarding HBV/HCV screening, diagnosis, treatment and prevention in resource-limited settings. Monitoring liver enzymes and markers indicative of HBV/HCV replication before and during treatment are mandatory for early diagnosis and treatment of viral reactivation. HBV is a preventable infection with an available affordable vaccine, but the continuous assessment of the ongoing efficacy of HBV vaccination programs is crucial. Eliminating HCV is possible with DAAs, and implementing of preventive measures and the involvement of stakeholders.

Funding remains a major barrier and most LICs lack suitable financial resources for hepatitis services. The difficulties associated with the procurement of enough data from different low-income settings are also barriers to detection, testing, treatment, and thus for proper intervention. However, sharing experiences may pave the way for the successful implementation of elimination strategies. Transfer of successful stories like the Egyptian model to interested countries is of value. The development of sustainable and resource-appropriate mitigation strategies focusing on reducing transmission in resource-limited settings is needed. The strategies should fulfill preventive measures that include expanding HCV testing, safe injection, HCV treatment coverage, and birth dose HBV vaccination. Meanwhile, the impact of host and viral genomic factors on dual HBV & HCV infection must also be investigated.

FOOTNOTES

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MINIREVIEWS

Alfapump® implantable device in management of refractory ascites: An update

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Abstract

Refractory ascites (RA) is a frequent and life-threatening complication of cirrhosis. In selected patients with RA, transjugular intrahepatic portosystemic shunt (TIPS) placement and liver transplantation (LT) are currently considered the best therapeutic alternatives to repeated large volume paracentesis. In patients with a contraindication to TIPS or LT, the alfapump® system (Sequana Medical, Ghent, Belgium) has been developed to reduce the need for iterative paracentesis, and consequently to improve the quality of life and nutritional status. We report here recent data on technical progress made since the first implantation, the efficacy and tolerance of the device, the position of the pump in the therapeutic arsenal for refractory ascites, and the grey areas that remain to be clarified regarding the optimal selection of patients who are potential candidates for this treatment.

Key Words: Alfapump; Refractory ascites; Automated low flow ascites pump; Cirrhosis; Liver

Core Tip: The alfapump® system (Sequana Medical, Ghent, Belgium) is a subcutaneous implantable device that allows the transfer of ascites from the peritoneal cavity to the bladder. In this review, we describe the practical aspects of the alfapump® device implantation, and discuss its effectiveness and safety as a treatment for refractory ascites in cirrhotic patients, based on the most recently published data.

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INTRODUCTION

Cirrhotic patients may develop a wide range of complications secondary to portal hypertension and/or liver insufficiency. Among them, ascites occurs in nearly 60% of patients with compensated cirrhosis within 10 years, during the course of their disease[1]. Approximately 10% of patients with ascites develop refractory ascites (RA), defined as ascites that cannot be mobilized by appropriate medical therapy (*i.e.*, a low salt diet combined with diuretic therapy), or whose early recurrence cannot satisfactorily be prevented[2]. The prognosis of RA is poor, with a transplant-free survival (TFS) rate of only 50% at 6 mo, notably because of an increased risk of type 2 hepatorenal syndrome (recently renamed HRS-non-acute kidney injury (AKI) by the European Association for the Study of the Liver[3, 4]). RA generally leads to severe malnutrition, deteriorated quality of life, and uncomfortable symptoms or complications (in particular anorexia, abdominal hernia, and dyspnea). Liver transplantation (LT) is the ultimate solution for RA and should be considered systematically. In patients who are not eligible for LT because of advanced age and/or comorbidities, or for whom access to LT remains limited [low or intermediate Model for End-stage Liver Disease (MELD) scores], alternative or "bridging" therapies should be proposed. The first-line treatment for RA consists of large volume paracentesis (LVP). This procedure, although easy to perform, is not risk-free (a risk of major complications of around 1%, especially in case of severe liver failure) and LVP does not improve the patient's quality of life because of the repeated hospitalizations[5]. Furthermore, albumin infusions, administered for the prevention of post-paracentesis circulatory dysfunction after each LVP, also contribute to a heavy healthcare burden. Transjugular portosystemic shunt (TIPS) placement reduces portal pressure and improves effective blood volume and renal function within 4 to 6 wk, making this procedure an effective treatment for RA. In the most recent series including patients with recurrent ascites, covered TIPS was associated not only with better control of ascites, but also with a significant improvement in 1-year TFS compared to patients treated with iterative paracentesis (93% *vs* 52%; $P = 0.003$) without increasing the incidence of hepatic encephalopathy[6]. However, careful selection of candidates for TIPS placement is necessary to prevent the occurrence of short- and medium-term complications, and TIPS can ultimately be implanted in only 40% of cirrhotic patients with ascites[7]. The Automated Low-Flow Ascites Pump (alfapump®) system is a therapeutic alternative to TIPS and LT for the treatment of RA[2,8]. In this review, we describe the practical aspects of the alfapump® device implantation and discuss its effectiveness and safety as a treatment for RA, according to the current literature.

DATA COLLECTION STRATEGY

A search of PubMed and Embase was performed by two independent investigators (D.W.V. and T.T), since inception. The search terms used were "alfapump" AND "ascites". Additionally, reference lists were manually searched for the relevant literature. The articles identified by the initial search were considered for further analysis if they contained original data relating to alfapump® use in patients with non-malignant ascites related to cirrhosis. The search for the terms "alfapump" AND "ascites" retrieved a total of 72 articles. Of these 72 publications, we excluded papers that were not in English ($n = 2$), articles not published in full ($n = 23$), articles that were off-topic ($n = 7$), as well as letters to the editor ($n = 7$), editorials ($n = 2$), errata/corrigenda ($n = 2$), reviews ($n = 11$), and guidelines ($n = 1$). Thus, a final total of 17 original articles reporting data on the use of the alfapump® in patients with refractory ascites related to cirrhosis were included in the review (see flowchart of study selection in Supplementary Figure 1).

TECHNICAL ASPECTS

Working principle of alfapump®

The basic working principle and surgical aspects of the implantation of alfapump® have been described elsewhere[9]. Briefly, the device is manufactured by Sequana Medical (Ghent, Belgium) and obtained the CE mark in July 2011. It comprises a battery-powered pump implanted subcutaneously in the abdominal wall, connected to a first catheter placed in the peritoneal cavity, and to a second catheter that is tunneled under the skin and connected to the bladder, thereby enabling the transfer of ascites to the bladder for elimination *via* urination (Figures 1 and 2). Sensors are used to adjust the pumping cycles according to the peritoneal and bladder pressures: The cycle is interrupted if the pressure becomes too low in the peritoneal cavity or too high in the bladder.

A consensus statement has recently been published by hepatologists and surgeons experienced in using alfapump®, which provides practical recommendations regarding patient selection, implantation procedure, and post-implantation care[10].

The absolute contraindications for the implantation of the alfapump® device are loculated ascites, untreatable obstructive uropathy, the presence of an active bacterial infection at the time of implantation (spontaneous bacterial peritonitis, urinary infection, or abdominal skin infection in particular), and an expected survival of less than 3 mo. Special caution is advised regarding frail patients, and nutritional status should be considered and optimized before implantation[10]. Once implanted, the patient must charge the pump battery by transcutaneous induction, twice a day for about 20 min, using a user-friendly charging device (Smart Charger) that is placed over the area of the pump. While charging, the charger also collects data from the pump, which are then transmitted anonymously to a central databank of Sequana Medical. The data are transferred to the treating physician by e-mail on a weekly basis and in the event of acute dysfunction. This makes it possible not only to provide an early warning in case of pump dysfunction, but also to adjust the operating time, the frequency of cycles, and the daily volume of ascites to be evacuated, and to check the correct charging of the device[9].

Implantation procedure, use, and follow-up of alfapump®

Consistent data are available in the literature and detailed procedures have been published in expert consensus statements[10] and in the article by Dembinsky *et al*[11]. The manufacturer provides technical instructions regarding the surgical procedure and advice regarding pre- and post-implantation care, that are consistent with expert recommendations. In accordance with these recommendations[10,11], the patient is hospitalized 24-48 h before implantation. Paracentesis is performed to ensure that there is no ongoing spontaneous bacterial peritonitis, and to drain the abdomen. It is mandatory to leave 1-2 liters of ascites prior to implantation in order to check that the pump is functioning adequately before surgical closure and to minimize the risk of ascitic fluid leakage. Intravenous antibiotic prophylaxis is started on the day of the implantation and continued for 48 to 72 h. Prior to the procedure, the daily volume, operating time, and frequency of the pumping cycles are determined and programmed (FlowControl™ software) by the clinician according to the volume and frequency of paracentesis required in the weeks prior to implantation. A target should be set that is 20% higher than the pre-implant rate, because a postoperative increase in ascites production is frequent. Alfapump® works in cycles of very small volumes (5-10 mL) that are pumped every 5-10 min into the bladder, enabling the removal of 500 mL to 4 L of ascites per day. Some inactive periods can be determined for the patient's comfort (for example to avoid nocturnal urination[9]). A detailed description of the surgical procedure has been published elsewhere[9-11]. Briefly, it consists of the following steps: (1) Skin incision; (2) Bladder catheter insertion; (3) Peritoneal catheter placement; (4) Pump pocket creation and catheter tunneling; (5) Catheter attachment to the pump; and (6) Closure of the surgical incisions[11].

As with any new surgical technology, there is an unavoidable learning curve before achieving an acceptable level of success. In Europe, implantation is usually performed surgically under general anesthesia and takes an average of 60 min[9]. In the United States and Canada, a less invasive method for implantation has been developed, using an interventional radiology technique. In the recently published North American multicenter MOSAIC study, most procedures (29 out of 30) were performed by interventional radiology, and 11 patients were implanted under conscious sedation or local anesthetic[12,13]. Briefly, the peritoneal catheter was inserted under ultrasound guidance into the right lower quadrant, and excess ascites was removed to prevent leakage and catheter migration. The bladder catheter was inserted above the pubis symphysis and correct placement was confirmed by aspiration of urine or dyed saline or contrast-enhanced fluoroscopy. A subcutaneous pocket was then created by an incision 5 cm in length at the midclavicular line, 5-6 cm below the costal border, mostly on the right quadrant (76% of patients). Both catheters were then tunneled to the pump pocket, connected to the pump, and fixed in place with sutures; the alfapump® was finally housed in the pocket before multilayer closure[13]. In this study, technical success was obtained in all patients. The median duration of hospitalization was 4 d (range: 2-69 d). After a 3-mo follow-up period, three serious adverse events were classified as "procedure-related" (one bleeding at the site of bladder catheter insertion, one fluid leakage at the implant site of the pump, and one bacterial peritonitis 26 d after implantation). At 3 mo, two pumps had been explanted for infectious complications (cellulitis and pump pocket infection). Four re-

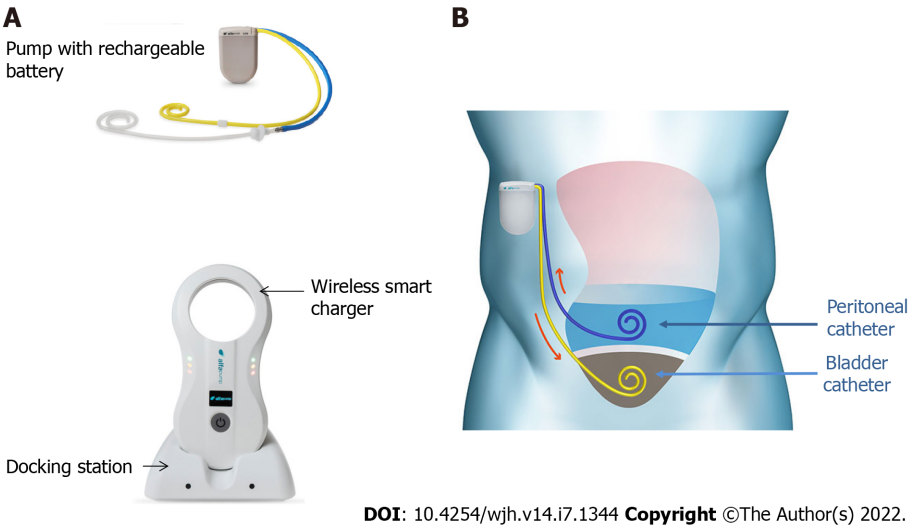

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Figure 1 Alfapump® device and principles of its implantation. A: The system consists of: (1) A pump, which contains a rechargeable battery and is connected to a peritoneal catheter and a bladder catheter; and (2) Charging accessories. The charger collects information and charges the pump through transduction; the docking station must be connected to the electrical network; B: The pump is positioned subcutaneously, under the costal margin (preferably on the right side), so that the patient is not hindered when sitting. The bladder must be full at the time of insertion of the bladder catheter; conversely, only a small amount of ascites is left in place for insertion of the peritoneal catheter, so that the pump can be tested before parietal closure. Images courtesy of Sequana Medical.

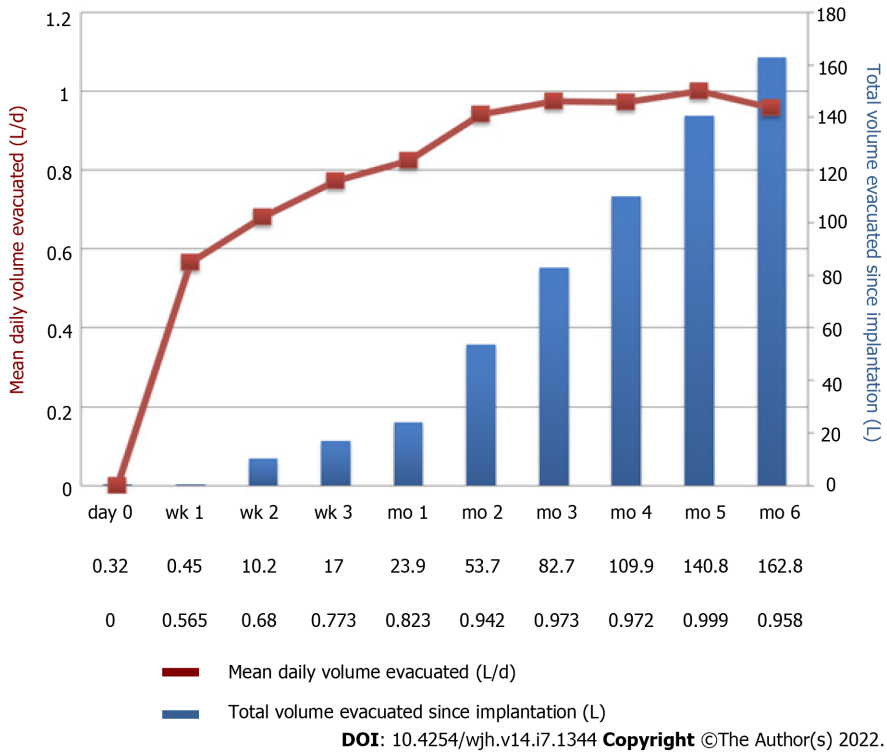

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Figure 2 Example of pump activity during the first 6 mo after implantation of alfapump®. A patient with refractory ascites was implanted with an alfapump®. The figure shows a progressive increase in the average daily volume of ascites evacuated (brown curve), resulting from adjustment of the pump by the clinician. The definitive rate is reached between the 1st and 2nd month. The bars (in blue) represent the total cumulative volume of ascites evacuated (Personal communication, Prof. Eric Nguyen-Khac, CHU Amiens, France).

interventions were performed, mostly because of peritoneal catheter dysfunction (three cases). This minimally invasive approach remains infrequent in European centers but a series of three cases reported by a team from Birmingham provided encouraging results[14]. Whatever the method used for implantation, a Sequana Medical implant specialist must be present during the procedure, to check that the pump is working properly, and in the event of a dysfunction, to have a back-up alfapump® available. During the hospitalization, which lasts approximately 4 to 7 d in the absence of complications, the patient must receive appropriate therapeutic education and training in the use of the pump. In

particular, the patient must be able to alert the physician immediately if symptoms occur, such as suture loosening, an inflammatory aspect at the surgical site, abdominal pain, reconstitution of abundant ascites, fever, or urinary symptoms. Notably, the presence of the alfapump® contraindicates the subsequent use of magnetic resonance imaging (risk of displacement of the pump and catheters, and damage to the system). Explantation of the pump may be necessary in some cases (death, LT, local complication, or pump dysfunction); this decision must be made on a case-by-case basis and in a multidisciplinary manner. The median life span of the device is around 2 years.

EFFICACY AND TOLERANCE OF THE DEVICE

Control of ascites

Most studies evaluating the efficacy of the alfapump® device included relatively small numbers of selected patients, generally not very old, with preserved liver function (Table 1). The international landmark PIONEER study performed in 40 patients showed a significant decrease in the number of monthly paracenteses in the "alfapump®" group compared to the "conventional treatment" group (0.2 *vs* 3.4; $P < 0.01$)[15]. More recently, a large prospective, multicenter, open-label, randomized, controlled study (RCT) was conducted in five European countries and aimed to evaluate the safety and efficacy of the alfapump® system in cirrhotic patients with RA in comparison with LVP[16]. This study included 60 patients (29 in the "alfapump®" group and 31 in the "SoC" (standard of care) group). Time to first LVP (primary endpoint) was significantly longer in the "alfapump®" group compared with "SoC" (hazard ratio: 0.13, $P < 0.001$). A total of 10/29 patients (37%) required LVP after pump implantation, mostly due to insufficient pumped volumes (4 patients) or device issues (5 patients). A recent meta-analysis of nine studies, including the European RCT[16] and eight observational studies[12,14,15,17-21], evaluated the efficacy of alfapump® in a total of 196 patients[22]. Despite significant heterogeneity between the studies (some of which were retrospective[17,21]), the proportion of patients receiving an alfapump® who no longer required paracentesis after pump implantation was 62%. This significant reduction in the need for paracentesis after pump implantation persisted over time (average follow-up time ranging from 6 to 24 mo)[22]. Interestingly, the reduced use of paracentesis is accompanied by an early and prolonged improvement in nutritional status[12,16]. In the study by Bureau *et al*[16], there was a significant improvement in brachial circumference, tricipital skinfold thickness, and hand grip strength in the first 3 mo after alfapump® placement compared to the control group.

The effect of alfapump® on quality of life was specifically studied in the RCT by Bureau *et al*[16] and in the MOSAIC study[12,23], and it was shown that quality of life, assessed by the Chronic Liver Disease Questionnaire, was significantly improved in patients with "alfapump®" compared to patients who underwent iterative paracentesis, in particular due to a reduction in ascites-related symptoms[12,16,23]. This benefit may be of interest in patients not eligible for LT.

Survival data

It is noteworthy that no prognostic impact of alfapump® has been demonstrated so far. In the European RCT, the overall survival at 6 mo was not different in the "alfapump®" group compared to the "iterative paracentesis" group (77% *vs* 87%, $P = 0.35$)[16]. In the series reported by Stirnimann *et al*[18], the median TFS of patients with alfapump® was only 9.8 mo, and the TFS rate was only 40% at 12 mo. The better TFS rate at 12 mo (57%) observed in the North American series could be explained, at least partially, by the lower severity of patients at inclusion. More insights should be provided by a European clinical trial that is currently recruiting subjects (NCT04326946), in which the primary endpoint is 6-mo post-implant survival.

A retrospective, single-centre, observational study compared the outcome of patients with RA treated with TIPS ($n = 19$) *vs* alfapump® ($n = 40$)[24]. As expected, patients receiving alfapump® had more impaired liver function (MELD-Na 16 *vs* 12; $P = 0.04$) and more frequently had encephalopathy (47% *vs* 16%; $P = 0.02$). Within the 6 mo following the procedure, the proportion of patients who did not require further paracentesis was 58% in the "TIPS" group *vs* 43% in the "alfapump®" group (P = NS). Two patients (10%) were transplanted in the "TIPS" group during the follow-up, *vs* 11 (27%) in the "alfapump®" group. In the subgroup of patients with a MELD-Na score below 15, 12-mo TFS was significantly higher in the "TIPS" group (65% *vs* 23% in the "alfapump®" group, $P = 0.02$), but the retrospective design of this study makes the results questionable. Two hypotheses can be proposed to explain the high mortality rate in patients from the "alfapump®" group who did not undergo LT. The first and major explanation is that, although alfapump® is an effective treatment to control ascites, it does not protect the patient against the other complications of persistent portal hypertension. The second hypothesis is related to the specific complications of the device, which are not rare (Tables 2 and 3) and may impact on prognosis *per se* or indirectly, if explantation of the pump is required.

Safety profile

Assessing the safety of the device remains challenging since most of the reported series do not include a control group. The heterogeneity of inclusion and non-inclusion criteria across studies (Table 1) hinders

Table 1 Characteristics and results of main studies evaluating alfapump®

Ref.	Study design	Main exclusion criteria	N patients	Mean age (yr)	MELD score[2]	Follow-up	Efficacy of the device	Mortality during follow-up, patients, *n* (%)	Liver transplantation after pump implantation (%)
Bellot *et al* [15], 2013	Observational Prospective	Life expectancy < 6 mo Creatinine > 176 µmol/L in the 7 d prior to inclusion Bilirubin > 85 µmol/L Malignancy (including HCC) HE and/or GI bleeding related to portal hypertension in the 2 wk prior to inclusion	40	59	12	6 mo	Number of paracentesis/mo/patient: 3.4 *vs* 0.24; $P < 0.01$	8 (25)	5 (12)
Thomas *et al* [20], 2015	Observational Prospective	Na	10	Na	16	Median: 165 d (maximum: 379 d)	Number of paracentesis/mo/patient: 3.4 ± 0.8 *vs* 0.4 ± 1.0 $P < 0.0001$	3 (30)	1 (10)
Bureau *et al* [16], 2017	RCT: alfapump (G1) *vs* iterative paracentesis (G2)	Creatinine > 176 µmol/L HCC outside Milan criteria Inability to use the device	G1: 27 G2: 31	61	12	6 mo	Median number of paracentesis on day 28 G1 *vs* G2: 0.3 *vs* 1.2; $P < 0.001$	G1 *vs* G2: 22 *vs* 13, P = NS	3 (11)
Stirnimann *et al*[18], 2017	Observational Prospective	Inability to use the device	56	62	13	Median: 5.8 mo (maximum: 26 mo)	Number of paracentesis/mo/patient: 2.9 ± 1.8 *vs* 0.3 ± 0.3, P = NA	23 (41)	9 (16)
Solà *et al*[19], 2017	Observational Prospective	Creatinine > 176 µmol/L Bilirubin > 85 µmol/L ≥ 2 urinary tract infections or SBP in the 6 mo prior to inclusion HCC outside Milan criteria	10	59	11	12 mo	Number of paracentesis/3 mo/patient 7.5 *vs* 2.4; P = NA	5 (50)	NA
Solbach *et al* [17], 2018	Retrospective	Na	21	56	15	Na	Number of paracentesis/wk/patient: 2.3 ± 2.7 *vs* 0; P = NA	Median survival: 153 d	4 (19)
Wong *et al* [20], 2020	Observational Prospective	MELD score > 21 HE stage > II in the 15 d prior to inclusion > 2 systemic or local infections in the 6 mo prior to inclusion Bilirubin > 85 µmol/L Creatinine > 132 µmol/L GFR < 30 mL/min/1.73 m^2	30[1]	60	11	12 mo	Number of paracentesis/mo/patient: 2.4 ± 1.4 *vs* 0.2 ± 0.4; $P < 0.05$	4 (13.3)	3 (10)
Will *et al*[24], 2020	Retrospective TIPS *vs* alfapump	Na	40	59	16	Median: 4.7 mo (maximum: 24 mo)	Number of paracentesis: no more paracentesis at 6 mo for 43% of patients	24 (60)	11 (28)

[1]Implant through interventional radiology (n = 29) or surgery (n = 1).

[2]Median values (ref. Bellot *et al*[15], Bureau *et al*[16], Stirnimann *et al*[18]) or mean values (ref. Thomas *et al*[20], Solbach *et al*[17], Wong *et al*[12], Will *et al*[24]) of the MELD score on the day of implantation. Main exclusion criteria without listing usual absolute contraindications. GFR: Glomerular filtration rate; GI: Gastrointestinal; HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; MELD: Model for End-stage Liver Disease; NA: Not available; NS: Not significant; RCT: Randomized control trial; SBP: Spontaneous bacterial peritonitis; TIPS: Transjugular intrahepatic portosystemic shunt.

the interpretation of the results.

Device-related complications

Complications directly related to the device are frequent. Among 100 patients with available data,

Table 2 General complications after implantation of alfapump®: Acute kidney injury and peritoneal and urinary tract infections

Ref.	Patients (*n*)	AKI occurrence during follow-up	Variation in creatinine levels before *vs* after implantation	Peritoneal infections (*n* episodes)	Urinary tract infections (*n* episodes)
Bellot *et al*[13], 2013	40	13 episodes, 11 patients	106 *vs* 127 μmol/L at 3 mo (*P* = NA) 105 μmol/L at 6 mo (*P* = NA)	12	3
Thomas *et al*[20], 2015	10	3 episodes	168 *vs* 221 μmol/L at 2 mo (*P* = NS)	NA	NA
Bureau *et al*[16], 2017[1]	27	After day 7: G1 *vs* G2: 17 *vs* 11 episodes; *P* = NS	G1 *vs* G2, at 3 mo: Increase of 18.1 *vs* 8.1 μmol/L (*P* = NS)	NA	NA
Stirnimann *et al*[18], 2017	56	NA	Increase of 46 μmol/L at 3 mo (*P* = NA)	5	1
Solà *et al*, 2017	10	18 episodes, 14 after day 7 in 7 patients	96 *vs* 105 μmol/L at 12 mo (*P* = NS)	3	8
Solbach *et al*[17], 2018	21	0	140 *vs* 168 μmol/L at 3 mo (*P* = NS)	11	4
Wong *et al*[20], 2020	30	11 episodes after day 7 in 9 patients	93 *vs* 107 μmol/L at 12 mo (*P* = NA)	1	3

[1]In this randomized controlled study, G1 and G2 correspond to alfapump® and iterative paracentesis groups, respectively. AKI: Acute kidney injury; NA: Not available; NS: Not significant.

Table 3 Device-related complications after alfapump® implantation

Ref.	Patients (*n*)	Peritoneal catheter dysfunction (*n* patients)	Bladder catheter dysfunction (*n* patients)	Pump dysfunction (*n* patients)	Pump pocket complication (*n* patients)	Explanted/replaced pumps
Bellot *et al*[15], 2013	40	5	9	2	Infection: 2 Wound: 2	13/NA
Thomas *et al*[20], 2015	10	0	Kinking: 1	1	Infection: 1 Wound: 2	1/0
Bureau *et al*[16], 2017	27	2	3	12	3	3/4
Stirnimann *et al*[18], 2017	56	Blockage: 13 Displacement: 2 Disconnection: 1 Twist: 2	Blockage: 1 Migration: 1	Clogging: 2 Humidity: 2 Communication: 4 Faulty sensor: 3	Infection: 2 Wound: 2	17/11
Solà *et al*[19], 2017	10	Migration: 2 Blockage: 1	2	Charging problem: 2 Transient blockage:2	1	2/1
Karkhanis *et al*[14], 2017	3	0	Migration: 1	1	2	
Solbach *et al*[17], 2018	21	Obstructions: 6	Dislocations: 5	4	4	4/2
Wong *et al*[20], 2020	30	13	1	3	4	10/9
Will *et al*[24], 2020	40	NA	Obstructions: 9	NA	NA	12/40

Lepida *et al* reported a pooled estimate rate of overall pump-related adverse events of 0.77 (95%CI: 0.64-0.87) with low heterogeneity[22]. Some of these events may require re-intervention, or even pump removal, which is not an uncommon event during follow-up (Table 2). We note, amongst others, the following events: Dysfunction of the peritoneal catheter due to blockage (debris, fibrin clots, or peritoneal aspiration) or displacement, more rarely dysfunction of the bladder catheter (occlusion and disconnection), migration or dysfunction of the pump, and infection of the pump pocket (Figure 3).

Concerns regarding renal function

Among the frequently reported adverse events of the pump, AKI may occur in up to 30% of patients during follow-up[22]. However, the heterogeneous definitions used for AKI and the widely varying

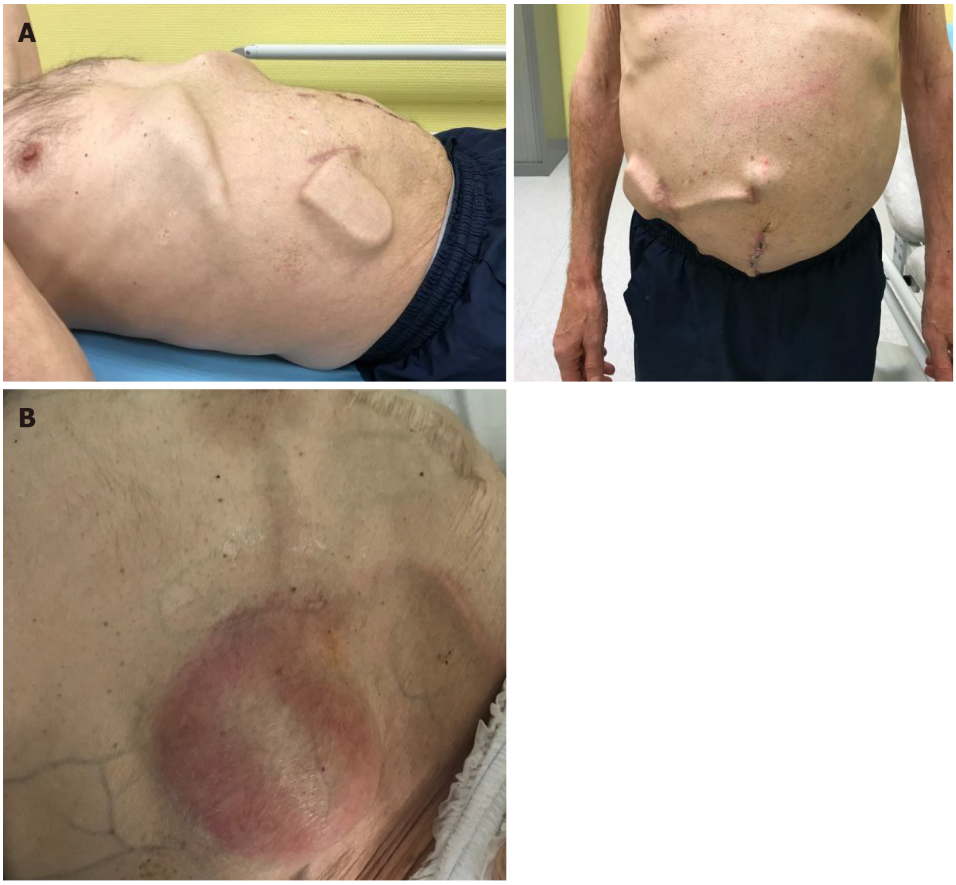

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Figure 3 Example of an alfapump complication. An alfapump® was implanted in July 2018, followed by omphalectomy in September 2018. A: October 2018: Increase in ascites after omphalectomy, leading to modification of the alfapump® settings and enabling subsequent deferral of paracentesis; B: February 2020: The patient was hospitalized for sepsis related to infection of the pump pocket, complicated with peritonitis and requiring pump explantation (Personal communication, Dr D. Weil-Verhoeven, CHU Besançon).

timeframe between pump implantation and assessment of renal function must be taken into consideration in the interpretation of this finding. It should be noted that the existence of chronic renal failure (based on serum creatinine values > 133 to 176 μmol/L or glomerular filtration rate < 30 to 50 mL/min depending on the series) was an ineligibility criterion for alfapump® in most studies (Table 1). An association between alfapump® and renal function deterioration at 6 mo was suggested in a series of ten patients followed for 1 year[19], but these results were not confirmed in the MOSAIC cohort[12]. In the European RCT, almost half of the patients experienced AKI, which was observed during the first week after implantation in 41% of them, but 75% of patients recovered their previous renal function[16]. In the meta-analysis, the mean increase in serum creatinine after implantation was 23 μmol/L (95%CI: 10-35)[22]. Several distinct and interrelated mechanisms may contribute to the deterioration of renal function in the postoperative period, such as changes in intra-abdominal pressure, systemic inflammation, and hemodynamic changes. In the medium term, it has been suggested that the continuous removal of ascites could cause circulatory dysfunction[19], thus favoring a deterioration of renal function. However, data regarding the impact of alfapump® implantation on the hemodynamic parameters are limited and conflicting[12,16,19] and this hypothesis has not been confirmed so far[25]. The issue of long-term albumin administration to prevent post-paracentesis circulatory dysfunction in these patients is not clear-cut, due to a lack of published data, and is therefore left at the discretion of the clinician in charge of the patient[26]. The ANSWER study provides some evidence that the benefits of long-term albumin administration in decompensated cirrhosis could be due to improved circulatory function and reduced proinflammatory cytokines[27]. However, the dosage, duration, and frequency of administration remain open to debate. Consequently, expert recommendations[10] advise following current guidelines regarding the use of albumin infusion after implantation, *i.e.*, whenever AKI occurs[2,8]; experts also considered albumin infusion whenever total daily volume of ascites removed exceeds one liter[10].

Bacterial infections

The second common adverse effect of pump implantation is the occurrence of bacterial infection. In the meta-analysis by Lepida *et al*, the incidence rates of ascites fluid infection and urinary tract infection

were 27% and 20%, respectively[22]. In the North American study, 15 bacterial infections occurred in 13 patients during the 12-mo follow-up, of whom 12 were considered to be related to the alfapump®[12]. Again, the absence of a control group limits the interpretation of these data. In the European RCT, the incidence of infectious events was similar in both the "alfapump®" and "standard treatment" groups [16]. Although the risk of developing multidrug-resistant infections related to long-term antibiotic prophylaxis remains a concern[7], patients receiving alfapump® have a particularly high risk of infection, and consequently long-term antibiotic prophylaxis should be maintained unless the patient's condition improves significantly (which occurs rarely). Norfloxacin at 400 mg/d remains the antibiotic of choice but, in the future, other molecules (such as rifaximin) with lower bacterial resistance and a better safety profile may be an alternative approach for long-term antibiotic prophylaxis[28]. Whatever the choice of antibiotic used for long-term prophylaxis, regular screening for multidrug-resistant organisms in these cirrhotic patients should be considered during antibiotic prophylaxis, in order to re-evaluate this strategy whenever multidrug-resistant Gram-negative bacteria or quinolone-resistant Gram-negative bacteria emerge[29]. However, two recent studies have provided more optimistic results regarding the long-term use of quinolones. The first observed that the incidence of infections caused by multidrug-resistant bacteria did not differ between the norfloxacin and placebo groups in patients with decompensated cirrhosis[30], while in the Global Study, no association was found between quinolone prophylaxis and multidrug-resistant bacterial infections, even when analysis was performed within different geographical areas[31].

UNRESOLVED ISSUES AND PERSPECTIVES

According to data on the efficacy and safety of the alfapump® device, it appears that the selection of candidates for insertion of an alfapump® as well as their pre-therapeutic evaluation must be rigorous (Figure 4). Multidisciplinary evaluation involving surgeons, anesthetists, and hepatologists is recommended. In fact, relative contraindications are frequent in these frail patients with RA (for example, pre-existing kidney injury, severe malnutrition or sarcopenia, cognitive impairment due to hepatic encephalopathy, significant peripheral oedema, and bed confinement[10]) and the risk-benefit ratio should be carefully considered. When LT is not possible, alfapump® implantation may be a satisfactory solution to improve the patient's quality of life, provided that there are no severe comorbidities that could threaten the short-term prognosis or compromise the success of the implantation procedure and/or the use of the device.

Patients awaiting a liver transplant

In patients who are candidates for LT, but with a long estimated waiting time until transplantation (notably when there is no possibility of prioritizing LT), alfapump® implantation may be discussed whenever TIPS is contraindicated. Few reports are available about the use of alfapump® in patients awaiting LT. A recent single-centre retrospective study among 22 patients listed for LT in Switzerland aimed to demonstrate the feasibility of LT in patients with an alfapump®[32]. In this cohort, the median (range) MELD score at alfapump® implantation was 15 (8-25), and only 14/22 patients underwent LT within an average of 6 mo after the pump implantation. The pump was removed before LT and at the end of the LT procedure in three and eight patients, respectively, and left in place in three patients for a limited period of time. No technical issues were attributed to the alfapump® during the LT procedure. The authors reported that eight patients died before LT, seven while on the waiting list, and one after being delisted due to progressive liver disease. The causes of death among the patients on the waiting list were progressive liver disease in four (of whom one had a bacterial infection of unknown focus and another suffered from peritonitis), and multi-organ failure in three patients (who respectively developed pump pocket empyema, an abdominal wall phlegmon with communication into the abdominal cavity, and septic shock associated with probable infected abdominal focus). A last patient died after small bowel perforation not directly related to the pump catheter. The lack of a control group of patients listed for LT with RA and treated by iterative LVP, precludes any firm conclusions. However, while these results suggest that alfapump® does not technically compromise LT, they also emphasize the high risk of severe infection in these patients carrying intra-abdominal foreign material.

Unproven benefits

The alfapump® offers interesting perspectives that warrant further evaluation.

Frailty: Frailty is recognized as a determining factor in the overall prognosis of cirrhotic patients and contributes to mortality on the LT waiting list[33,34]. By enabling an improvement in nutritional status and a return to physical activity, we may speculate that the alfapump® device could limit sarcopenia and frailty, but data regarding this potential benefit are scarce and this point warrants specific evaluation in dedicated studies.

Percutaneous treatment of hepatocellular carcinoma: By reducing the quantity of ascites, alfapump®

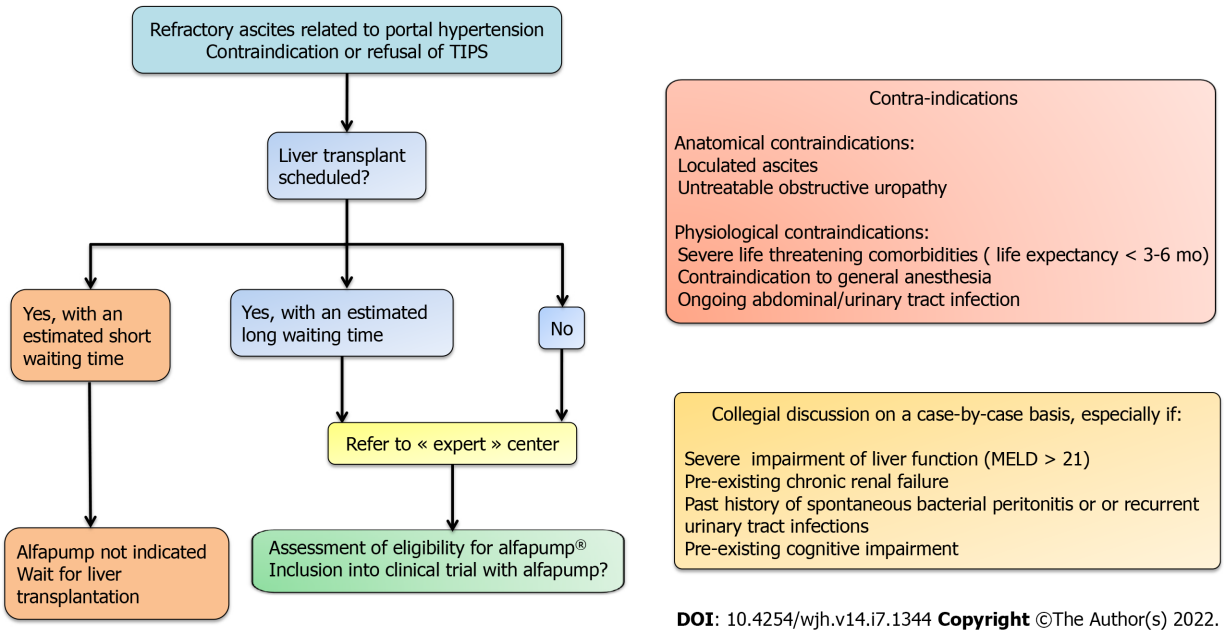

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Figure 4 Decision-making algorithm and key evaluation criteria for eligibility for alfapump® implantation. MELD: Model for end-stage liver disease; TIPS: Transjugular intrahepatic portosystemic shunt.

renders the percutaneous treatment of hepatocellular carcinoma possible. To date, this was reported in only one case report[35], but this therapeutic approach warrants further study.

Cure of hernia: A retrospective study of European multicenter data recently showed that patients who had concomitant umbilical or inguinal hernia repair and alfapump® placement had a shorter hospital stay, fewer complications, and better survival without paracentesis than patients undergoing emergency hernia surgery[36]. Hernia surgery concomitant with the implantation of the alfapump® enables the patient to undergo programmed surgery and to avoid the usual postoperative drainage, since the pump achieves ascites control. However, these data must be confirmed prospectively before this "concomitant" approach can be recommended. In the current state of knowledge, experts discourage concomitant repair of hernias[10].

Prevention of multidrug-resistant bacterial infections: Due to the decrease in hospitalizations for paracentesis, patients with alfapump® may be less exposed to nosocomial bacterial infections, which mainly involve multi-drug resistant bacteria. This may be of interest for patients who are candidates for LT. However, this potential benefit has not yet been evaluated in the long-term, and must be balanced against the risk of infections related to the procedure.

Cost-effectiveness: The overall cost of the procedure (implantation and patient follow-up), compared with that of standard treatment (iterative paracentesis), is a crucial point for the routine use of alfapump®. This cost in the first 6 mo after implantation is higher than that of the SoC treatment, mainly due to the cost of the device and the surgical intervention (about 30000 Euros), but tends to stabilize thereafter[16]. The ongoing French multicenter randomized medico-economic study (ARIAPUMP protocol, NCT03506893) comparing two management approaches for RA, namely, alfapump® implantation and iterative paracentesis, will make it possible to compare the costs of the long-term care for both these strategies, taking into account whether or not there is programmed LT. The radiological approach offers interesting perspectives in reducing the perioperative risk of morbidity in frail patients. Whether this mini-invasive technique can significantly reduce the duration of the post-procedure hospital stay, or the rate of local complications, has not yet been demonstrated, due to insufficient data and a lack of head-to-head studies.

CONCLUSION

Alfapump® is a device that has proven its effectiveness in reducing the need for iterative paracentesis and in improving the quality of life of cirrhotic patients with refractory ascites. It should be considered in particular for patients contraindicated for a TIPS, regardless of the patient's eligibility for LT. To minimize the risk of complications after implantation, careful selection of these frail patients is essential. The concerns related to the cost of the device, the surgical procedure of implantation, as well as the

potential complications that can occur are not fully resolved yet, but the implantation technique could evolve towards a "minimally invasive" approach, with a view to reducing the risks and improving the cost-effectiveness of the implantation. Patient information and active participation of the patient are two prerequisites for successful management. Additional studies, particularly real-world data from large heterogeneous populations with long-term follow-up, are required to clarify some unresolved issues, notably concerning the acceptable limits of liver and kidney function, age, forms of albumin compensation, or cost-effectiveness. There are currently several ongoing observational studies (NCT04326946, NCT03973866, and NCT03506893) that will hopefully provide a more complete picture of the advantages and disadvantages of this innovative device.

FOOTNOTES

Author contributions: Weil-Verhoeven D designed the research and wrote the paper; Weil-Verhoeven D and Thé venot T analyzed the data; Thévenot T, Di Martino V, Stirnimann G, Cervoni J-P, and Nguyen-Khac E made critical revisions related to important content of the revised manuscript; Weil-Verhoeven D, Thévenot T, Di Martino V, Stirnimann G, Cervoni J-P, and Nguyen-Khac E provided the final approval of the version to be published.

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ORIGINAL ARTICLE

Basic Study

Tissue pad degradation of ultrasonic device may enhance thermal injury and impair its sealing performance in liver surgery

Masatoshi Kajiwara, Takahisa Fujikawa, Suguru Hasegawa

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Abstract

BACKGROUND

Ultrasonic devices are widely used in many surgical fields, including hepatectomy; however, the negative effects of tissue pad degradation of ultrasonic devices, including those in liver surgery, remain unknown. The Harmonic® 1100 (H-1100) scalpel has advanced heat control technology than previous models, such as the Harmonic® HD1000i (H-HD1000i). We hypothesized that, because of its advanced temperature-control technology, the H-1100 scalpel would show less tissue pad degradation, resulting in superior sealing performance, compared to that with the H-HD1000i scalpel.

AIM

To elucidate ultrasonic device tissue pad degradation effects on instrument temperature and sealing performance using *ex vivo* porcine liver/vessel models.

METHODS

Two different harmonic scalpels were used and compared: A newer model, the H-1100 scalpel, and an older model, the H-HD1000i scalpel. Using *ex vivo* porcine livers, each instrument was activated until the liver parenchyma was dissected. The device temperature (passive jaw temperature) was measured after every 10 consecutive activations, until 300 transections of the porcine liver were performed. Tissue pad degradation was evaluated after 300 activations. Sealing performance was evaluated using excised porcine carotid vessels; vessel sealing speed and frequency of vessel burst pressure below 700 mmHg were determined after 300 transections of porcine liver parenchyma.

RESULTS

The temperature of the H-HD1000i scalpel was approximately 10°C higher than

that of the H-1100 scalpel, and gradually increased as the number of activations increased. The median passive jaw temperature of the H-HD1000i scalpel was significantly higher than that of the H-1100 scalpel (73.4°C *vs* 65.1°C; $P < 0.001$). After 300 transections of porcine liver parenchyma, less tissue pad degradation was observed with the H-1100 scalpel than with the H-HD1000i scalpel (0.08 mm *vs* 0.51 mm). The H-1100 scalpel demonstrated faster vessel-sealing speed (4.9 sec. *vs* 5.1 sec.) and less frequent vessel burst pressure < 700 mmHg (0% *vs* 40%) after 300 activations than the H-HD1000i scalpel; however, the difference did not reach statistical significance ($P = 0.21$ and $P = 0.09$, respectively).

CONCLUSION

In an *ex vivo* porcine hepatectomy model, the H-1100 scalpel shows lower passive jaw temperature and maintains its sealing performance by avoiding tissue pad degradation compared to that with the H-HD1000i.

Key Words: Ultrasonic device; Harmonic scalpel; Tissue pad degradation; Hepatectomy; Device temperature; Vessel sealing

Core Tip: The present study showed that the tissue pad of the Harmonic® 1100 (H-1100) ultrasonic scalpel, with improved heat control technology, was preserved even in a hepatectomy model, which is a severe condition for ultrasonic devices. It can be inferred that by using the H-1100 scalpel, even surgeons-in-training inexperienced in handling ultrasonic devices do not need to worry about device issues related to tissue pad degradation. Furthermore, use of the H-1100 scalpel may eventually reduce hospital costs.

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INTRODUCTION

Advanced-energy devices have become indispensable in modern surgery for tissue dissection and vascular control. In hepatectomy, these devices are known to shorten the liver transection time[1] and reduce blood loss during liver transection[2]. Ultrasonic devices, such as the harmonic scalpel (Ethicon, Cincinnati, Ohio, United States), are representative of the various energy devices that provide precise tissue dissection and secure hemostasis during meticulous procedures and improve surgical outcomes [3-5]. However, the tissue pad on the passive jaw of ultrasonic devices is reported to degrade with repeated long activations, or with activations without any tissue intervention[6].

To maintain a satisfactory burst pressure, a higher compression force is important[7,8]. Tissue pad degradation in an ultrasonic device generates a substantial gap between the active blade and the tissue pad, leading to decreased compression pressure of the targeted tissue and increased sealing time. With longer sealing time, the device temperature becomes higher[6,8]. Consequently, tissue pad degradation can result in undesirable thermal damage to the adjacent tissues.

The Harmonic® 1100 (H-1100) scalpel is a state-of-the-art ultrasonic device equipped with an improved heat control algorithm compared to that of the Harmonic® HD1000i (H-HD1000i), a previous model. The H-1100 scalpel actively lowers the blade heat as soon as the targeted tissue is dissected. This advanced H-1100 technology prevents overheating of the blade, which may protect the surrounding tissue from thermal injury and enhance tissue pad life throughout the procedure[9,10]. However, the advantage of the H-1100 scalpel in the field of liver surgery remains understudied.

As the liver is a solid organ, liver parenchymal dissection may burden ultrasonic devices with more mechanical stress compared to that with digestive tract surgery (in which, membrane and fat are the main dissection targets) and demand the enhanced tissue pad life. We hypothesized that, because of its advanced temperature-control technology, the H-1100 scalpel would show less tissue pad degradation, resulting in superior sealing performance, compared to that with the H-HD1000i scalpel. Therefore, we compared tissue pad degradation between H-1100 and H-HD1000i devices and examined the negative effects of tissue pad degradation on the device temperature and sealing capability (vessel sealing speed and burst pressure) using *ex vivo* porcine liver and vessel models, respectively.

MATERIALS AND METHODS

Ultrasonic devices

Two different models of harmonic scalpels were used: a newer Harmonic® 1100 (H-1100) model and an older Harmonic® HD1000i (H-HD1000i) model. Both devices were powered by a GEN11 generator (Ethicon, Cincinnati, Ohio, United States), and the power level was set to five for all experiments. The H-1100 scalpel employs a new version of intelligent heat control technology (adaptive tissue technology), which prevents inadvertent activation when tissue is not on the device by cooling down the blade temperature immediately after tissue dissection[9,10]. With the exception of this difference in thermal control technology, the H-1100 and H-HD1000i models are considered as mechanically identical products[10].

Each experiment was performed twice (n = 2), and a new set of harmonic devices was used in each experiment. All experiments were conducted by a single attending surgeon familiar with hepatectomy using ultrasonic devices.

Device temperature measurement

We measured the temperature of the back of the passive jaw as the device temperature. *Ex vivo* porcine liver (Tokyo-Shibaura-Zouki Co., Ltd., Tokyo, Japan) was used for this experiment. Commercially available porcine liver was harvested from living bodies at six days before the experiment, maintained and transported at 4°C, and then returned to room temperature on the day of the experiment. The harmonic scalpel was applied to the edge of the porcine liver with an almost full tissue bite and gradually clamped with continuous activation (Figure 1A). Activation was stopped when the liver tissue was completely dissected. A thermocouple temperature probe (Multichannel Recorder, MCR-4TC; T&D Corporation, Nagano, Japan) was attached to the back side of the passive jaw as soon as activation was completed (Figure 1B). The device temperature was measured repeatedly after every 10 consecutive activations, until 300 porcine liver transections were performed. A sufficient instrument cooling time was set after each device temperature measurement.

Tissue pad degradation

The extent of tissue pad degradation of the harmonic scalpel was measured using a digital indicator with a resolution of 0.01 mm (ID-S1012X, Mitutoyo Corporation, Kanagawa, Japan), after 300 activations.

Vessel sealing speed and burst pressure

Commercially available excised porcine carotid arteries (5-7 mm diameter; Tokyo-Shibaura-Zouki Co., Ltd., Tokyo, Japan) were used to evaluate the vessel sealing speed and burst pressure. The evaluated vessel size has been reported as safely sealed and cut using an ultrasonic device[11,12]. A catheter, securely ligated to the carotid vessel, was connected to a syringe and a manometer (Artfreak, Tokyo, Japan). Vessel sealing time was defined as the duration from harmonic scalpel activation to complete transection of the vessels. After sealing the vessel, saline was gradually infused into the vessel lumen at a constant rate by manually pushing a syringe. The burst pressure was identified as the pressure at the moment that the sealed vessel ruptured. Five replicates were performed for each harmonic device after 300 activations of the porcine liver parenchyma. We counted the frequency of burst pressure below 700 mmHg, which is considered to be a reliable measurement threshold for the manometer, although the scale of the manometer was up to 760 mmHg.

Statistical analyses

Continuous variables, such as device temperature and vessel sealing speed, are expressed as medians with ranges, and were non-parametrically compared between groups using the Mann-Whitney U test. The frequency of vessel burst pressure below 700 mmHg was compared between devices using the Fisher's exact test.

All P values were two-sided, and P values < 0.05 were considered statistically significant. All statistical analyses were performed in EZR (Saitama Medical Centre, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria)[13].

RESULTS

Device temperature

Figure 2 shows the representative trend in device temperature, which was measured after every 10 consecutive activations, until 300 porcine liver dissections were performed. The temperature of the H-HD1000i scalpel was approximately 10°C higher than that of the H-1100 scalpel, and gradually increased as the number of activations increased. All device temperatures measured in the two independent experiments are shown in Figure 3. The median device temperature of the HD-1000i

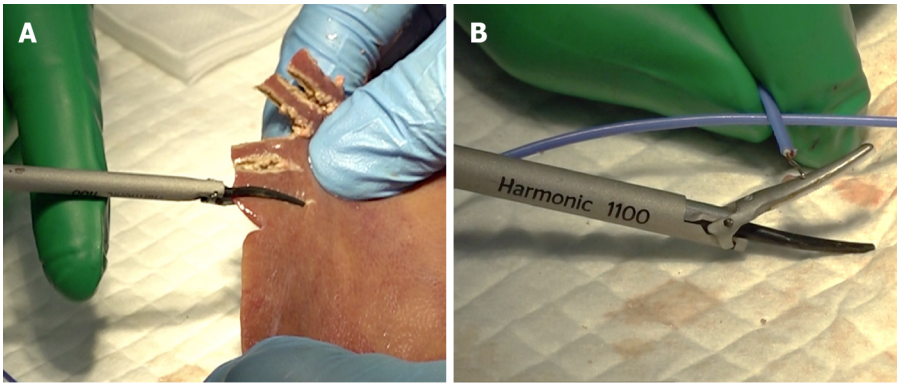

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Figure 1 Device temperature measurement. A: The harmonic scalpel was applied to the edge of the porcine liver with an almost full tissue bite, and gradually clamped with continuous activation; B: A thermocouple temperature probe was attached to the back side of the passive jaw after every 10 consecutive activations.

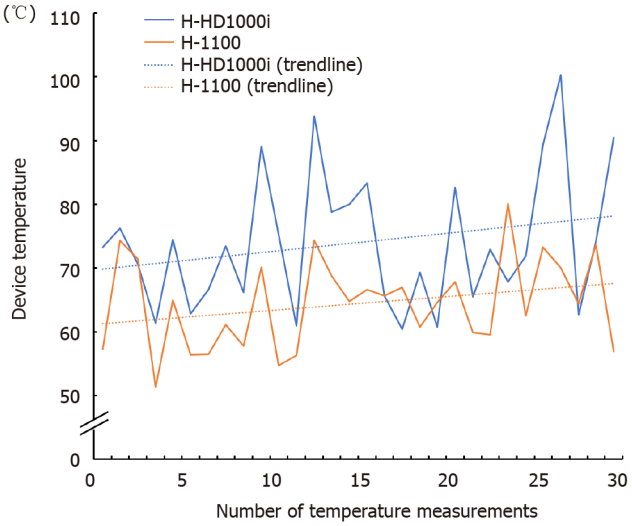

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Figure 2 Representative trend in device temperature measured after every 10 consecutive activations, until 300 porcine liver dissections were performed. H-HD1000i (blue, solid line) and H-1100 (orange, solid line) are shown. The equations of linear trend line are as follows: H-HD1000i (blue, dot line), y = 0.285x + 69.6; H-1100 (orange, dot line), y = 0.217x + 61.1.

scalpel was significantly higher than that of the H-1100 scalpel (73.4°C [range, 57.0°C-125.4°C] *vs* 65.1°C [range, 48.3°C-84.9°C]; $P < 0.001$).

Tissue pad degradation

Figure 4 shows the tissue pads of both devices, before use and after 300 repeated activations. More prominent tissue pad degradation was observed with the H-HD1000i scalpel than with the H-1100 scalpel in both experiments. The mean depth of tissue pad degradation was 0.51 mm and 0.08 mm for the H-HD1000i and H-1100 scalpels, respectively (Table 1).

Vessel sealing performance

The vessel sealing speeds of the H-1100 and H-HD1000i scalpels after 300 transections of porcine liver were 4.9 sec. and 5.1 sec., respectively. To evaluate the vessel burst pressure, we counted the frequency of vessel burst pressure below 700 mmHg after 300 activations (Table 2). Four of 10 (40%) vessel burst trials (five replicates per independent experiment) showed a burst pressure below 700 mmHg with the H-HD1000i scalpel (650, 600, 600, and 500 mmHg), whereas zero of 10 (0%) vessel burst trials with the H-1100 device exhibited a vessel sealing pressure less than 700 mmHg. Although the H-1100 device demonstrated superior vessel sealing performance in both experiments, the difference did not reach statistical significance ($P = 0.21$ and $P = 0.09$, respectively).

Table 1 Tissue pad degradation of H-HD1000i and H-1100 after 300 repeated activations

	H-HD1000i	H-1100
1st experiment (mm)	0.59	0.06
2nd experiment (mm)	0.42	0.09
Mean (mm)	0.51	0.08

Table 2 Vessel sealing performance after 300 repeated activations. Values are presented as median (range) or *n* (%)

	H-HD1000i	H-1100	*P* value
Vessel sealing spead (sec.)	5.1 (3.9-5.9)	4.9 (3.2-5.2)	0.21
Vessel burst pressure < 700 mmHg	4 (40)	0 (0)	0.09

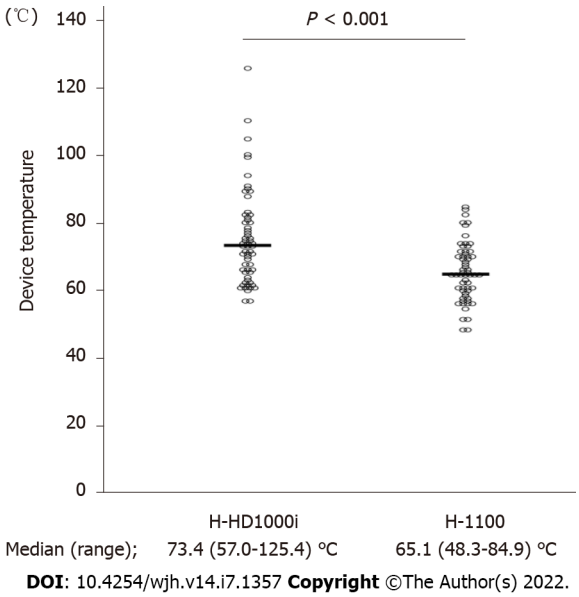

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Figure 3 All device temperatures measured in the two independent experiments.

DISCUSSION

The tissue pad on the passive jaw of ultrasonic devices is made of polytetrafluoroethylene, also known as Teflon, which is durable, resistant to heat, and chemically stable, but begins to degrade when it reaches approximately 260°C[6]. The tissue pad degrades and melts with repeated long or inadvertent activation without any tissue intervention[6]. Possible reasons for tissue pad degradation are considered to comprise both thermal and mechanical stresses generated by the frictional motion of ultrasonic energy.

To the best of our knowledge, no study has focused on tissue pad degradation in ultrasonic devices. The results of the present study demonstrated that the H-1100 scalpel consistently maintained a lower passive jaw temperature and sustained its sealing capability by minimizing tissue pad degradation compared to that with the H-HD1000i scalpel in an *ex vivo* hepatectomy model. When using the H-HD1000i scalpel, which uses an older version of heat control technology, tissue pad degradation must be prevented manually by stopping the activation as soon as the target tissue is dissected. All experiments in the present study were performed by a single experienced surgeon who was familiar with hepatectomy using ultrasonic devices. Thus, the present results suggest that even a well-trained surgeon was not able to completely eliminate "air-activation" (inadvertent activation without tissue between the jaws) when using a harmonic scalpel, and thermal and mechanical stresses derived from an accumulation of minute air-activations following every single transection led to degradation of the tissue pad on the passive jaw.

The device temperature after 10 consecutive activations of the H-HD1000i scalpel gradually increased as the number of liver dissections increased. This finding likely indicates that the H-HD1000i tissue pad steadily degraded in accordance with the increase in the number of activations. The H-HD1000i scalpel

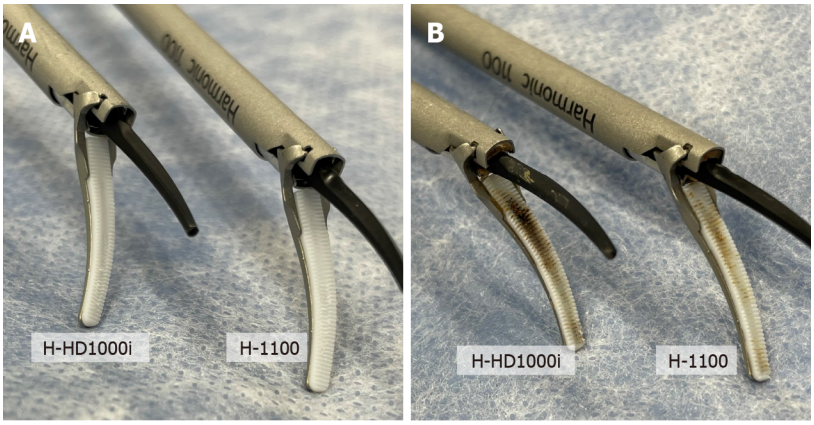

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Figure 4 Tissue pad of H-HD1000i and H-1100. A: Before use; B: After 300 repeated activations.

was approximately 10°C higher than that of the H-1100 scalpel, on average, and became well above 60°C, which has been reported as the critical temperature for causing irreversible tissue denaturation (*e.g.* recurrent laryngeal nerve injury during esophagectomy)[14,15]. Interestingly, sporadic extremely high temperatures above 90°C were only observed with the H-HD1000i scalpel, which was more affected by prominent tissue pad degradation (Figures 3 and 4). To be more precise, the temperature accounting for lateral thermal damage should be measured a few millimeters from the point of instrument application, not at the device itself. Nevertheless, physicians should be aware of the potential risk of thermal injury when using ultrasonic devices and avoid it by ensuring an adequate cooling time for the device[6,16].

Regarding vessel sealing performance, the H-1100 scalpel showed sufficient sealing performance even after repeated activations of the porcine liver parenchyma. By preventing significant tissue pad degradation, the H-1100 scalpel can maintain the optimal clamping force. However, it must be noted that the burst pressure obtained by the H-HD1000i device after dissecting the liver parenchyma as many as 300 times was also well above the physiological level of blood pressure and, thus, is adequately within the safety limit for clinical purposes.

It has been reported that the temperature of liver tissue at 1 mm away from the harmonic scalpel is significantly higher than that of thyroid tissue or muscle tissue in a live porcine model, which could be a reflection of the tissue consistency, size, and relatively higher volume of blood flow through the liver [17]. Accordingly, the liver may constitute a heavy load in ultrasonic device dissection. In cases of major anatomical liver resection, many parenchymal dissections are required. The present study showed that the H-1100 tissue pad was preserved even in a hepatectomy model, which is a severe condition for ultrasonic devices. Accordingly, it can be inferred that by using the H-1100 scalpel, even surgeons-in-training inexperienced in handling ultrasonic devices do not need to worry about device issues related to tissue pad degradation. Furthermore, use of the H-1100 scalpel may eventually reduce hospital costs.

The present study has several limitations. First, the data were obtained from benchtop experiments using porcine tissues at room temperature, which may not reflect actual human liver surgery. Second, the real targets to be sealed with energy devices during hepatectomy are the intrahepatic Glissonian triads and hepatic veins. In the present study, vessel sealing was investigated using only on porcine carotid arteries without blood flow. Therefore, care should be taken when applying our results to the clinical setting of human hepatectomy. Finally, the data were obtained from duplicate experiments. The reason for the lack of a statistically significant difference in vessel sealing performance (even though the H-1100 scalpel exhibited superior sealing performance after repeated activations compared to that with the H-HD1000i scalpel) may be attributed to the small number of experiments.

CONCLUSION

In conclusion, the cutting-edge H-1100 scalpel, with improved heat control technology, maintains a lower passive jaw temperature than the previous model (H-HD1000i), and may reduce undesirable collateral thermal damage. Moreover, the H-1100 scalpel maintains its sealing performance by avoiding tissue pad degradation better than the previous model.

ARTICLE HIGHLIGHTS

Research background

Ultrasonic devices are widely used in many surgical fields including hepatectomy in the modern era, while the negative effects of tissue pad degradation of ultrasonic devices in liver surgery still remain unknown.

Research motivation

As the liver is a solid organ, liver parenchymal dissection may burden ultrasonic devices with more mechanical stresses compared to the digestive tract surgery (in which, membrane and fat are the main dissection targets) and demand the enhanced tissue pad life. Therefore, we chose liver surgery for evaluating the effect of the tissue pad degradation.

Research objectives

To elucidate ultrasonic device tissue pad degradation effects on instrument temperature and sealing performance using *ex vivo* porcine liver/vessel models.

Research methods

Two different harmonic scalpels were used and compared: Harmonic® 1100 (a new model; H-1100) and Harmonic® HD1000i (a previous model; H-HD1000i). The device temperature (passive jaw temperature), tissue pad degradation after 300 repeated activations, vessel sealing speed and burst pressure were measured.

Research results

H-1100 scalpel consistently maintained a lower passive jaw temperature and sustained its superior sealing performance by avoiding tissue pad degradation compared to that with the H-HD1000i scalpel.

Research conclusions

In an *ex vivo* porcine hepatectomy model, the cutting-edge H-1100 scalpel maintains excellent performance throughout the procedure with the enhanced tissue pad life.

Research perspectives

This study provides a new insight into understanding the negative influence of tissue pad degradation of ultrasonic devices on device temperature and sealing performance. H-1100 scalpel solves issues related to tissue pad degradation. Furthermore, use of the H-1100 scalpel may eventually reduce hospital costs.

FOOTNOTES

Author contributions: Kajiwara M, Fujikawa T, and Hasegawa S designed and coordinated the study; Kajiwara M, and Fujikawa T performed the experiments, acquired and analyzed data; Kajiwara M, Fujikawa T, and Hasegawa S interpreted the data; Kajiwara M, and Fujikawa T wrote the manuscript; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Basic Study

Regulation of PPAR-γ activity in lipid-laden hepatocytes affects macrophage polarization and inflammation in nonalcoholic fatty liver disease

Xiao-Yun Li, Pei-Xuan Ji, Xi-Xi Ni, Yu-Xin Chen, Li Sheng, Min Lian, Can-Jie Guo, Jing Hua

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Abstract

BACKGROUND

Lipid metabolism disorder and inflammatory-immune activation are vital triggers in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). Various studies have shown that PPAR-γ exerts potent anti-inflammatory and immunomodulatory properties. However, little is known about the regulation of PPAR-γ activity in modulating cell crosstalk in NAFLD.

AIM

To investigate whether the regulation of PPAR-γ activity in lipid-laden hepatocytes affects macrophage polarization and inflammation.

METHODS

Primary hepatocytes were isolated from wild-type C57BL6/J mice or hepatocyte-specific PPAR-γ knockout mice and incubated with free fatty acids (FFAs). Macrophages were incubated with conditioned medium (CM) from lipid-laden hepatocytes with or without a PPAR-γ agonist. Wild-type C57BL/6J mice were fed a high-fat (HF) diet and administered rosiglitazone.

RESULTS

Primary hepatocytes exhibited significant lipid deposition and increased ROS production after incubation with FFAs. CM from lipid-laden hepatocytes promoted macrophage polarization to the M1 type and activation of the TLR4/NF-κB pathway. A PPAR-γ agonist ameliorated oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes and subsequently prevented M1 macrophage polarization. Hepatocyte-specific PPAR-γ deficiency

aggravated oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes, which further promoted M1 macrophage polarization. Rosiglitazone administration improved oxidative stress and NLRP3 inflammasome activation in HF diet-induced NAFLD mice in vivo.

CONCLUSION

Upregulation of PPAR-γ activity in hepatocytes alleviated NAFLD by modulating the crosstalk between hepatocytes and macrophages *via* the reactive oxygen species-NLRP3-IL-1β pathway.

Key Words: Nonalcoholic fatty liver disease; Hepatocyte; Macrophage polarization; PPAR-γ; NLRP3; Oxidative stress

Core Tip: Nonalcoholic fatty liver disease (NAFLD) is currently one of the most endemic chronic liver diseases worldwide. We aimed to investigate whether the regulation of PPAR-γ activity in lipid-laden hepatocytes affects macrophage polarization and to explore the underlying mechanism. Our study revealed that lipid-laden hepatocytes skewed macrophage polarization to the M1 phenotype. Regulation of PPAR-γ activity alleviates NAFLD by modulating the crosstalk between hepatocytes and macrophages *via* the reactive oxygen species-NLRP3-IL-1β signaling pathway. Strategies that manipulate PPAR-γ activity to regulate cell crosstalk will be beneficial for treating NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently one of the most common liver diseases, with a high morbidity presently exceeding 25% worldwide[1,2]. Manifesting from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH) and even to cirrhosis and hepatocellular carcinoma, NAFLD has already posed heavy public and financial burdens worldwide[3]. Given the lack of effective medications for treatment, there is a need to deeply explore the pathogenesis of NAFLD and to look for potential therapeutic targets for alleviating NAFLD[4].

Lipid metabolism disorder and inflammatory immune activation are two main triggers in the pathogenesis of NAFLD[5]. The widely accepted "two-hit theory" suggests that excess free fatty acids (FFAs) act as the first hit, causing abnormal lipid accumulation and insulin resistance and increasing the susceptibility of the liver to inflammatory damage[5,6]. Based on the first hit, the second hit involves activation of immune cells and oxidative metabolite production, leading to oxidative stress and an inflammatory response[7-9]. Hence, the accumulation of lipotoxic agents in hepatocytes is key to the onset and progression of NAFLD. Lipotoxicity can directly induce endoplasmic reticulum stress and pyroptosis in steatotic hepatocytes[10,11]. Among the many factors that trigger the progression of NAFLD to NASH, activation of immune cells plays a prominent and indispensable role[12].

Activation of macrophages, including hepatic resident Kupffer cells and peripherally recruited monocytes, plays an important role in the progression of NAFLD[12,13]. It is now widely considered that macrophages can be classified into two types: the classically activated M1 phenotype and the alternatively activated M2 phenotype. M1 phenotype macrophages are mainly induced by interferon-γ and lipopolysaccharide and secrete proinflammatory factors (IL-1, 6, 12, 23, CXCL 10, NO, peroxides, *etc.)*, which participate in the Th1 immune response and exert proinflammatory, bactericidal and antitumor effects. M2 macrophages are mainly induced by IL-4 and IL-10 and participate in the Th2 immune response with anti-inflammatory and tissue remodeling effects[14,15]. Under normal conditions, macrophages in the liver predominantly exhibit an M2 phenotype[16]. However, in NAFLD mice induced by a high-fat diet, the number of macrophages increases dramatically, and the polarity of macrophages appears to shift toward the M1 type[17]. These M1 phenotype macrophages contribute to the progression and prolongation of liver inflammation[13]. However, there is no certainty as to what exactly drives the activation of macrophages. Recent studies have revealed that various factors, including high levels of free fatty acids and the gut microbiota, may lead to macrophage activation[18, 19]. Furthermore, the crosstalk or interaction between parenchymal and nonparenchymal cells in the liver may reciprocally regulate macrophage phenotype or function.

PPAR-γ is a ligand-activated nuclear transcription receptor that mainly participates in adipocyte differentiation, lipogenesis, and insulin resistance[20]. Recently, much attention has been focused on the immunomodulatory and anti-inflammatory properties of PPAR-γ[21]. It has been demonstrated that activation of PPAR-γ synergistically upregulates the NRF2/HO-1 signaling pathway, thereby ameliorating methotrexate-induced hepatotoxicity[22]. Our previous study demonstrated that regulation of PPAR-γ activity in macrophages and HSCs could modulate their activation and alleviate the development of NAFLD/NASH[17,23]. Most previous studies have focused on the anti-inflammatory properties of PPAR-γ in nonparenchymal cells, such as macrophages and HSCs, in NASH; thus, the role of PPAR-γ in hepatocytes and the interaction between hepatocytes and macrophages remain to be explored.

In the current study, we aimed to investigate whether the regulation of PPAR-γ activity in lipid-laden hepatocytes affects macrophage polarization and explore the underlying mechanism. We found that upregulation of PPAR-γ activity could alleviate NAFLD through modulation of the crosstalk between hepatocytes and macrophages *via* the ROS-NLRP3-IL-1β signaling pathway.

MATERIALS AND METHODS

Primary hepatocyte isolation and treatment

Primary hepatocytes were isolated from wild-type C57BL/6 mice or hepatocyte-specific PPAR-γ knockout mice *via* two-step collagenase in situ perfusion of the liver[24] and then cultured in DMEM containing 10% FBS (Gibco, Waltham, MA, United States) with 100 U/mL penicillin G and 100 U/mL streptomycin sulfate at 37 °C with 5% CO_2 on collagen I-coated plates. The viability of primary hepatocytes was assessed using a trypan blue exclusion test and was greater than 95%. Mixed free fatty acids (FFAs) with a final concentration of 1 mmol/L were prepared with palmitic acid (PA, 0.66 mmol/L, Sigma Aldrich) and oleic acid (OA, 0.33 mmol/L, Sigma Aldrich)[25]. After overnight culture, primary hepatocytes were treated with FFAs for 24 h to induce a cell model of NAFLD in vitro. In some experiments, primary hepatocytes were pretreated with the PPAR-γ agonist GW1929 (20 μmol/L, Sigma Aldrich) for 3 h, followed by incubation with FFAs for 6 h or 24 h. Cell lysates were collected for RT–PCR and western blot analyses.

RAW264.7 macrophage culture and treatment

RAW264.7 macrophages were purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM containing 10% fetal bovine serum with 100 U/mL penicillin G and 100 U/mL streptomycin sulfate at 37 °C with 5% CO_2. All experimental interventions were conducted on the third passage of cells.

Primary hepatocyte and RAW264.7 macrophage conditional coculture system

As mentioned above, primary hepatocytes were incubated with FFAs or with GW1929 for 3 h followed by FFAs. Then, the cell culture supernatants were collected, centrifuged, and filtered to remove impurities. RAW264.7 macrophages were incubated with different types of conditioned medium (CM) from primary hepatocytes to establish conditional coculture systems for 6 h or 24 h, which were called CM-NC, CM-FFA, and CM-GW1929+FFA.

Animal experiments

The animal protocol was designed to minimize pain or discomfort to the animals. $Pparg^{fl/fl}$ mice and Alb-cre mice on the C57BL6/J background were purchased from GemPharmatech (Nanjing, China) to breed and obtain hepatocyte-specific PPAR-γ knockout ($PPAR\text{-}\gamma^{\blacktriangle hep}$) male mice (Supplementary Figure 2). Wild-type C57BL/6 male mice (aged 6-8 weeks) were obtained from the Experimental Animal Center (Renji Hospital, Shanghai Jiao Tong University). Wild-type mice were fed either a normal control (NC) diet (15% kilocalories from fat, n = 10) or a high-fat (HF) diet (60% kilocalories from fat, n = 10) for 16 weeks. For the rosiglitazone intervention experiment, mice received rosiglitazone (30 mg/kg/day, Sigma Aldrich) by oral gavage once daily for 28 consecutive days after 12 weeks of HF diet feeding (n = 10). All animal experiments fulfilled the Shanghai Jiao Tong University criteria for the humane treatment of laboratory animals and were approved by the Renji Hospital Animal Care and Use Committee (Permit number: RJ2018-0930).

Oil Red O staining

Free fatty acid-treated hepatocytes were fixed with 4% paraformaldehyde for 1 h and then stained with 5 mg/mL Oil Red O (Sigma Aldrich) for 60 min to examine lipid accumulation.

Assay of lipid contents, IL-1β concentration and oxidative stress markers

The cell culture supernatant of primary hepatocytes was collected for further analysis. Triglyceride (TG) and total cholesterol (T-CHO) were measured using a triglyceride assay kit and a total cholesterol assay

kit, respectively (Nanjing Jiancheng Bioengineering Institute, China). The IL-1β concentration was measured using an IL-1β ELISA kit (Lianke Biotechnology Company, China). Plasma from mice was centrifuged, separated and stored at -80 °C for further analysis. Plasma levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) were measured using MDA, SOD and GSH assay kits, respectively (Nanjing Jiancheng Bioengineering Institute, China). The plasma level of reactive oxygen species (ROS) was measured using an ROS ELISA kit (Nanjing JiaBeiSen Biotechnology, China). ROS generation in the cell culture supernatant was assayed using a DCFH-DA fluorescent probe kit (Beyotime Biotechnology, China). Total protein was extracted from mouse liver tissues. Caspase-1 activity in liver tissues was assessed with a Caspase-1 activity assay kit (BioVision, Milpitas, CA, United States). All procedures were performed according to the manufacturers' instructions.

Total RNA isolation and real-time PCR

Total RNA was extracted from mouse liver tissues, RAW264.7 macrophages and primary hepatocytes using TRIzol reagent (TaKaRa, Kusatsu, Japan). Complementary DNA was generated from 1 μg of RNA using a cDNA synthesis kit (Nanjing Vazyme Biotech, China). For real-time PCR, 10 ng of template was added to a 10-μL reaction system containing each primer and SYBR Green PCR Master Mix (TaKaRa, Kusatsu, Japan). The PCR thermocycling parameters were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, performed with an ABI Prism 7300 system (Applied Biosystems, Foster City, CA). All reactions were performed in triplicate. The expression levels of target genes were quantified using the double-delta method ($2^{-\Delta\Delta Ct}$). The murine primers (provided by Sangon Biotech Co., Shanghai, China) are shown in Table 1.

Western blotting

Total proteins extracted from mouse liver tissues, RAW264.7 macrophages and primary hepatocytes were assessed using a Pierce BCA protein assay kit (Thermo Fisher Scientific). The proteins were separated by SDS–PAGE (Epizyme Biotech), transferred to polyvinylidenedifluoride membranes (Bio-Rad, Hercules, CA) and incubated with primary antibody in TBST containing 5% (wt/vol) BSA at 4 °C overnight. The blots were then incubated with HRP-conjugated secondary antibody (1:10000, KangChen Biotech, Shanghai, China) at room temperature for 1 h. Immunoreactive bands were detected with an ECL chemiluminescence kit (Thermo Scientific Pierce, Waltham, MA). The density of the bands on the immunoblots was measured using ImageJ software (National Institutes of Health) and was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (1:10000, KangChen Biotech, Shanghai, China) or β-actin (1:5000, Cell Signaling Technology). In this study, the total expression levels of TLR4, IκBα, p-IκB α, NF-κB and p-NF-κB (1:1000, all from Cell Signaling Technology) in macrophages were measured and normalized to the β-actin (1:5000, Cell Signaling Technology) expression level. The total expression levels of NLRP3, IL-1β, Caspase-1, Nrf2, Keap1 and HO-1 (1:1000, all from Cell Signaling Technology) in hepatocytes were measured and normalized to the GAPDH (1:10000, KangChen Biotech, Shanghai, China) expression level.

Statistical analysis

All statistical analyses were carried out with GraphPad Prism v7.03 software (GraphPad, La Jolla, CA, United States). All the data are expressed as the mean ± SE of the mean. Statistical differences among multiple groups were determined by one- or two-way analysis of variance. Differences between two groups were analyzed using Student's *t* test. A P value < 0.05 was considered statistically significant.

RESULTS

Lipid-laden primary hepatocytes have direct effects on M1/M2 macrophage polarization and inflammation

Because of their high plasticity and heterogeneity, macrophages can be skewed into the M1 phenotype or M2 phenotype under different microenvironments[26]. However, whether lipid-laden hepatocytes can affect macrophage polarization is uncertain. Here, we isolated primary hepatocytes and incubated them with FFAs. After 24 h of culture, most primary hepatocytes adhered to the plate and exhibited centered dual nuclei and a polyhedral shape under the microscope, indicating that the primary hepatocytes were in good condition (Figure 1A). Moreover, the hepatocytes displayed excess lipid accumulation after incubation with FFAs, as shown by Oil Red O staining (Figure 1B). At the same time, the TG and T-CHO contents generated in hepatocytes were significantly increased (Figure 1C). The mRNA expression levels of the lipid synthesis genes Fasn and Srebp1c were upregulated, and the mRNA expression levels of the lipid decomposition genes Acox1 and Cpt1a were downregulated (Figure 1D). These results suggested that the NAFLD hepatocyte model was successfully established. Next, a supernatant transfer experiment between lipid-laden hepatocytes and macrophages was established. We found that CM from FFA-treated hepatocytes induced M1-polarized macrophages with significant upregulation of all M1 markers, including Nos2, Tnf and Il-6, and partial downregulation of

Table 1 Murine primers

Primer	Forward (5'-3')	Reverse (5'-3')
Nos2	GTGTTCCACCAGGAGATGTTG	CTCCTGCCCACTGAGTTCGTC
Tnf	TCTTCTCATTCCTGCTTGTGG	GGTCTGGGCCATAGAACTGA
Il-6	GTTCTCTGGGAAATCGTGGA	GGAAATTGGGGTAGGAAGGA
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Mrc2	TACAGCTCCACGCTATGGATT	CACTCTCCCAGTTGAGGTACT
Il-10	GTTACTTGGGTTGCCAAG	TTGATCATCATGTATGCTTC
Acox1	ACCAGCCCAACTGTGACTTC	ACAAAGGCATGTAACCCGTA
Cpt1a	CTTCCCATTTGACACCTTTG	ATACGTGAGGCAGAACTTGC
Srebp1c	ACAGCAACCAGAAGCTCAAG	TGCCCTCCATAGACACATCT
Fasn	TTGGGTGCTGACTACAACCT	TGGATGATGTTGATGATGGA
Keap1	AGAGCGGGATGAGTGGCA	GCTGAATTAAGGCGGTTTGTC
Nrf2	CTTTAGTCAGCGACAGAAGGAC	AGGCATCTTGTTTGGGAATGTG
Ho-1	AGACCGCCTTCCTGCTCAACAT	TCTGACGAAGTGACGCCATCTGT
Nlrp3	GAGTTCTTCGCTGCTATGT	ACCTTCACGTCTCGGTTC
Caspase-1	TGGAGAGAAACAAGGAG	TTGAAGAGCAGAAAGCAAT
Il-1β	TCTTTGAAGTTGACGGACCC	TGAGTGATACTGCCTGCCTG
Ppar-γ	GCCCTTTACCACAGTTGATTTCT	GTGATTTGTCCGTTGTCTTTCCT
β-actin	TGTTACCAACTGGGACGACA	CTGGGTCATCTTTTCACGGT

Acox1: Acyl-CoA oxidase 1; Arg1: Arginine-1; Caspase-1: Cysteinyl aspartate-specific proteinase-1; Cpt1a: Carnitine palmitoyltransferase 1A; Fasn: Fatty acid synthase; Ho-1: Heme oxygenase-1; Il: Interleukin; Nos: Nitric oxide synthase; Keap1: Kelch-like ECH-associated protein 1; Mrc2: Mannose receptor 2; Nlrp3: NLR family pyrin domain-containing 3; Nrf2: NF-E2-related factor 2; Ppar-γ: Peroxisome proliferators-activated receptor-γ; Srebp1c: Sterol-regulatory element-binding protein 1C; Tnf: Tumor necrosis factor.

M2 markers, such as Il-10 (Figure 1E). In addition, the NF-κB signaling pathway in macrophages was activated by CM-FFA, as demonstrated by significant increases in the protein expression levels of TLR4, p-NF-κB and p-IκBα (Figure 1F). These results demonstrate that lipid-laden hepatocytes exert direct roles in M1 macrophage polarization and inflammation.

Lipid-laden hepatocytes induce macrophage M1 polarization and inflammation via IL-1β signaling

Lipid-laden hepatocytes promoted M1 macrophage polarization and inflammation; however, the possible pathways of signal exchange between the primary hepatocytes and macrophages were unclear. We found that incubation with FFAs obviously increased the mRNA and protein expression levels of the inflammatory factor IL-1β in hepatocytes (Figure 2A and B). Similarly, lipid-laden hepatocytes secreted a high level of IL-1β into the cell culture supernatant (Figure 2C). To further investigate whether IL-1β participates in the signaling between hepatocytes and macrophages, we pretreated macrophages with an interleukin-1 receptor antagonist (IL-1Ra) to block IL-1β receptors. Then, a supernatant transfer experiment between lipid-laden hepatocytes and macrophages was conducted. The results showed that IL-1β expression in macrophages was significantly decreased with IL-1Ra pretreatment (Figure 2D). As expected, we found that inhibition of IL-1β signaling with IL-1Ra significantly prevented macrophage M1 polarization induced by CM-FFAs, as shown by the downregulation of M1-type markers and the upregulation of M2-type markers (Figure 2E). Simultaneously, IL-1Ra suppressed the protein expression levels of TLR4, p-NF-κB and p-IκBα in macrophages induced by CM-FFA (Figure 2F). These results indicate that NAFLD hepatocytes induce M1 macrophage polarization and inflammatory signal activation *via* IL-1β signaling.

Upregulation of PPAR-γ activity ameliorates oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes

PPAR-γ is a nuclear receptor that is firmly involved in lipid metabolism and the inflammatory-immune response[27]. To further explore the role and properties of PPAR-γ in lipid-laden primary hepatocytes, the PPAR-γ agonist GW1929 was added to the hepatocyte culture system for 3 h before incubation with FFAs. The results showed that GW1929 administration significantly decreased the ROS content and IL-1

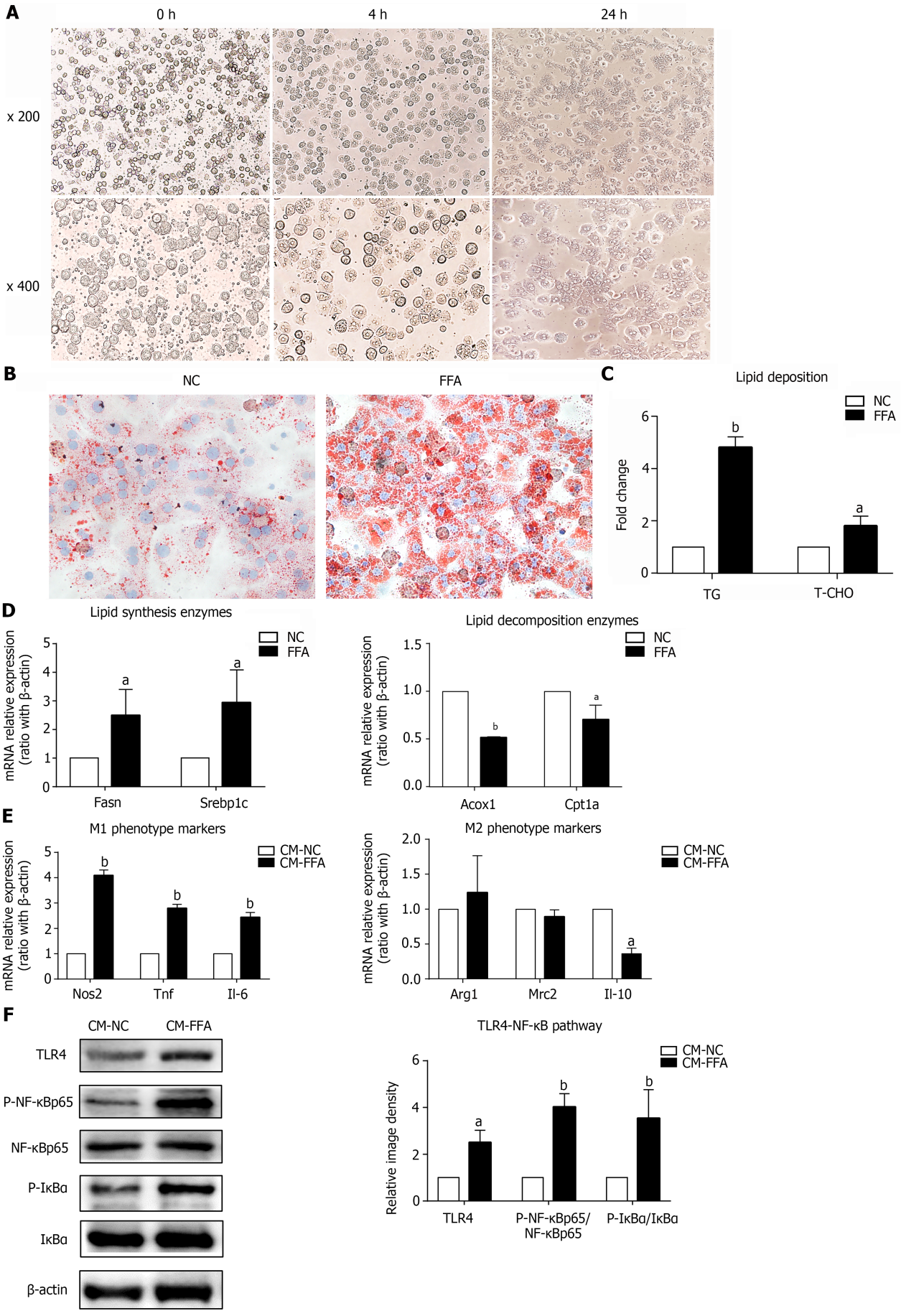


Figure 1 Lipid-laden primary hepatocytes have direct effects on macrophage M1/M2 polarization and inflammation. Primary hepatocytes were

incubated with free fatty acids for 24 h to induce the nonalcoholic fatty liver disease hepatocyte model. Cell culture supernatants of hepatocytes were collected and prepared for different conditioned mediums (CMs). RAW264.7 macrophages were treated with different CMs for 6 h for RT–PCR or 24 h for western blotting. A: Primary hepatocytes isolated by in situ perfusion of collagenase (inverted microscope, × 200, × 400); B: Lipid accumulation in hepatocytes measured by Oil Red O staining (× 400); C: Triglyceride and total cholesterol contents in primary hepatocytes; D: mRNA expression of lipid-related genes in primary hepatocytes; E: mRNA expression of M1/M2 markers in macrophages treated with CM; F: Protein expression of the TLR4/NF-κB pathway in macrophages treated with CM. Values are expressed as the mean ± SE of the mean, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal control (NC) or CM-NC, $n = 3$ experiments. NC: Normal control; CM: Conditioned medium; Fasn: Fatty acid synthase; Srebp1c: Sterol-regulatory element-binding protein 1C; Acox1: Acyl-CoA oxidase 1; Cpt1a: Carnitine palmitoyltransferase 1A; Nos: Nitric oxide synthase; Tnf: Tumor necrosis factor; Il: Interleukin; Arg1: Arginine-1; Mrc2: Mannose receptor 2; TLR4: Toll-like receptor 4; NF-κB: Nuclear factor kappa-B; IκBα: Inhibitor of nuclear factor kappa-B.

β secretion level in hepatocytes treated with FFAs (Figure 3A and B). In addition, GW1929 significantly downregulated both the mRNA and protein expression levels of NLRP3 inflammasome-related genes, including Nlrp3, Caspase-1 and IL-1β, in lipid-laden hepatocytes (Figure 3C and D). Furthermore, GW1929 markedly reduced the mRNA and protein expression levels of the oxidative injury marker Keap1 but enhanced the mRNA and protein expression levels of the antioxidant-related genes Nrf2 and Ho-1 (Figure 3E and F). These results indicate that upregulation of PPAR-γ activity in NAFLD hepatocytes can ameliorate oxidative stress and NLRP3-IL-1β pathway activation.

Hepatocyte-specific PPAR-γ knockout aggravates oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes

To further confirm the anti-inflammatory and antioxidant effects of PPAR-γ on lipid-laden hepatocytes, we isolated primary hepatocytes from hepatocyte-specific PPAR-γ knockout mice and treated them with FFAs in vitro. The mRNA expression level of Ppar-γ in primary hepatocytes from hepatocyte-specific PPAR-γ knockout mice was fully knocked out (Figure 4A). As expected, the loss of PPAR-γ in hepatocytes enhanced IL-1β secretion and ROS generation after incubation with FFAs (Figure 4B and C). In addition, PPAR-γ deficiency in lipid-laden hepatocytes increased the mRNA and protein expression levels of the Nlrp3, Caspase-1 and IL-1β genes (Figure 4D and E). Furthermore, PPAR-γ deficiency markedly increased the mRNA and protein expression levels of Keap1 but decreased the mRNA and protein expression levels of Nrf2 and Ho-1 (Figure 4F and G). These results further confirm that PPAR-γ exerts a protective effect against lipid peroxidation and inflammation in lipid-laden hepatocytes.

Regulation of PPAR-γ activity in lipid-laden hepatocytes affects macrophage polarization and inflammation

Next, we further explored whether regulation of PPAR-γ activity in lipid-laden hepatocytes would subsequently affect macrophage polarization shifts and inflammation. Macrophages were incubated with CM derived from hepatocytes that were pretreated with GW1929 or FFAs alone or with CM from PPAR-γ-deficient hepatocytes treated with FFAs. Interestingly, the increase in M1 marker expression was significantly downregulated and the expression levels of the M2 markers Arg1 and Il-10 were markedly upregulated in macrophages incubated with CM from lipid-laden hepatocytes that were pretreated with GW1929 (CM-GW1929+FFA) compared with the CM-FFA group (Figure 5A). In addition, the protein expression levels of TLR4, p-NF-κB and p-IκBα were significantly decreased in macrophages from the CM-GW1929+FFA group (Figure 5B). In contrast, depletion of PPAR-γ in lipid-laden hepatocytes significantly upregulated the mRNA expression levels of all M1 markers but decreased the mRNA expression levels of Arg1 and Mrc2 in macrophages (Figure 5C). Furthermore, depletion of PPAR-γ in lipid-laden hepatocytes further promoted activation of the TLR4/NF-κB signaling pathway in macrophages (Figure 5D). These results illustrate that regulation of PPAR-γ activity in NAFLD hepatocytes can modulate macrophage polarization and inflammation.

Rosiglitazone improved NLRP3 inflammasome activation and oxidative stress in high-fat diet-induced NAFLD mice

Our in vitro experiments demonstrated that PPAR-γ exerts anti-inflammatory and antioxidant effects in lipid-laden hepatocytes, which can further affect macrophage polarization and inflammation. Next, we further explored whether these effects also occur in vivo. Our previous studies showed that rosiglitazone administration improves hepatic steatosis and Kupffer cell activation in high-fat diet-induced NAFLD mice. Here, we found that rosiglitazone significantly decreased the mRNA expression levels of the Nlrp3, Caspase-1 and Il-1β genes in bulk cells from the livers of high-fat diet-induced NAFLD mice (Figure 6A). Furthermore, rosiglitazone markedly downregulated the mRNA expression level of Keap1 but increased the mRNA expression levels of Ppar-γ, Nrf2 and Ho-1 (Figure 6B). The IL-1β level in plasma and the Caspase-1 activity in the liver both declined after rosiglitazone administration (Figure 6C and D), while the SOD activity and GSH content were significantly enhanced in rosiglitazone-treated mouse plasma (Figure 6E). In contrast, the levels of oxidative injury metabolites, such as ROS and MDA, in plasma were decreased after rosiglitazone intervention (Figure 6F). Therefore, we concluded that rosiglitazone not only alleviated hepatic steatosis and Kupffer cell activation but also

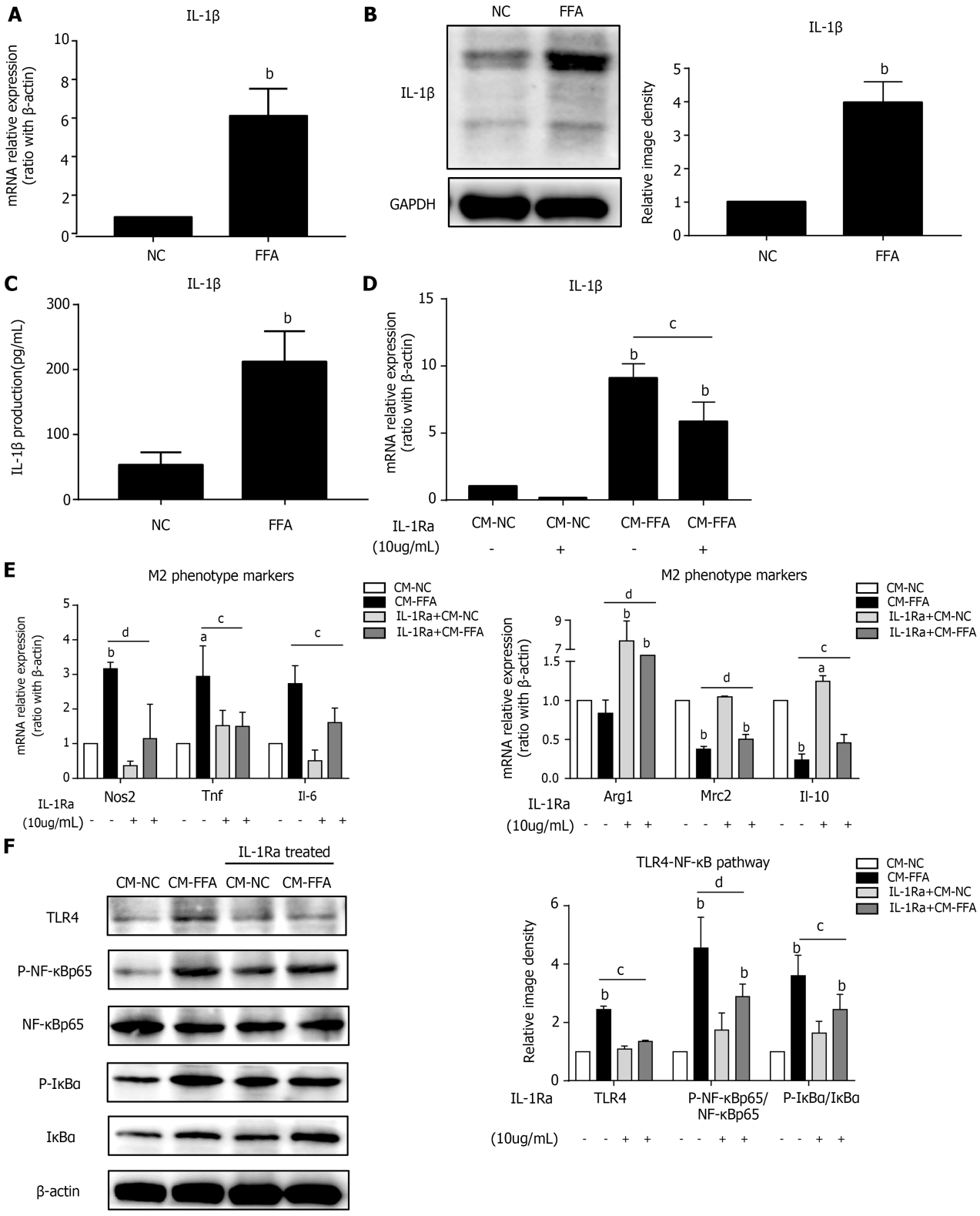


Figure 2 Lipid-laden hepatocytes induce macrophage M1 polarization and inflammation *via* IL-1β signaling. Primary hepatocytes were incubated with free fatty acids for 6 h for RT–PCR or 24 h for western blot and ELISA. Cell culture supernatants of hepatocytes were collected and prepared for different conditioned mediums (CMs). RAW264.7 macrophages were pretreated with interleukin-1 receptor antagonist and treated with different CMs for 6 h for RT–PCR or 24 h for western blotting. A: mRNA expression of Il-1β in primary hepatocytes; B: Protein expression of IL-1β in primary hepatocytes; C: IL-1β production in the hepatocyte culture supernatant; D: mRNA expression of Il-1β in macrophages; E: mRNA expression of M1/M2 markers in macrophages; F: Protein expression of the TLR4/NF-κB pathway in macrophages. Values are expressed as the mean ± SE of the mean, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal control (NC) or CM-NC; $^{c}P < 0.05$, $^{d}P < 0.01$ comparison of the designated two groups, $n = 3$ experiments. NC: Normal control; CM: Conditioned medium; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL-1Ra: Interleukin-1 receptor antagonist; Nos: Nitric oxide synthase; Tnf: Tumor necrosis factor; Il: Interleukin; Arg1: Arginine-1; Mrc2: Mannose receptor 2; TLR4: Toll-like receptor 4; NF-κB: Nuclear factor kappa-B; IκBα: Inhibitor of nuclear factor kappa-B.

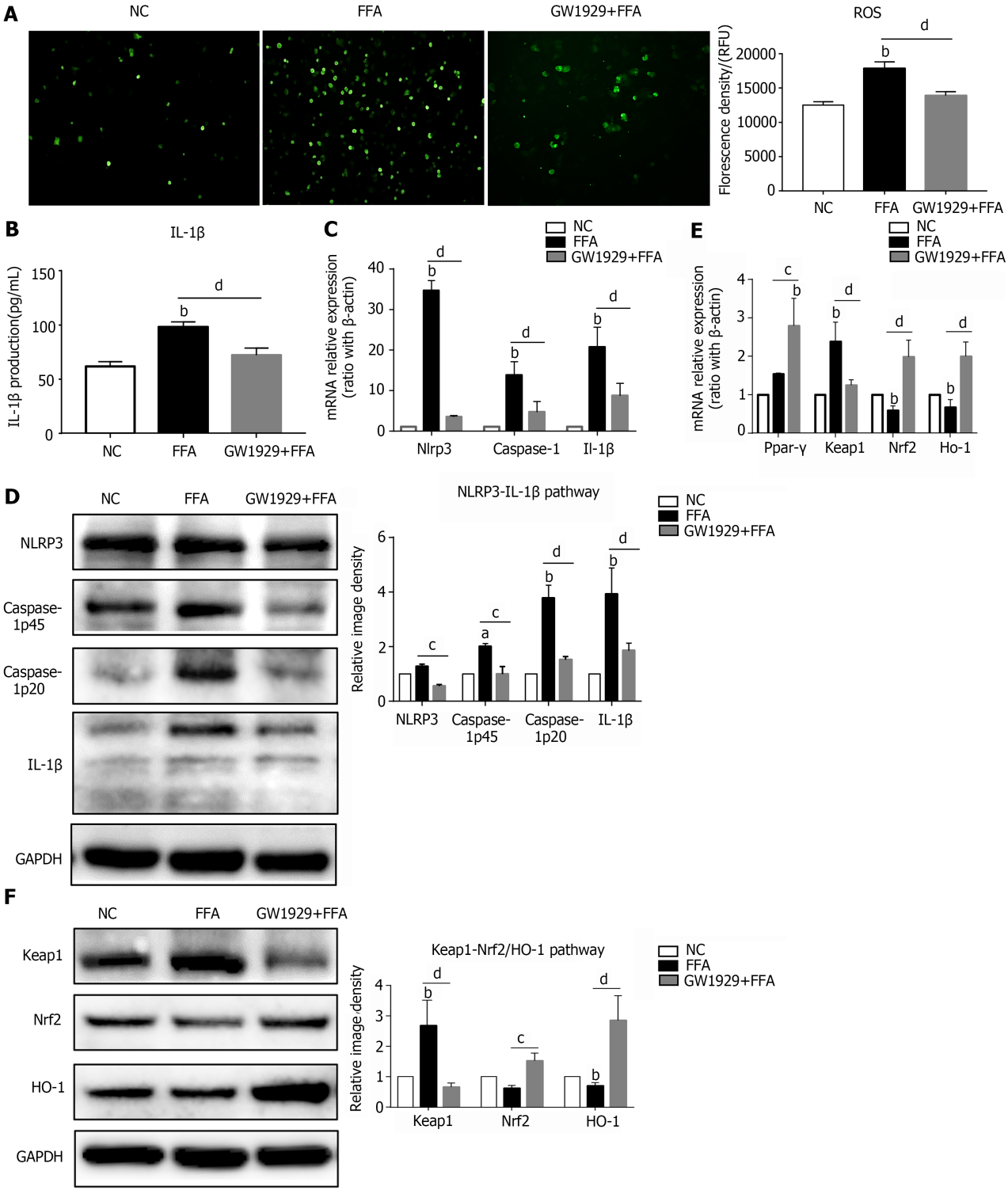
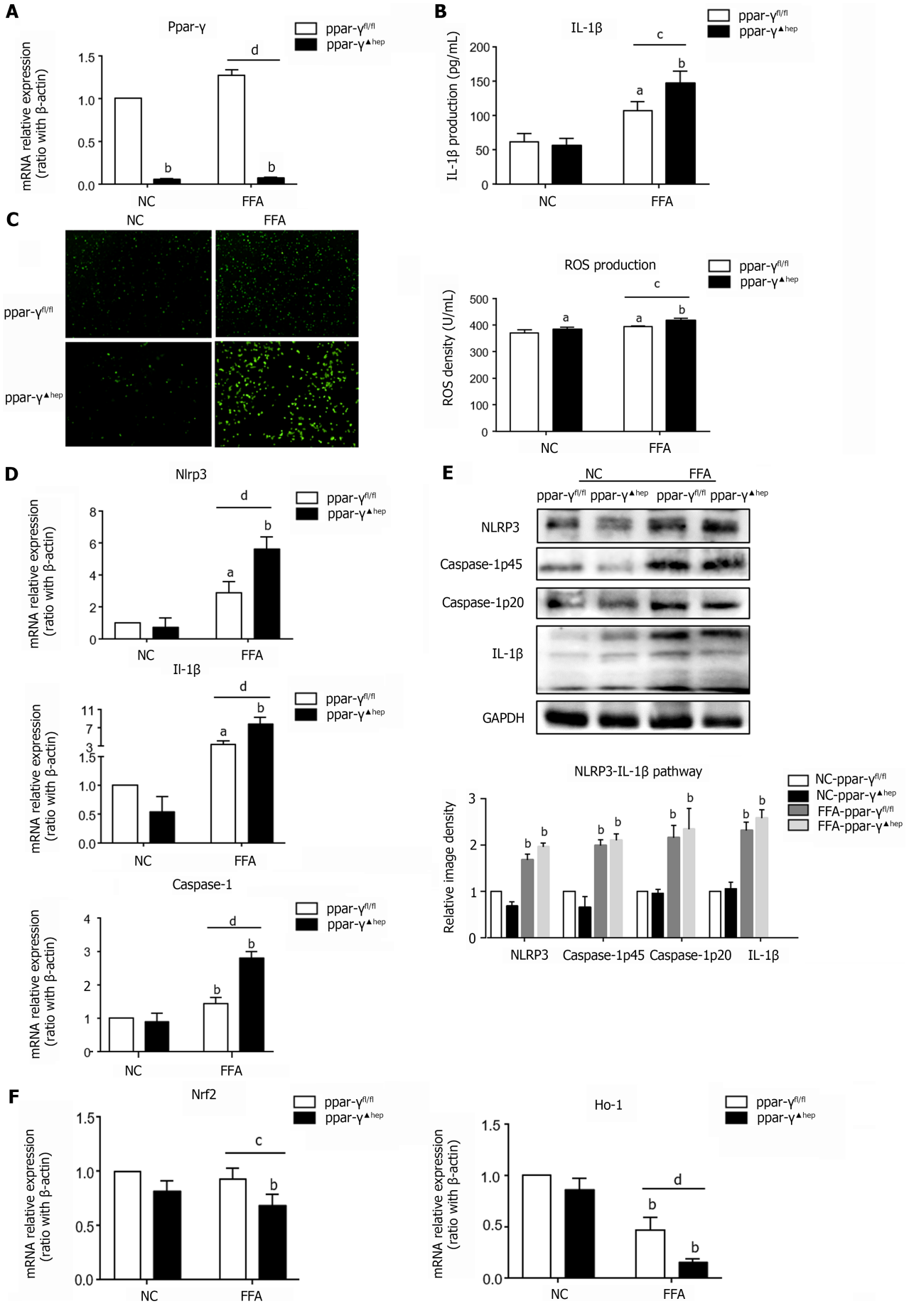


Figure 3 Upregulating PPAR-γ activity ameliorates oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes. Primary hepatocytes were preincubated with the PPAR-γ agonist GW1929 for 3 h, followed by treatment with free fatty acids for 6 h for RT–PCR or 24 h for western blot and reactive oxygen species (ROS) detection. A: ROS production in primary hepatocytes; B: IL-1β production in the hepatocyte culture supernatant; C: mRNA expression of NLRP3 inflammasome-related genes in hepatocytes; D: Protein expression of NLRP3 inflammasome-related genes in hepatocytes; E: mRNA expression of Ppar-γ and oxidative stress-related genes in hepatocytes; F: Protein expression of oxidative stress-related genes in hepatocytes. Values are expressed as the mean ± SE of the mean, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal control; $^{c}P < 0.05$, $^{d}P < 0.01$ comparison of the designated two groups, $n = 3$ experiments. NC: Normal control; ROS: Reactive oxygen species; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; Keap1: Kelch-like ECH-associated protein 1; Nrf2: NF-E2-related factor 2; Ho-1: Heme oxygenase-1; Nlrp3: NLR family pyrin domain-containing 3; Caspase-1: Cysteinyl aspartate-specific proteinase-1; Ppar-γ: Peroxisome proliferators-activated receptor-γ.

improved NLRP3 inflammasome activation and oxidative stress in HF diet-fed NAFLD mice.


A
Ppar-γ
ppar-γfl/fl
ppar-γ▲hep
mRNA relative expression (ratio with β-actin)
NC
FFA
B
IL-1β
IL-1β production (pg/mL)
C
NC
FFA
ppar-γfl/fl
ppar-γ▲hep
ROS production
ROS density (U/mL)
D
Nlrp3
Il-1β
Caspase-1
E
NC
FFA
NLRP3
Caspase-1p45
Caspase-1p20
IL-1β
GAPDH
NLRP3-IL-1β pathway
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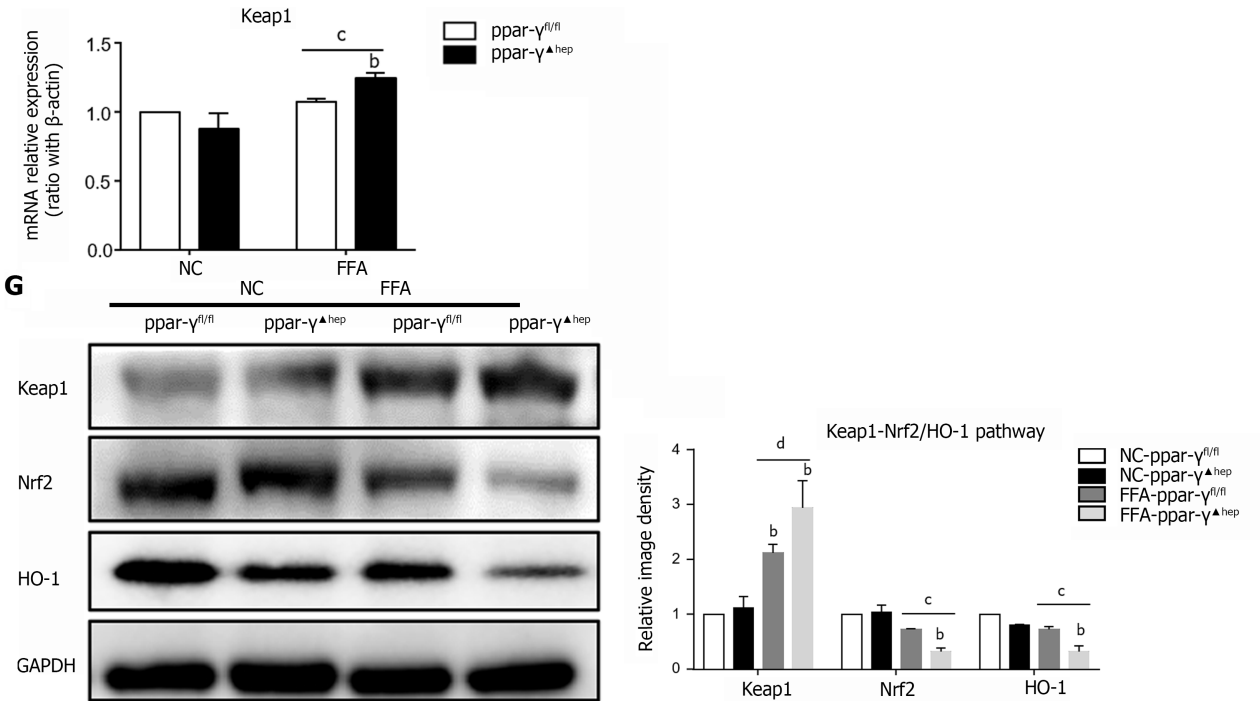


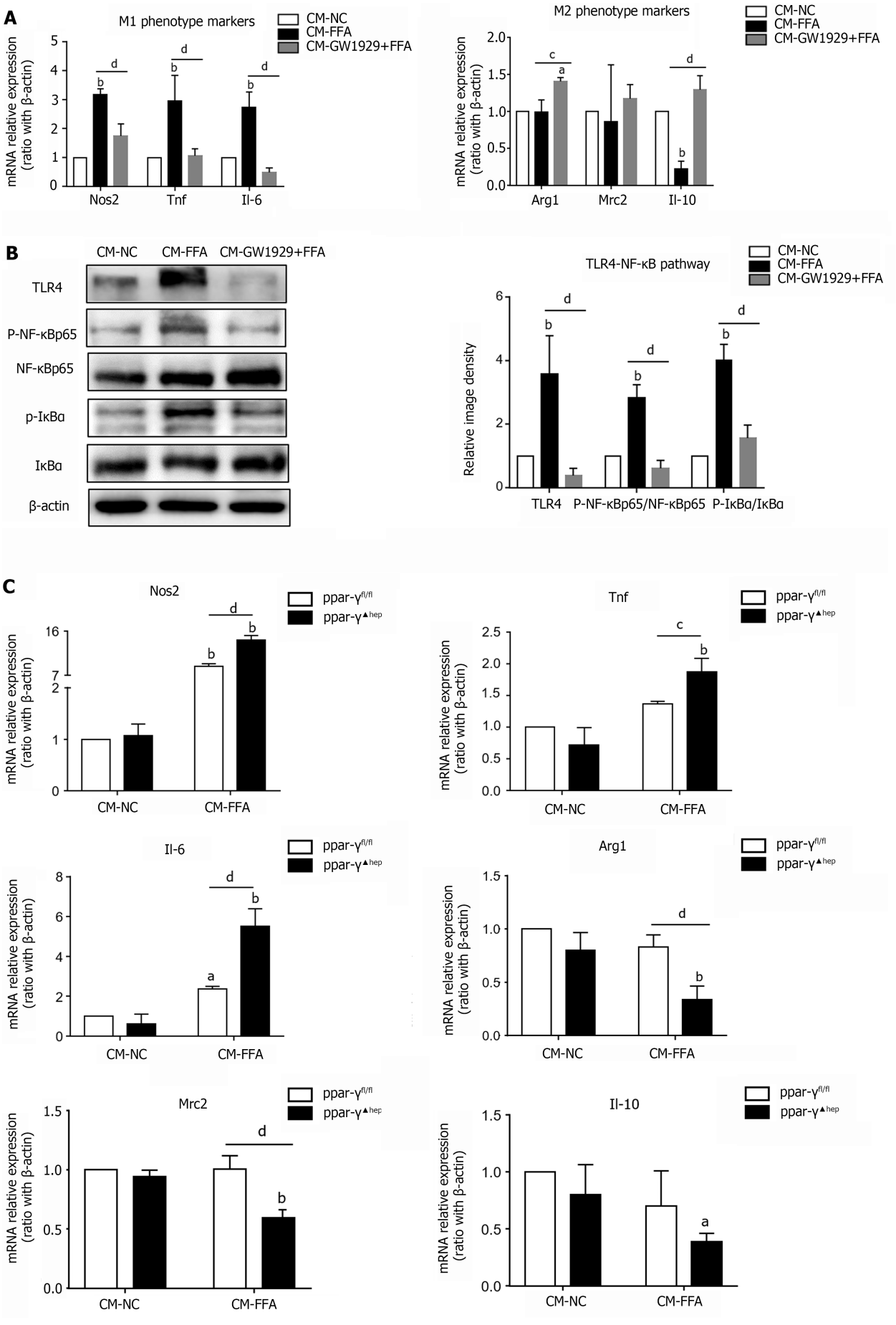
Figure 4 Hepatocyte-specific PPAR-γ knockout aggravates oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes. Primary hepatocytes were isolated from PPAR-γ$^{fl/fl}$ and PPAR-γ$^{▲hep}$ mice and treated with free fatty acids for 6 h for RT–PCR or 24 h for western blot, ELISA and reactive oxygen species (ROS) detection. A: mRNA expression of Ppar-γ in PPAR-γ-deficient hepatocytes; B: IL-1β production in PPAR-γ-deficient hepatocyte culture supernatant; C: ROS production in PPAR-γ deficiency hepatocytes; D: mRNA expression of NLRP3 inflammasome-related genes in PPAR-γ-deficient hepatocytes; E: Protein expression of NLRP3 inflammasome-related genes in PPAR-γ-deficient hepatocytes; F: mRNA expression of oxidative stress-related genes in PPAR-γ-deficient hepatocytes; G: Protein expression of oxidative stress-related genes in PPAR-γ-deficient hepatocytes. Values are expressed as the mean ± SE of the mean, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* ppar-γ$^{fl/fl}$ or normal control-ppar-γ$^{fl/fl}$; $^{c}P < 0.05$, $^{d}P < 0.01$ comparison of the designated two groups, $n = 3$ experiments. NC: Normal control; ROS: Reactive oxygen species; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Keap1: Kelch-like ECH-associated protein 1; Nrf2: NF-E2-related factor 2; Ho-1: Heme oxygenase-1; Nlrp3: NLR family pyrin domain-containing 3; Caspase-1: Cysteinyl aspartate-specific proteinase-1; Ppar-γ: Peroxisome proliferators-activated receptor-γ; IL: Interleukin.

DISCUSSION

Lipid metabolism disorder is a critical initiator of the inflammatory response and immune activation in NAFLD[10]. How lipids lead to innate immune activation is poorly understood. Here, we demonstrated that lipid-laden hepatocytes can transmit inflammatory signals to macrophages through the release of IL-1β, directly inducing M1 macrophage polarization and inflammatory activation. Regulation of PPAR-γ activity in lipid-laden hepatocytes further affected macrophage polarization and inflammation. In addition, an in vivo study showed that a PPAR-γ agonist improved NLRP3 inflammasome activation and oxidative stress in high-fat diet-induced NAFLD mice, expanding our understanding of the underlying mechanisms of PPAR-γ in NAFLD.

Macrophage activation is considered to be a prominent hallmark of NASH[28]. The polarization of macrophages is firmly related to the inflammatory state. Previous studies have suggested that macrophages are more likely to transform from the M1 to the M2 phenotype if activation of the NF-κB signaling pathway is inhibited[15]. TLR4 deficiency directly alters the polarization of adipose tissue macrophages toward alternative activation[29]. Upon activation, macrophages not only release inflammatory cytokines and chemokines but also regulate the status or function of surrounding cells[13,30]. Recent studies have demonstrated that selective depletion of Kupffer cells reduced liver steatosis and monocyte infiltration in NASH[31-33]. Our previous study confirmed that macrophages/Kupffer cells polarized by fatty acids can regulate lipid metabolism in hepatocytes[30]. This result suggests a potential interaction between macrophages and hepatocytes in NAFLD. In the current study, our results revealed that lipid-laden hepatocytes directly induced macrophage M1 polarization and TLR4/NF-κB pathway activation. Through an in-depth study, we found that IL-1β secreted by lipid-laden hepatocytes mediates the communication between hepatocytes and macrophages. Interestingly, we confirmed that when macrophages were blocked with an IL-1β receptor inhibitor, M1 macrophage polarization induced by lipid-laden hepatocytes was weakened. Furthermore, we verified that residual fatty acids in hepatocyte supernatant alone were not sufficient to induce macrophages toward M1 polarization (Supplementary Figure 1).

PPAR-γ is a ligand-activated nuclear receptor with potent anti-inflammatory properties and is involved in the regulation of immune and inflammatory responses[34]. A recent study demonstrated


A
M1 phenotype markers
CM-NC
CM-FFA
CM-GW1929+FFA
mRNA relative expression (ratio with β-actin)
Nos2
Tnf
Il-6
M2 phenotype markers
Arg1
Mrc2
Il-10
B
CM-NC
CM-FFA
CM-GW1929+FFA
TLR4
P-NF-κBp65
NF-κBp65
p-IκBα
IκBα
β-actin
TLR4-NF-κB pathway
Relative image density
TLR4
P-NF-κBp65/NF-κBp65
P-IκBα/IκBα
C
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Tnf
Il-6
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Mrc2
Il-10
ppar-γfl/fl
ppar-γ▲hep
CM-NC
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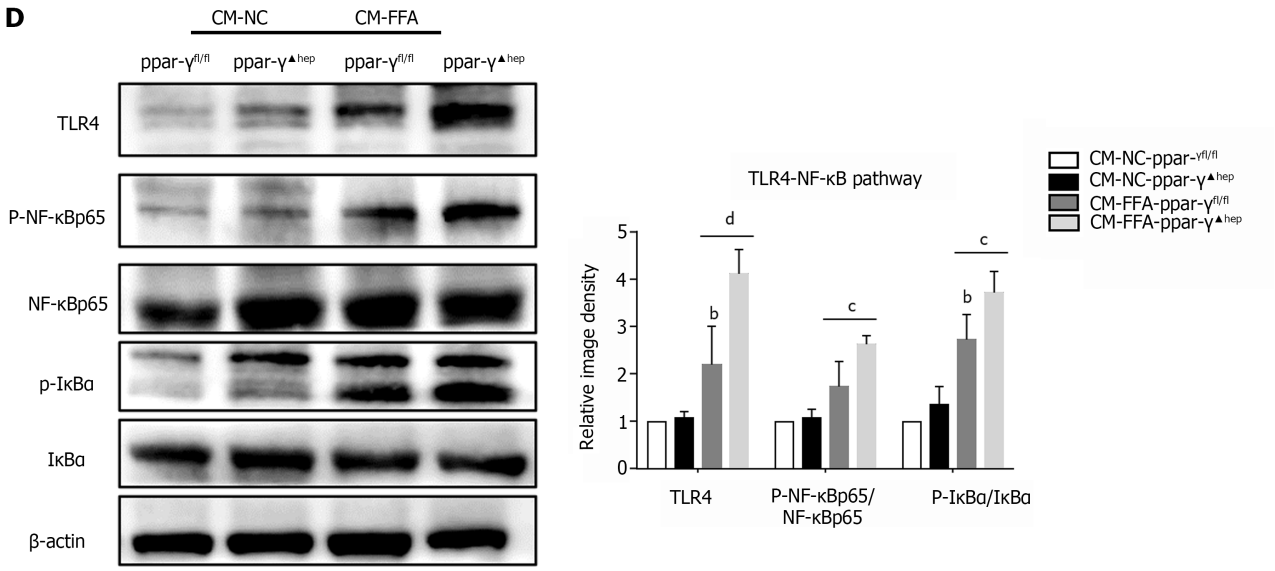

Figure 5 Regulation of PPAR-γ activity in lipid-laden hepatocytes affects macrophage polarization and inflammation. Primary hepatocytes were preincubated with the PPAR-γ agonist GW1929 for 3 h, followed by treatment with free fatty acids (FFAs) for 24 h (GW1929+FFA). Primary hepatocytes were isolated from PPAR-$\gamma^{fl/fl}$ and PPAR-$\gamma^{\blacktriangle hep}$ mice and treated with FFA for 24 h. Cell culture supernatants of hepatocytes were collected and prepared for different conditioned mediums (CMs). Macrophages were treated with different CMs for 6 h for RT–PCR or 24 h for western blotting. A: mRNA expression of M1/M2 markers in macrophages treated with CM; B: Protein expression of the TLR4/NF-κB pathway in macrophages treated with CM; C: mRNA expression of M1/M2 markers in macrophages treated with CM from PPAR-γ knockout hepatocytes; D: Protein expression of the TLR4/NF-κB pathway in macrophages treated with CM from PPAR-γ knockout hepatocytes. Values are expressed as the mean ± SE of the mean, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* CM- normal control (NC) or CM-NC-ppar-$\gamma^{fl/fl}$; $^{c}P < 0.05$, $^{d}P < 0.01$ comparison of the designated two groups, $n = 3$ experiments. NC: Normal control; CM: Conditioned medium; Nos: Nitric oxide synthase; Tnf: Tumor necrosis factor; Il: Interleukin; Arg1: Arginine-1; Mrc2: Mannose receptor 2; TLR4: Toll-like receptor 4; NF-κB: Nuclear factor kappa-B; IκBα: Inhibitor of nuclear factor kappa-B.

that modulation of PPAR-γ activity attenuated HFD-induced NAFLD by regulating lipid metabolism and oxidative stress in hepatocytes *via* Nrf2 activation[35]. Due to the low expression of PPAR-γ in hepatocytes, the role of PPAR-γ in hepatocytes is not fully understood[36]. In the current study, we found that ROS generation and IL-1β secretion in lipid-laden hepatocytes were significantly reduced when cells were pretreated with the PPAR-γ agonist GW1929. Recently, a study reported that PPAR-γ exerted an anti-inflammatory effect by suppressing NLRP3 inflammasome activation in macrophages [37]. Here, we revealed that a PPAR-γ agonist exerted an anti-inflammatory effect by inhibiting NLRP3 inflammasome activation in lipid-laden hepatocytes. In contrast, PPAR-γ deficiency in hepatocytes enhanced ROS generation, NLRP3 inflammasome activation, and IL-1β secretion after FFA treatment in vitro.

Interestingly, we further found that upregulation of PPAR-γ activity in lipid-laden hepatocytes subsequently reversed macrophage M1 polarization and reduced activation of the TLR4/NF-κB pathway. Conversely, PPAR-γ depletion in lipid-laden hepatocytes exacerbated macrophage M1 polarization and TLR4/NF-κB pathway activation. This result suggests that the regulation of PPAR-γ activity in hepatocytes plays an important role in the interaction between lipid-laden hepatocytes and macrophages. Our previous study revealed that rosiglitazone, a PPAR-γ agonist, significantly alleviated hepatic lipid deposition and Kupffer cell activation in HFD-induced NAFLD mice[17]. In the present study, our results clearly demonstrated that rosiglitazone mitigated NLRP3 inflammasome activation and oxidative stress in HFD-induced NAFLD mice.

A recent study showed that hepatocyte-specific PPAR-γ disruption reduced hepatic steatosis but increased hepatic neutrophil infiltration after HFD feeding plus binge ethanol[38]. PPAR-γ deletion in hepatocytes highly augmented PA- or TNF-α-induced production of Cxcl1. This result indicates that PPAR-γ activation in hepatocytes exerted an anti-inflammatory effect, which was consistent with our findings. Another study found that hepatocyte-specific loss of PPAR-γ reduced the progression of high fat, cholesterol, and fructose (HFCF)-induced NASH in mice[39]. These findings seem to suggest that hepatocyte PPAR-γ contribute to the development of inflammation and fibrosis in NASH. However, the hepatocyte-specific loss of PPAR-γ did not reduce hepatic steatosis in HFCF- or MCD-induced NASH [39]. These contradictory experimental results may be due to the use of different animal models in the studies. In the current study, lipid-laden hepatocytes were established by incubation excess FFAs in vitro. The classical lipogenic property of PPAR-γ is significantly involved in this condition[40]. In addition, we further found that PPAR-γ activation exerted obvious antioxidant and anti-inflammatory effects in lipid-laden hepatocytes. We assumed that FFA overload directly led to lipotoxicity in hepatocytes, which contributed to lipid peroxidation. Thus, PPAR-γ was upregulated and exerted antioxidant effects against ROS to mitigate cell injury and downstream inflammatory events. Therefore, the role of PPAR-γ in hepatocytes is firmly related to different models, diet patterns and cellular

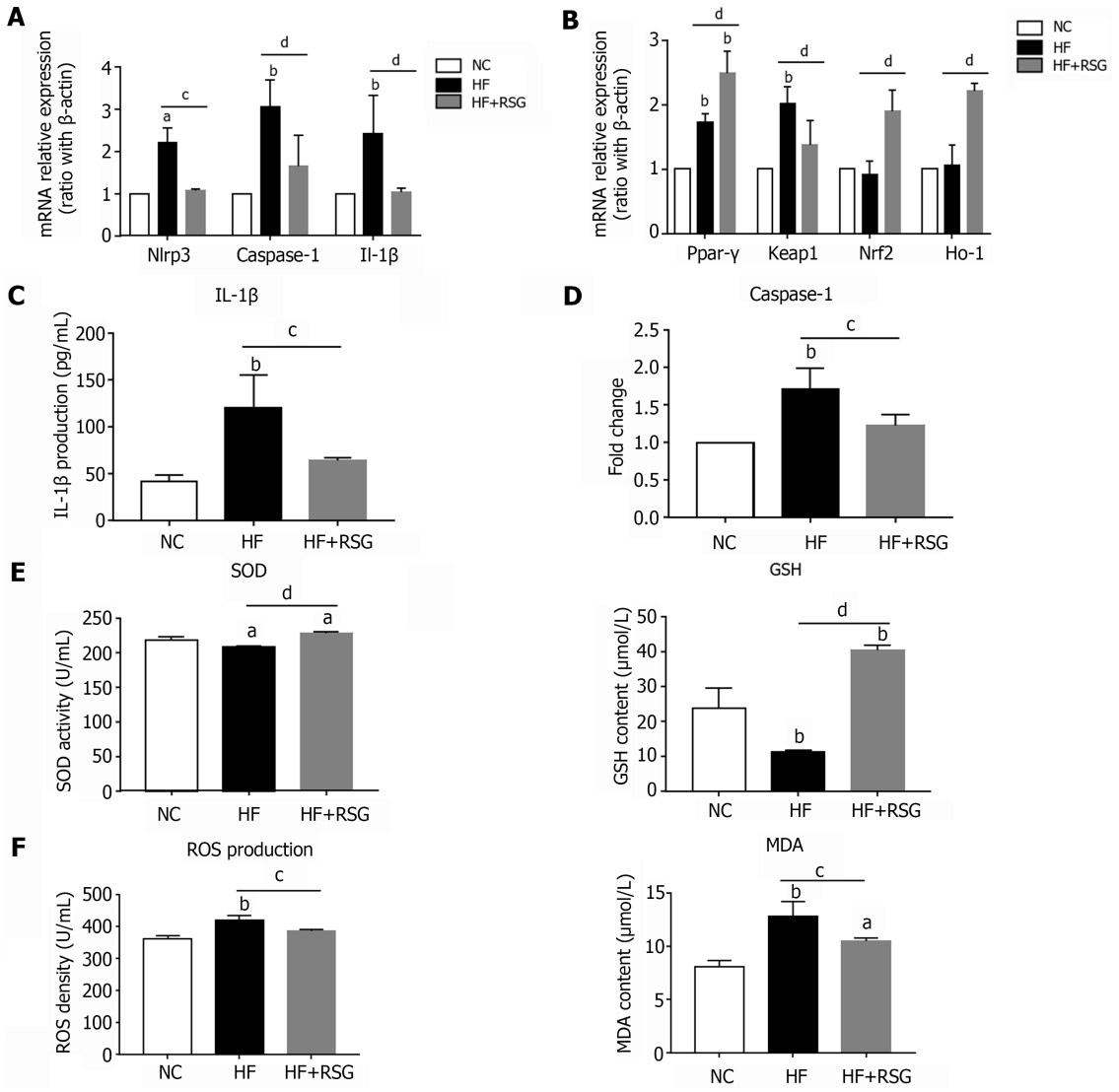

Figure 6 Rosiglitazone improved NLRP3 inflammasome activation and oxidative stress in high-fat diet-induced nonalcoholic fatty liver disease mice. Wild-type C57BL/6 mice were fed either an normal control diet or an high-fat diet for 16 wk. Rosiglitazone was administered by oral gavage once daily for 28 consecutive days after 12 wk of HF diet feeding. A: mRNA expression of NLRP3 inflammasome-related genes in the bulk cells of liver; B: mRNA expression of Ppar-γ and oxidative stress-related genes in the liver; C: IL-1β Level in the plasma; D: Caspase-1 activity in the liver; E: SOD activity and GSH content in the plasma; F: ROS and MDA production in the plasma. Values are expressed as the mean ± SE of the mean, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* NC; $^{c}P < 0.05$, $^{d}P < 0.01$ comparison of the designated two groups, $n = 10$ animals per group. HF: High-fat; NC: Normal control; RSG: Rosiglitazone; Keap1: Kelch-like ECH-associated protein 1; Nrf2: NF-E2-related factor 2; Ho-1: Heme oxygenase-1; Nlrp3: NLR family pyrin domain-containing 3; Caspase-1: Cysteinyl aspartate-specific proteinase-1; Ppar-γ: Peroxisome proliferators-activated receptor-γ; IL: Interleukin; SOD: Superoxide dismutase; GSH: Glutathione; MDA: Malondialdehyde; ROS: Reactive oxygen species.

stimulations. PPAR-γ is a complex nuclear receptor with specific dominant properties in various disease courses.

CONCLUSION

In conclusion, our study revealed that lipid-laden hepatocytes significantly skewed macrophage polarization to the M1 phenotype. Upregulation of PPAR-γ activity in lipid-laden hepatocytes improved macrophage M1 polarization and inflammation by attenuating hepatocyte oxidative stress and NLRP3 inflammasome activation. Strategies that target the regulation of PPAR-γ activity to modulate cell crosstalk will be beneficial for treating NAFLD.

ARTICLE HIGHLIGHTS

Research background

Lipid metabolism disorder and inflammatory-immune activation are two vital triggers in nonalcoholic fatty liver disease (NAFLD). Little is known about the regulation of PPAR-γ activity in modulating cell crosstalk in NAFLD.

Research motivation

The role of PPAR-γ in hepatocytes and in the interaction between hepatocytes and macrophages in NAFLD remain unknown.

Research objectives

To investigate whether the regulation of PPAR-γ activity in lipid-laden hepatocytes affects macrophage polarization and inflammation and explore the potential mechanisms.

Research methods

Primary hepatocytes were isolated from wild-type C57BL6/J mice or hepatocyte-specific PPAR-γ knockout mice and incubated with free fatty acids (FFAs). Macrophages were incubated with conditioned medium from lipid-laden hepatocytes with or without the PPAR-γ agonist. Wild-type C57BL/6J mice were fed a high-fat diet and administered rosiglitazone.

Research results

Primary hepatocytes exhibited significant lipid deposition and increased ROS production after incubation with FFAs. Conditioned medium from lipid-laden hepatocytes promoted macrophage polarization to the M1 type and activation of the TLR4/NF-κB pathway. A PPAR-γ agonist ameliorated oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes and subsequently prevented M1 macrophage polarization. Hepatocyte-specific PPAR-γ deficiency aggravated oxidative stress and NLRP3 inflammasome activation in lipid-laden hepatocytes, which further promoted M1 macrophage polarization. Rosiglitazone administration improved oxidative stress and NLRP3 inflammasome activation in HF diet-induced NAFLD mice in vivo.

Research conclusions

Upregulation of PPAR-γ activity alleviated NAFLD through modulation of the crosstalk between hepatocytes and macrophages *via* the ROS-NLRP3-IL-1β signaling pathway.

Research perspectives

To elaborate the underlying pathogenesis of NAFLD from the perspective of inflammation and immune activation.

FOOTNOTES

Author contributions: Li XY, Ji PX, and Ni XX contributed equally to this work; Hua J designed the research; Li XY, Ji PX and Ni XX performed the research; Chen YX, Sheng L, Lian M and Guo CJ analyzed the data; Li XY and Hua J wrote the paper.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Renji Hospital.

Institutional animal care and use committee statement: All animal experiments fulfilled the Shanghai Jiao Tong University criteria for the humane treatment of laboratory animals and were approved by the Renji Hospital Animal Care and Use Committee (Permit number: RJ2018-0930). All methods were carried out in accordance with relevant guidelines and regulations.

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Data sharing statement: No additional data are available.

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

Country/Territory of origin: China

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P-Editor: Cai YX

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ORIGINAL ARTICLE

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Transcriptome changes in stages of non-alcoholic fatty liver disease

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the United States and globally. The currently understood model of pathogenesis consists of a 'multiple hit' hypothesis in which environmental and genetic factors contribute to hepatic inflammation and injury.

AIM
To examine the genetic expression of NAFLD and non-alcoholic steatohepatitis (NASH) tissue samples to identify common pathways that contribute to NAFLD and NASH pathogenesis.

METHODS
We employed the Search Tag Analyze Resource for Gene Expression Omnibus platform to search the The National Center for Biotechnology Information Gene Expression Omnibus to elucidate NAFLD and NASH pathology. For NAFLD, we conducted meta-analysis of data from 58 NAFLD liver biopsies and 60 healthy liver biopsies; for NASH, we analyzed 187 NASH liver biopsies and 154 healthy liver biopsies.

RESULTS
Our results from the NAFLD analysis reinforce the role of altered metabolism, inflammation, and cell survival in pathogenesis and support recently described contributors to disease activity, such as altered androgen and long non-coding RNA activity. The top upstream regulator was found to be sterol regulatory element binding transcription factor 1 (SREBF1), a transcription factor involved in lipid homeostasis. Downstream of SREBF1, we observed upregulation in CXCL10, HMGCR, HMGCS1, fatty acid binding protein 5, paternally expressed imprinted gene 10, and downregulation of sex hormone-binding globulin and insulin-like growth factor 1. These molecular changes reflect low-grade inflammation secondary to accumulation of fatty acids in the liver. Our results from the NASH analysis emphasized the role of cholesterol in pathogenesis. Top canonical pathways, disease networks, and disease functions were related to cholesterol synthesis, lipid metabolism, adipogenesis, and metabolic disease. Top upstream regulators included pro-inflammatory cytokines tumor necrosis factor and IL1B, PDGF BB, and beta-estradiol. Inhibition of beta-estradiol was shown to be related to derangement of several cellular downstream processes including metabolism, extracellular matrix deposition, and tumor suppression. Lastly, we found riciribine (an AKT inhibitor) and ZSTK-474 (a PI3K inhibitor) as potential drugs that targeted the differential gene expression in our dataset.

CONCLUSION
In this study we describe several molecular processes that may correlate with NAFLD disease and progression. We also identified ricirbine and ZSTK-474 as potential therapy.

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Bioinformatics; AKT inhibitor; Therapy

Core Tip: Our results from the non-alcoholic fatty liver disease analysis reinforce the role of altered metabolism, inflammation, and cell survival in pathogenesis and support recently described contributors to disease activity, such as altered androgen and lncrna activity. The top upstream regulator was found to be sterol regulatory element binding transcription factor 1 (SREBF1), a transcription factor involved in lipid homeostasis. Downstream of SREBF1, we observed upregulation in CXCL10, HMGCR, HMGCS1, FABP5, PEG10, and downregulation of SHBG and IGF1. These molecular changes reflect low-grade inflammation secondary to accumulation of fatty acids in the liver. Our results from the NASH analysis emphasized the role of cholesterol in pathogenesis. Top upstream regulators included pro-inflammatory cytokines TNF and IL1B, PDGF BB, and beta-estradiol. Inhibition of beta-estradiol was shown to be related to derangement of several cellular downstream processes including metabolism, extracellular matrix deposition, and tumor suppression. Lastly, we found riciribine (an AKT inhibitor) and ZSTK-474 (a PI3K inhibitor) as potential drugs that targeted the differential gene expression in our dataset.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease that is characterized by the accumulation of triglycerides within hepatocytes. This process strongly resembles alcohol-induced fatty liver damage but occurs in the absence of excessive alcohol consumption. Akin to obesity, rates of NAFLD are burgeoning and represent a growing health burden; it is estimated that the global disease prevalence is between 20-30%[1]. There is growing evidence that NAFLD is a multisystem disease with both intra- and extra-hepatic manifestations, with strong association between NAFLD and type 2 diabetes mellitus and metabolic syndrome[2].

NAFLD comprises of a spectrum of disease that includes simple steatosis, non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC). While hepatic steatosis is seen as a generally benign state, NASH is considered a progressive disease state with increased risk of intra- and extra-hepatic disease complications, including cirrhosis[3]. The gold-standard to diagnose NASH is an invasive liver biopsy. As there are no effective non-invasive diagnostic techniques, which makes estimating the true prevalence of NASH difficult; however, it has been estimated that up 25% of patients with NAFLD have concurrent NASH[4]. As rates of NAFLD continue to increase, it is estimated that NAFLD-related cirrhosis will soon surpass chronic hepatitis as the leading indication for liver transplantation[5].

The increasing prevalence and health burden of NAFLD has made it imperative to understand the pathogenesis of this disease process. The most current, best understood model of NAFLD conceptualizes a 'multiple hit' hypothesis in which interactions between genetics and environmental factors promote inflammation, cellular injury, and liver damage[6]. These 'hits' include lipid accumulation secondary to diet and lifestyle, obesity, and insulin resistance, all of which predispose the liver to inflammation and fibrosis. However, the mechanisms by which these hits promote disease progression are still poorly understood. In this meta-analysis, we aim to use bioinformatics of publicly available data to elucidate the most common genetic pathways involved in NAFLD and identify potential therapeutic targets for intervention.

MATERIALS AND METHODS

The National Center for Biotechnology Information Gene Expression Omnibus (GEO) is one of the largest databases available to researchers. The Search, Tag, Analyze, Resource GEO, or STARGEO, was developed to tag samples from the GEO database and produce robust meta-analyses. The GEO database is a genomics repository comprised of all published samples from omics studies. Briefly, STARGEO uses a standard random model for meta-analysis to generate both meta *P* values and effects size across studies[7]. Study weight percentages were calculated using the inverse variance method *via* the DerSimonian-Laird estimate[8]. The STARGEO "Tagging" interface was used to gather samples under the "NAFLD," "NASH," and "NASH_NAFLD_Control" tag to conduct two separate meta-analyses: one comparing liver biopsies from NAFLD patients to healthy liver controls and the other comparing liver biopsies from NASH to healthy controls.

Series GSE48452, GSE63067, GSE66676, and GSE107231 were used to gather NAFLD, NASH, and healthy liver samples[9-12]. Studies were found by searching NAFLD or NASH under human samples on stargeo.org. Studies selected for analysis had to meet the following criteria: expression analysis was conducted on liver biopsies, the study included contained patients meeting NAFLD or NASH criteria and had matched healthy controls, and biopsies met definitive diagnosis of liver steatosis as below.

In these studies, liver biopsies were performed to diagnose liver disease and healthy liver biopsies were defined as having less than < 5% steatosis and patients with evidence of viral hepatitis, alcoholic consumption, and hemochromatosis were excluded. Standard histopathological analysis by blinded pathologists were used to defined NASH, NAFLD, and healthy liver samples[13]. For example, GSE48452 investigated intra-individual biopsies taken pre and post-bariatric surgery meeting NAFLD, NASH, and healthy liver criteria as above. Only pre-bariatric samples were tagged. For the NAFLD analysis, there was a total of 58 NAFLD liver biopsies and 60 healthy liver biopsies. The NASH analysis featured 187 NASH liver biopsies and 154 healthy liver biopsies.

We were able to extract approximately 20000 genes for each of the meta-analyses conducted using STARGEO. We analyzed gene signature outputs with Ingenuity Pathway Analysis (IPA) to genes showing statistical significance ($P < 0.05$) and an absolute experimental log ratio greater than 0.1 between case and control samples[14]. The genes included in our analysis are further detailed in Tables 1 and 2. IPA allowed us to define top canonical pathways, disease functions, disease networks, and potential upstream regulators that define NAFLD and NASH pathogenesis. Regulator analysis identifies upstream regulators that best explain the genetic expression in our dataset with *P* values reflecting the degree of overlap of known effector targets and the gene signature analyzed in IPA. We also used the global molecular network feature of IPA to identify top disease networks. IPA ranks networks from the Global Molecular Network based on the number of focus genes from given networks that match with our analysis. Significance is represented by the p-score, as previously described[14].

Table 1 Top canonical pathways for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis identified by Ingenuity Pathway Analysis

	Overlap	*P* value
Top canonical pathways in NAFLD *vs* healthy control		
Liver X receptor / retinoid X receptor activation	5/121	4.35E-05
Superpathway of cholesterol biosynthesis	3/29	1.08E-04
Granulocyte adhesion and diapedesis	5/173	2.34E-04
CREB signaling	8/596	6.25E-04
Mevalonate pathway I	2/14	8.96E-04
Top canonical pathways in NASH *vs* healthy control		
Cholesterol biosynthesis I	4/13	5.48E-05
Cholesterol Biosynthesis II (*via* 24,25-dihydrolanosterol)	4/13	5.48E-05
Cholesterol biosynthesis III (*via* desmosterol)	4/13	5.48E-05
IGF-1 signaling	9/106	9.16E-05
Superpathway of cholesterol biosynthesis	5/28	1.05E-04

NAFLD: Non-alcoholic fatty liver disease; CREB: cAMP response element binding protein.

Table 2 Summary of the list genes that are the most upregulated and downregulated in our meta-analysis of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis liver samples compared to healthy controls

Top upregulated genes				Top downregulated genes			
NAFLD *vs* Healthy		NASH *vs* Healthy		NAFLD *vs* Healthy		NASH *vs* Healthy	
XIST	0.326	Crystallin alpha A	1.185	LINC02535	-0.198	MT1L	-0.454
PEG10	0.267	CYP7A1	0.409	GPR88	-0.194	CYR61	-0.386
SUCO	0.252	BBOX1	0.381	CYP1A1	-0.170	FOSB	-0.339
CBWD5	0.239	TAF4B	0.355	IGFBP2	-0.168	IGFBP2	-0.326
TMEM154	0.228	FNDC5	0.346	P4HA1	-0.166	FOS	-0.275
HMGCR	0.225	MROH2A	0.293	TSPAN13	-0.159	CAPZA3	-0.254
LINC00885	0.216	Fc alpha and mu receptor	0.265	NR4A2	-0.148	CSRNP1	-0.254
Chitinase 3 Like 1	0.186	IL13RA2	0.252	PER3	-0.145	PCDHB19P	-0.252
MEP1B	0.181	ABHD1	0.250	SHBG	-0.135	Nicotinamide phosphoribosyltransferase	-0.240
Phosphodiesterase 11A	0.180	Muscular LMNA interacting protein	0.229	CENPO	-0.131	RASD1	-0.237

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis, PEG10: Paternally expressed imprinted gene 10; SHBG: Sex hormone-binding globulin.

To find potential drug interactions, we used clue.io to analyze our dataset[15]. We inversed the gene expression pattern from the meta-analysis and used the "list-maker" function to identify drugs (Table 3). We focused on HEPG2 cell lines given they are immortalized HCC cells that relate most closely to the cells studied in our analysis.

All data analyzed were taken from Gene Expression Omnibus. There was no interaction or intervention with human subjects and no involvement with access to identifiable private patient information. As such, no Institutional Review Board approval was necessary.

Table 3 Top disease functions for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis identified by Ingenuity Pathway Analysis

Top disease functions in NAFLD *vs* healthy control	*P* values
Inflammatory response	1.67E-03
Liver lesion	6.59E-05
Cell movement of epithelial cells	3.88E-04
Activation of cells	5.30E-04
Synthesis of lipid	5.49E-08
Accumulation of lipid	6.12E-04
Concentration of lipid	2.38E-06
Fibrosis	3.75E-05
Secretion of lipid	1.06E-03
Hepatic injury	1.54E-04
Organismal injury and abnormalities	5.10E-16
Cancer	3.47E-15
Dermatologic diseases and conditions	5.20E-11
Metabolic disease	6.69E-10
Lipid metabolism	8.09E-12
Molecular transport	7.06E-12
Small molecule biochemistry	9.86E-11
Cell death and survival	5.05E-8
Cellular movement	6.28E-8
Adipogenesis	1.31E-7

NAFLD: Non-alcoholic fatty liver disease.

RESULTS

Top canonical pathways and genes of interest from NAFLD and NASH analysis

From STARGEO, we were able to extract approximately 20000 genes from our analysis of NAFLD and NASH liver biopsies compared to normal biopsy controls. Table 1 summarizes top upregulated and downregulated genes from the two analyses. Only genes that demonstrated statistically significant ($P < 0.05$) differences in up-and down-regulation and absolute experimental log ratios of 0.1 were analyzed in IPA. Additionally, we used IPA to classify the top canonical pathways for NAFLD and NASH. P values and experimental log ratios are included in Tables 1 and 2.

For the NAFLD analysis, the genetic changes and top canonical pathways illustrate several disease processes such as dysregulated metabolism, immune cell recruitment, and altered signal transduction. IPA identified liver X receptor/retinoid X receptor activation (P = 4.35E-05), superpathway of cholesterol biosynthesis (P = 1.08E-04), granulocyte adhesion and diapedesis (2.34E-04), cAMP response element binding protein (CREB) signaling (P = 6.35E-04), and mevalonate pathway (P = 8.96E-04) as top canonical pathways. Among the most upregulated genes are the long non-coding RNAs (lncrna), X-inactive specific transcript (XIST), and LINC00885, with the role of lncrna in liver disease playing an increasing role[16,17]. Additionally, we found upregulation of tumorigenic proteins such as paternally expressed imprinted gene 10 (PEG10) and phosphodiesterase 11A[18,19]. We also noted dysregulated metabolism and increased lipogenesis through upregulation of inositol hexakisphosphate kinase (IP6K3), flavin containing monooxygenase 1, perilipin 1, 3-hydroxy-3-methylglutaryl coenzyme A synthase, HMG-CoA reductase, fatty acid binding protein 5 (FABP5), and downregulation of insulin-like growth factor binding protein 2, and insulin-like growth factor 1 (IGF1)[20-22]. Upregulation of steroid 5-alpha reductase 2 and downregulation of sex hormone-binding globulin (SHBG) and nuclear receptor subfamily 0 group B member 2 leads to higher androgen activity with implication in liver disease[23]. Interestingly, we found downregulation of the circadian rhythm gene period circadian regulator 3 (PER3)[24]. There was also upregulation of several chemoattractants, including CXCL10. Lastly, we noted downregulation of the glycoprotein chitinase 3 Like 1, which regulates several cellular

processes including proliferation, differentiation, inflammation, and others[25].

Similarly, the gene expression changes and top canonical pathways from the NASH analysis detailed several pathologic processes. IPA identified cholesterol biosynthesis and insulin-like growth factor 1 (IGF-1) signaling as top canonical pathways. We found upregulation of genes involved in bile acid synthesis and carnitine synthesis including cholesterol 7 alpha hydroxylase (CYP7A1) and gamma-butyrobetaine hydroxylase 1 (BBOX1), respectively[26-28]. Notably, we saw upregulation of the novel myokine fibronectin type 3 (FNDC5), which correlated with NAFLD severity and extracellular matrix deposition[29]. Interestingly, we found upregulation of the lamin-associated gene muscular LMNA interacting protein. Lamins and lamin-associated proteins have implications in liver disease[30].We also found upregulation of several pro-inflammatory genes including the interleukin 13 receptor and the immunoglobin receptor Fc alpha and mu receptor[31]. Our genetic analysis also highlighted dysregulated apoptosis through the downregulation of pro-apoptotic regulators such as the matricellular protein cysteine-rish angiogenic inducer 61 (CYR61), FOS protein (modulates JUN signaling), and Ras related dexamethasone induced 1 (RASD1) from the RAS family[32-24]. Lastly, we found downregulation of insulin-like growth factor binding protein-2 (IGFBP2), similar for our NAFLD analysis above, and the nicotinamide phosphoribosyltransferase, a rate-limiting enzyme in the NAD+ pathway[35].

Top disease function and networks

NAFLD and NASH are the result of several complex disease processes in tandem. To define these processes, we used IPA to identify top disease function and networks of interest. In the NAFLD analysis, disease processes were largely related to lipid regulation, inflammation, and hepatic fibrosis and injury (Table 2). Similarly, the disease functions in NASH included processes related to lipid metabolism in addition to other functions such as cancer and cell death and survival. Figure 1 illustrates one of the disease functions, adipogenesis, in the NASH analysis.

Next, we employed the IPA Disease Network feature to further elucidate the pathologic changes in NAFLD and NASH. IPA takes genes from the analyzed dataset and superimposes it onto curated information from the Ingenuity Knowledge base[14]. In Table 3, we detail the top disease networks identified for NAFLD and NASH. Figure 2 details the lipid metabolism network from the NAFLD analysis.

Top upstream regulators and causal analysis

To propose potential drivers of NAFLD and NASH pathogenesis and their downstream effector genes, we used IPA Upstream Regulator analysis[14]. In the NAFLD analysis, beta-estradiol (P = 9.42E-12), cholesterol (P = 1.79E-11), tumor necrosis factor (TNF) (P = 8.73E-10), nuclear receptor coactivator (P = 1.22E-09), and sterol regulatory element binding transcription factor 1 (SREBF1) (P = 12.8E-08) were top upstream regulators. Of these regulators, SREBF1 demonstrated the highest z-score (2.200), demonstrating how the gene expression signature reflects known downstream SREBF1 gene signaling. Next, we investigated how the genes described above are affected by SREBF family (Figure 3). We see activation of SREBF1 and SREBF2 is linked to the changes in expression noted in CXCL10, FABP5, IGF1, HMGCR, HMGCS1, sex hormone binding globulin, and PEG10.

In the NASH analysis, TNF (P = 1.22E-19), lipopolysaccharide or LPS (P = 6.27E-16), beta estradiol (P = 1.42E-15, with predicted inhibition), interleukin 1B or IL1B (P = 1.78E-14), and platelet-derived growth factor BB or PDGF BB (P = 1.90E-14) were top upstream regulators. Beta-estradiol demonstrates anti-fibrotic effects in the liver, so we investigated its downstream effects in our dataset (Figure 4). Inhibition of beta-estradiol activity is reflected by the changes we noted in the top upregulated genes including crystallin alpha A, BBOX1, CYP7A1, and FNDC5 and top downregulated genes including IGFBP2, nicotinamidenphosphoribosyltransferase pseudogene 1 (NAMP1), and RASD1 genes in our dataset described above. In addition, IPA related inhibition of beta-estradiol to other gene expression changes of interest including upregulation of the PEG10, squalene epoxidase (SQLE), IP6K3 and downregulation of the tumor suppressor Kruppel-like factor 6 (KLF6)[36,37].

Therapeutic analysis

To investigate potential drug targets from our dataset, we utilized clue.io. We inputted genes that were both upregulated and downregulated in our NAFD and NASH dataset (see Figure 5). We used the query tool from the platform and focused on HEPG2 cell lines, immortalized HCC cells. By looking at compounds that inverse the pathologic expression patterns in our meta-analyses, we identified riciribine (an AKT inhibitor) and ZSTK-474 (a PI3K inhibitor) as potential therapeutic compounds that target the genes in our investigation (see Table 3).

DISCUSSION

NAFLD represents a growing health burden, with an astonishing prevalence of 25% of the global population[38]. A better understanding of pathogenesis is needed to tackle this herculean disease. Here, we use meta-analysis of public data using our STARGEO platform in search of insights to disease and

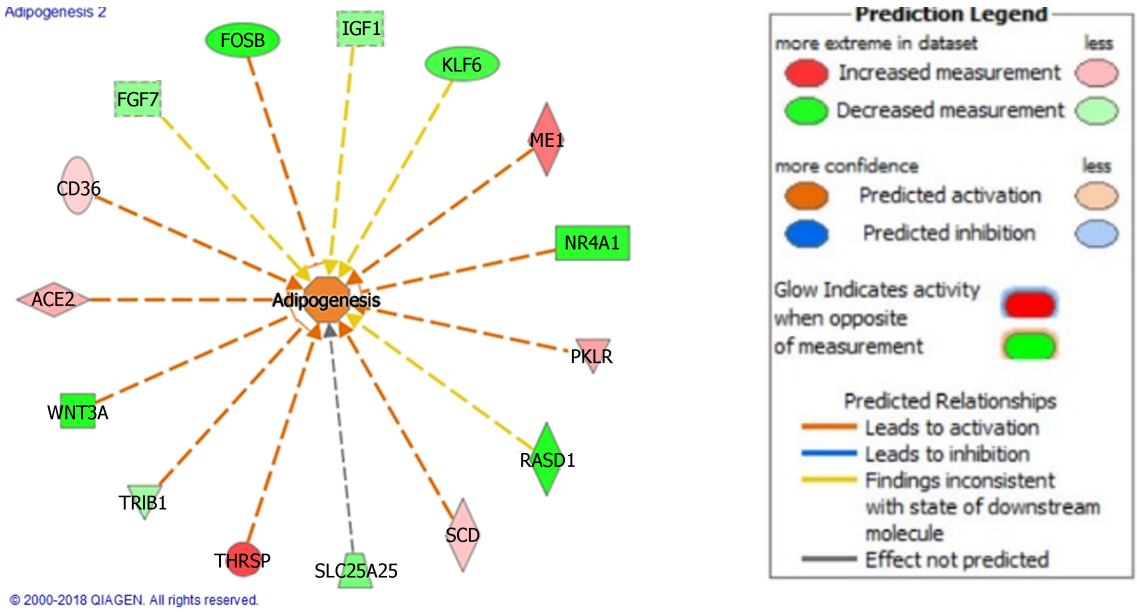

Figure 1 We used Ingenuity Pathway Analysis, gene function feature to define pathologic processes in our non-alcoholic steatohepatitis analysis. This Figure highlights the adipogenic changes in hepatocytes from non-alcoholic steatohepatitis patients. Prediction legend illustrates relations of molecules and Figure generated using Ingenuity Pathway Analysis.

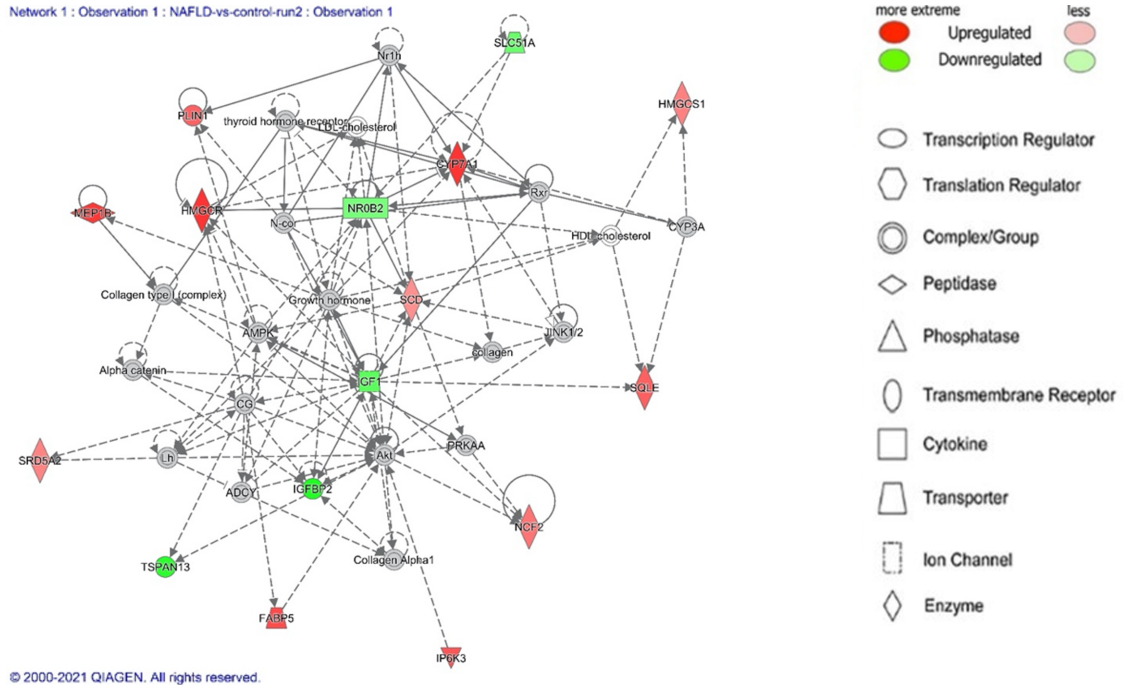

Figure 2 Top network (Lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism) identified by Ingenuity Pathway Analysis Network analysis of non-alcoholic fatty liver disease. Legend illustrates class of the gene. Red indicates upregulation and green downregulation, with shade depicting magnitude of change. Solid and dashed lines depict direct and indirect, respectively, relationship between genes. Figure generated using Ingenuity Pathway Analysis.

potential therapeutic targets. The gene expression profiles from our analyses can elucidate function and regulatory patterns to disease[39]. Our results from the NAFLD analysis reinforce the role of altered metabolism, inflammation, and cell survival and supports recently described contributors to disease such as altered androgen and lncrna activity[17,23,40].

Our results demonstrated several changes that are implicated in altered lipid and metabolic homeostasis. It is the accumulation of lipids that lead to several downstream effects that characterized NAFLD development and progression[20,41]. For example, lipid droplets in hepatocytes can lead to hepatic insulin resistance, decreased autophagy, oxidative stress, and interaction with several

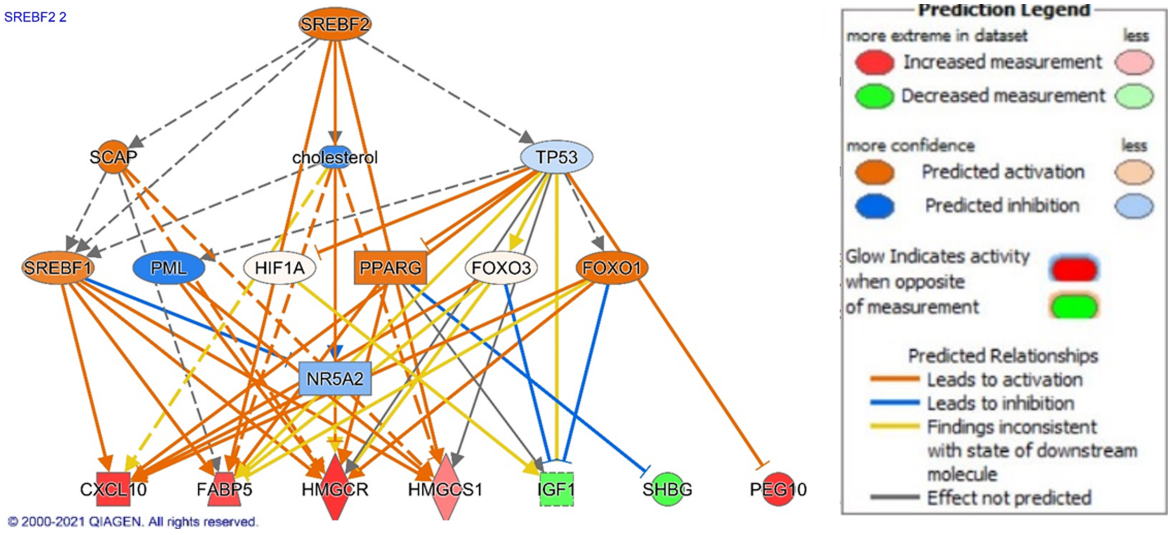

Figure 3 Ingenuity Pathway Analysis of SREBF1 signaling in non-alcoholic fatty liver disease. Genes are implicated in several potential disease processes including the inflammation, metabolism, and transport. Legend illustrates relationship between genes. See Figure 2 legend for identification of shapes. Figure generated using Ingenuity Pathway Analysis.

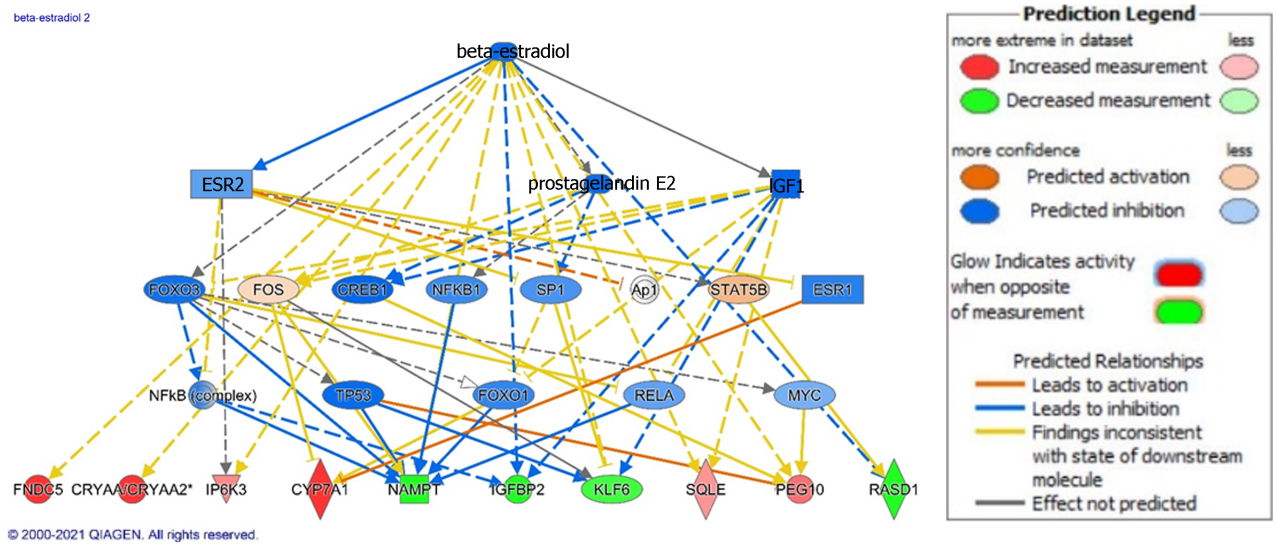

Figure 4 Ingenuity Pathway Analysis of beta-estradiol signaling in non-alcoholic steatohepatitis. Genes are implicated in several potential disease processes including the metabolism, cancer development, bile acid synthesis, and cell survival. Legend illustrates relationship between genes. See Figure 2 legend for identification of shapes. Figure generated using Ingenuity Pathway Analysis.

transcription factors such as SREBF[20]. These lipid droplets can form through the activity of proteins from the perilipin family, such as perilipin 1 (PLN1), which was upregulated in our dataset (Table 2)[20]. "Superpathway of Cholesterol Biosynthesis" was one of the top canonical pathways, and several top disease functions and networks were related to lipid accumulation (Tables 1-3). In addition, cholesterol and SREBF1, a transcription factor involved in lipid homeostasis, were top upstream regulators[42]. SREBF1 stimulates accumulation of lipids in hepatocytes through activation of patatin-like phospholipase domain-containing 3 (PNPAL3)[43]. In our results, we illustrate how downstream signaling of SREBF1 and SREBF2 Leads to fatty acid accumulation and other disease functions. Downstream of SREBF1 and SREBF2 signaling, we noted upregulation of CXCL10, HMGCR, HMGCS1, FABP5, and PEG10 in addition to downregulation of SHBG and IGF1. HMGCR catalyzes the first reaction of cholesterol synthesis and HMGCS1 also contributes to hepatic cholesterol synthesis. Increased activity of HMGCSR and HMGCS1 was associated with NAFLD and with fatty acid accumulation[44]. Additionally, FABP5 is a fatty acid binder normally expressed in adipocytes, but expression in hepatocytes was correlated with fatty acid infiltration in NAFLD[45]. In addition to fatty acid changes, we found downregulation of IGF-1, which leads to hyperglycemia and increases risk for diabetes seen as in NAFLD[46,47]. IGF-1 also has anti-fibrotic effects through attenuation of hepatic stellate cell (HSC) activation in murine models[48]. Furthermore, SHBG was downregulated in analysis,

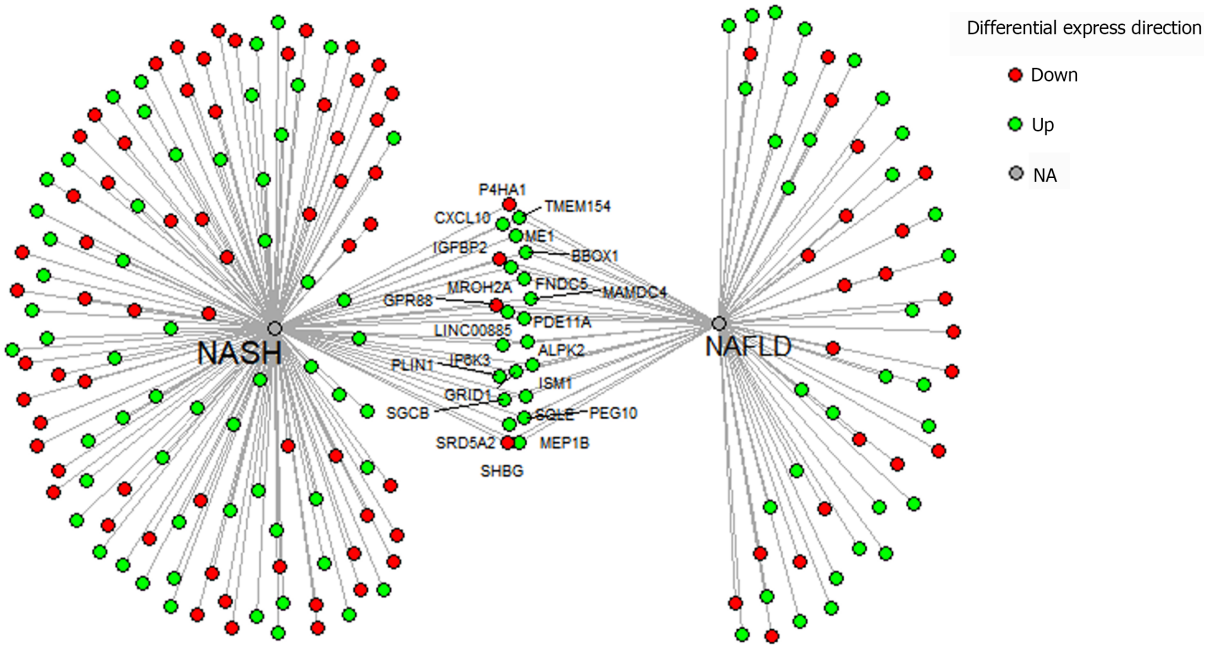

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Figure 5 Pathologic gene patterns shared in the non-alcoholic fatty liver disease and non-alcoholic steatohepatitis meta-analyses are highlighted above. This dataset was inputted in clue.io to identify potential drug targets. We found riciribine (an AKT inhibitor) and ZSTK-474 (a PI3K inhibitor) as drugs that best targeted the gene expression above.

with decreased SHBG levels being associated with increased insulin resistance in NAFLD patients[49]. Higher levels of SHBG are also associated with lower odds for NAFLD and may have some protective effect[50]. In addition to fatty acid accumulation and glycemia, we related SREBF activity to malignant changes through upregulation of PEG10. PEG10 is a transcription factor that was found to be an oncogene in several solid cancers such as HCC, gastric cancer, and breast carcinoma[36]. PEG10 is upregulated in NASH and NAFLD and may be associated with increased risk for HCC seen in this patient population[18]. Furthermore, our results fortified other changes in NAFLD that are implicated in lipolytic changes that may induce NAFLD. CREB signaling was identified as a top canonical pathway. Awaad, *et al* showed elevated cAMP and CREB levels in a NAFLD murine model and suggest the role of cAMP and CREB as a marker of early NAFLD[51].

Accumulation of fatty acids in the liver induces chronic, low-grade inflammation, and subsequently, progression of NAFLD to NASH. Our results illustrated the inflammatory changes in NAFLD. The inflammatory response was a top disease function in our analysis (Table 3) and the pro-inflammatory cytokine TNF was a top upstream regulator. In murine models, TNF plays an essential role in NAFLD development through upregulation of inflammatory mediators and genes associated with liver fibrosis [52]. TNF also induces hepatic steatosis in murine models through upregulation of SREB proteins[53]. We also noted upregulation of several pro-inflammatory cytokines in our analysis including CXCL10 (Table 1). CXCL10 recruits T cells and macrophages and is an independent risk factor for NASH[54]. Since fatty acids lead to inflammatory changes, it is expected that SREB signaling would lead to downstream pro-inflammatory changes such as upregulation of CXCL10 (Figure 3).

Aside from inflammation and metabolic derangements, our results illustrated several other signaling and cellular processes of interest in NAFLD. One such cellular process is protein prenylation. Protein prenylation is a protein post-translational modification where farnesyl (farnesylation) or geranylgeranyl (geranylgeranylation) side chain is added to a C-terminal cysteine residue[55]. The mevalonate pathway, a top canonical pathway in our analysis, affects the ratio of farnesylation and geranylgeranylation. Alteration in this ratio is implicated in NAFLD and NAFLD-associated fibrosis[56]. In addition to post-translational protein modification, our results suggest a role for lncRNAs in NAFLD. LnRNAs are critical mediators of normal liver physiology, with aberrant expression being observed in metabolic, fibrotic, and malignant hepatic changes[17]. We found upregulation of lncRNAs in our analysis, including XIST and LINC0085. XIST is one of the earliest described lnRNAs and assists in the formation of silenced heterochromatin[57]. While not well-described in NAFLD and NASH, XIST has been shown to promote HCC and colorectal cancer[58,59]. Additionally, LINC0085 is a positive cell growth regulator in breast cancer models and may, alongside XIST, cause proliferative and pathologic changes in hepatocytes in NAFLD and NASH[16]. Lastly, recent research has connected the link between circadian rhythm genes with NAFLD[24]. Asynchronization of circadian rhythms, such as from shift work, are correlated with higher prevalence and NAFLD[60]. Per3 is a circadian rhythm gene that regulates adipogenesis, with deletion leading to increased adipogenesis in animal models[24]. Thus,

downregulation of Per3 in our results may suggest dysregulation of circadian rhythm and consequent changes in regulation of adipogenesis.

NASH is a subset of NAFLD characterized by steatosis inflammation and fibrosis[61]. It typically takes years for NAFLD to progress for NASH, and while the mechanisms behind this progression are not clear, our current understanding suggests a "multi-hit hypothesis" where multiple modes of fatty acid accumulation and oxidative stress synergistically induce liver inflammation and fibrosis[61]. Aside from lifestyle modifications, obeticholic acid is the only FDA-approved treatment of NASH[62]. The growing burden of NASH necessitates new therapeutics and our analysis of NASH offers insight into potential treatment.

Ingenuity Pathway Analysis of our NASH dataset reinforces the role of cholesterol. Several of our top canonical pathways, disease network, and disease functions were related to cholesterol synthesis, lipid metabolism, adipogenesis, and metabolic disease (Figure 1, and Tables 1, 3 and 4). The role of lipids in liver injury have been described above[20,40]. Other disease functions and disease networks of note involved cell death and survival, cancer, digestive system disease, and organismal injury (Tables 3 and 4). The top upstream regulators in addition to upregulated and downregulated genes reflect activity related to these disease functions. Among our top upstream regulators were pro-inflammatory cytokines TNF and IL1B, PDGF BB, and beta-estradiol (with predicted inhibition).

As already described, inflammation is a major contributor to liver disease. It has been long shown that patients with NASH, and more so those with severe NASH, have elevated levels of TNF[63]. Elevated serum levels of TNF in NASH patients was linked to increased major adverse hepatic events [64]. While TNF inhibition reduces steatosis and fibrosis in murine models, their role in select NAFLD and NASH patient populations has still not been proven effective[52,65-67]. Similarly, IL1B signaling has pro-fibrotic and lipogenic effects in murine models and may have promise as directed therapy in NASH patients[68-70]. Lastly, the cytokine PDGF BB exerts its pro-fibrotic effects through activation of hepatic stellate cells and, consequently, is another potential drug target[71].

Experimental models have shown that estrogen has protective, anti-fibrotic activity through attenuation of HSC activation and generation of reactive oxygen species[72]. Additionally, estrogen receptor agonism in a NASH murine model had therapeutic effects through modulating bile acid receptor signaling and inhibiting fibrosis and adipogenesis[73]. Interestingly, decreased estrogen levels and other hormone changes in menopause may be related to increase risk for NAFLD and NASH[74]. Since beta-estradiol was a top upstream regulator with predicted inhibition in our NASH analysis, we applied IPA to investigate beta-estradiol signaling and its downstream genetic effects (Figure 4).

Our analysis related inhibition of beta-estradiol to derangement of several cellular processes downstream including metabolism, extracellular matrix deposition, and tumor suppression. In regard to metabolism, we related inhibition of estradiol to upregulation of IP6K3, CYP7A1, and SQLE and to downregulation of NAMP1 and IGFBP2. IP6K3 produces inositol pyrophosphates and regulates metabolic control[75]. Deletion in murine models leads to improved glucose tolerance, reduced body weight, and protection from fatty liver disease[75,76]. SQLE is involved in cholesterol synthesis and has been shown in both human and animal studies to promote development of HCC in fatty liver disease [77]. CYP7A1 is a rate-limiting enzyme in the classical pathway of bile acid synthesis with upregulated gene expression in NAFLD and NASH patients alike, but discrepancies exist in post-transcriptional protein levels[27]. The effects of fatty liver disease on CYP7A1 are inconsistent, but bile acid dysregulation is a growing hallmark in this disease[27]. NAMP1 is a critical enzyme in the synthesis of nicotinamide adenine dinucleotide (NAD+). NAD+ functions in mitochondrial oxidative phosphorylation and protection of cells from reactive oxygen species[78]. Depletion of hepatic NAD+ has been shown to be a risk factor for NAFLD in a murine model[35]. There is growing interest in targeting NAD+ in NAFLD[79]. Lastly, IGFBP2 binds to IGF1 and has a positive effects in glucose control[80]. Early epigenetic silencing, *via* methylation, of IGBFP2 predicts development of fatty liver later in mice[81].

In addition to metabolic changes, our analysis showed pro-oncogenic and fibrotic genetic changes in NASH that may relate to inhibition of beta-estradiol signaling. Through IPA, we correlated inhibition of beta-estradiol signaling to upregulation of PEG10 and FNDC5 and to downregulation of RASD1 and KLF6. Interestingly, we found upregulation of PEG10 in our NAFLD analysis and discussed its pro-oncogenic activity. RASD1 is a member of the Ras superfamily of G proteins that regulate signal transduction through G-protein coupled receptors[82]. RASD1 prevents aberrant cell growth, and its downregulation may lead to increased risk for HCC seen in fatty liver disease.[34,83] Additionally, KFL6 is a zinc finger transcriptional protein with tumor suppressor function that is inhibited in various cancers, including HCC[37]. Lastly, FNDC5 is a novel myokine that controls extracellular matrix deposition. Higher expression of FNDC5 in HSCs correlated to severity of fibrosis in NAFLD patients [29]. Our results illustrate malignant and fibrotic gene expression changes in both NAFLD and NASH stages of disease and its possible relation with inhibition of beta-estradiol signaling.

Lastly, our analysis suggests potential use of riciribine and ZSTK-474 in the treatment of NAFLD. Dysregulation of the PI3KT/AKT pathway in hepatocytes has been described in NAFLD[84]. Such dysregulation is implicated in hepatic steatosis and fibrosis. While the mechanisms underpinning pathogenesis through the PI3KT/AKT pathway are still under investigation, our results add further evidence of targeting this pathway for therapeutic benefit.

Table 4 Top five molecular networks associated with genetic differences in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis liver biopsies compared to healthy controls. Disease networks were identified using Ingenuity Pathway Analysis

Top molecular networks in NAFLD *vs* healthy control	
Lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism	34
Cell-to-cell signaling and interaction, cellular movement, hematological system development and function	23
Connective tissue disorders, inflammatory disease, organismal injury and abnormalities	19
Cellular development, connective tissue development and function, skeletal and muscular system development and function	16
Cell death and survival, neurological disease, organismal injury and abnormalities	16
Amino acid metabolism, molecular transport, small molecule biochemistry	34
Cellular development, skeletal and muscular system development and function, tissue development	34
Hereditary disorder, neurological disease, organismal injury and abnormalities	32
Digestive system development and function, lipid metabolism, small molecule biochemistry	29
Cell cycle, cell death and survival, cellular movement	29

NAFLD: Non-alcoholic fatty liver disease.

Our meta-analysis approach offers insights into NAFLD and NASH, but this approach is not without limitations. Biological samples in Gene Expression Omnibus have limitations in terms of description of samples. Some details that may present confounding variables are the co-morbidities in patients and differing stages in fatty liver disease, including degree of fibrosis. Other patient characteristics may also influence results such as medications, age, gender, and ethnicity. Samples were also taken under different conditions such as diagnosis of undifferentiated liver disease or in bariatric patients, which may lead to further differences between samples. Though there are set diagnostic criteria for hepatic steatosis on biopsy, the diagnoses were made by separate pathologists across these studies and a meta-analysis approach would not be able to account for these differences. Additionally, while transcriptomic and meta-analysis studies can offer a global view of disease function and regulatory signaling using gene expression patterns, causality necessitates more direct functional experimentation[39]. This approach itself does not offer direct experimental or clinical evidence. Nonetheless, our results offer a foundation to future studies in NAFLD and NASH that warrant further investigation with experimental and human models.

CONCLUSION

We utilized our platform STARGEO to produce genetic signatures from GEO datasets that provide molecular insights to fatty liver disease. We conducted to separate analysis of NAFLD and NASH liver biopsies to investigate genetic changes that define stages of fatty liver disease. Our analyses buttresses how the dysregulation in lipid homeostasis, though such regulators as the transcription factor SREBF1, contribute to steatosis. We also noted upregulation of genes implicated in oncogenesis, such as PEG10, that may partly explain the increased risk of HCC in these patients. We also describe the potential contribution on long noncoding RNAs in NAFLD pathogenesis. From our NASH analysis, we explored how beta-estradiol dysregulation may mechanistically contribute to steatosis and its several consequences such as fibrosis and oncogenesis. Lastly, we used out dataset and clue.io to identify genes that target pathologic genetic changes and signaling, such as PI3KT/AKT signaling, and found ricirbine and ZKST-474 as possible therapeutic targets. Overall, our analysis illustrates several changes that may explain progression of NAFLD pathogenesis and promising directions that warrant further investigation.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) pathogenesis is poorly understood but may result from a mix of exogenous and genetic factors that lead to fatty infiltration and inflammation.

Research motivation
NAFLD is a growing cause for liver transplant with limited therapeutic options.

Research objectives
To define genetic changes that underlie NAFLD and progression to non-alcoholic steatohepatitis (NASH) in pursuit of identifying promising therapeutic targets.

Research methods
We employed our STARGEO platform to conduct meta-analyses of publicly available liver biopsies from NAFLD and NASH patients.

Research results
We identified various genes implicated in inflammation and fatty infiltration, as well as signaling processes that lead to these changes. We also identified riciribine and ZSTK-474 as potential drugs.

Research conclusions
NAFLD and its progression to NASH is likely led by several genetic changes detailed in our manuscript. The genetic changes in our dataset are targeted by ricirbine and ZSTK-474 and warrants further study.

Research perspectives
As NAFLD becomes an increasing clinical burden, a bioinformatics approach is valuable in understanding causes and elucidating treatment avenues.

FOOTNOTES

Author contributions: All authors contributed to conception or designing of the work, data collection, drafting manuscript, critical revision of manuscript, final edits and approval the manuscript.

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Data sharing statement: Data was taken by already available public pathologic samples.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Cardiac risk factors limiting survival to liver transplantation in patients with nonalcoholic fatty liver disease

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) describes the hepatic manifestations of metabolic syndrome, which is estimated to affect 25% of adults, and currently represents the second most common indication for liver transplant in the United States. Studies have shown that patients with NAFLD are at an increased risk for heart failure, arrhythmia, and coronary artery disease (CAD), which may impact outcomes of liver transplantation. However, it remains unclear whether the presence of cardiac disease affects survival prior to liver transplant. If so, this would represent an important opportunity to optimize cardiac status and improve outcomes before liver transplant.

AIM

To identify cardiac factors that impact survival to liver transplantation in patients with NAFLD and on the transplant waitlist.

METHODS

The aim of this study was to identify cardiac risk factors that limit survival to transplant in patients with NAFLD. We performed a retrospective analysis of patients with NAFLD listed for liver transplant at a tertiary academic medical center in the United States from January 2015 to January 2021, identified through United Network of Organ Sharing registry. Exclusion criteria included a concurrent etiology of liver disease and removal from the transplant list due to chemical dependency, lack of social support, improvement in liver disease, or being lost to follow-up. We manually reviewed patient charts including electrocardiogram, echocardiogram, and cardiac catheterization reports as well as physician notes to identify cardiac disease states (*i.e.*, heart failure, arrhythmia, valvular disease and CAD) and other related diagnoses. We performed a survival analysis by Cox proportional hazards regression model to analyze the association between cardiac factors at the time listed for transplant and death or clinical deterioration prior to transplant.

RESULTS

Between January 2015 and January 2021, 265 patients with nonalcoholic fatty liver disease were listed for liver transplant at our institution. Our patient sample had a median age of 63 and an even distribution between sexes. The median Model for End-Stage Liver Disease (MELD) score was 17 and the median body mass index was 31.6. Of these 265 patients, 197 (74.3%) survived to transplant and 68 (25.7%) died or clinically deteriorated prior to transplant. The presence of mild or moderate CAD represented a hazard ratio of 2.013 (95%CI 1.078-3.759, P = 0.029) for death or clinical deterioration when compared to patients without CAD, after adjustment for age, sex, and MELD. MELD represented an adjusted hazard ratio of 1.188.

CONCLUSION

Mild or moderate CAD represents a hazard for waitlist mortality prior to liver transplant in patients with NAFLD. Aggressive management of CAD may be needed to improve patient outcomes.

Key Words: Nonalcoholic fatty liver disease; Liver transplant; Cardiovascular disease; Pre-transplant outcomes; Coronary artery disease; Risk factors

Core Tip: Nonalcoholic fatty liver disease (NAFLD) continues to rise in prevalence as a leading indication for liver transplantation. Due to its metabolic features, NAFLD is a risk factor for cardiovascular disease such as coronary artery disease (CAD), atrial fibrillation and heart failure. In our study, we examined the impact of cardiac factors on survival to liver transplant, once listed, in patients with NAFLD. We observed that even mild or moderate CAD represents an independent hazard for waitlist mortality before liver transplant after adjustment for confounding variables. This compels improved treatment of less severe forms of CAD in patients undergoing liver transplant.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) describes the hepatic manifestations of metabolic syndrome. NAFLD encompasses a spectrum of disease that ranges from simple steatosis to nonalcoholic steatohepatitis and cirrhosis. The prevalence of nonalcoholic fatty liver disease is increasing in Europe and the United States, becoming one of the most frequent causes of end-stage liver disease and hepatocellular carcinoma. NAFLD is now the second most common etiology of liver disease among patients listed for liver transplant (LT) in the United States, with an increase in the prevalence of NAFLD as an indication for liver transplant by 170% between 2004 and 2013[1].

Due to its metabolic features, NAFLD is a reported risk factor for cardiovascular disease such as coronary artery disease, atrial fibrillation, diastolic dysfunction, and heart failure[2-6]. Cardiovascular disease is the leading cause of early mortality after liver transplant, accounting for over 40% of early deaths related to both coronary and non-coronary events[7]. However, whether cardiovascular disease influences outcomes while on the waitlist for liver transplantation remains to be established. The purpose of this retrospective cohort study is to identify cardiac factors that impact patient survival to liver transplantation in patients with NAFLD and on the transplant waitlist.

MATERIALS AND METHODS

Study design

We performed a retrospective cohort study assessing the impact of cardiac risk factors on death or clinical deterioration prior to liver transplant among patients with nonalcoholic fatty liver disease.

Subject identification

Our study was approved by the institutional review board prior to subject identification. We identified all patients listed for LT at a tertiary academic referral center in the Midwest United States from January 1st 2015 to January 31st 2021 *via* review of United Network of Organ Sharing (UNOS) registry. Adult patients (> 18 years) with a clinical diagnosis of NAFLD, as listed by UNOS, were included in our study. Exclusion criteria included a concurrent etiology of liver disease and removal from the transplant list due to chemical dependency, medical non-adherence, or clinical improvement. Patients who remained active on the transplant list during the study period were also excluded due to a lack of outcome at the time of investigation. We reviewed all patient charts to confirm the etiology of liver disease and reasons for removal from the liver transplant list.

Data collection

We extracted demographics and clinical information from UNOS that included patient name, medical record number, date of birth, date listed for transplant, liver disease diagnosis, indication for transplant, Model for End-Stage Liver Disease (MELD) score, body mass index (BMI), date removed from the transplant list, and reasons for removal. We reviewed all patient charts to confirm the reason for removal from the transplant list. Patients were classified into two categories: transplanted *vs* death or clinical deterioration. Patients who successfully received LT were categorized into the transplanted group. Clinical deterioration was defined as acute illness, progression of hepatocellular carcinoma, and incidence of concurrent illness that prompted removal from the LT list. Patients removed from the LT list because of chemical dependency, medical non-adherence or improvement in liver disease were excluded from analysis.

Study data were collected and managed using REDCap electronic data capture system hosted at our home institution. All most recently available data were collected at the time of listing for LT. We utilized natural language processing and electronic medical record coding to extract numerical data from patient charts. The data extracted included high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride count, hemoglobin A1c, troponin, NT Pro BNP and left ventricular ejection fraction. We performed a random manual review of these data to confirm accuracy.

We also performed manual chart reviews including review of diagnostic reports and physician documentation to identify and assess cardiac risk factors. Electrocardiogram reports were reviewed to identify QTc and arrhythmia defined as atrial fibrillation or atrial flutter. Echocardiogram reports were reviewed to identify left ventricular ejection fraction, estimated right ventricular systolic pressure and valvular abnormality (*i.e.*, aortic stenosis, aortic insufficiency, mitral regurgitation and tricuspid regurgitation). Valvular abnormalities described as moderate or severe were included; trivial or mild valvular abnormalities were not included. Cardiac catheterization reports were reviewed to identify coronary artery disease (CAD). Severe CAD was defined as luminal stenosis of 70% or greater in a main coronary vessel (*i.e.*, left main, left circumflex, left anterior descending, right coronary artery or posterior descending artery), and/or history of myocardial infarction (MI), percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG)[8]. Moderate CAD was defined as luminal stenosis of 50-69% in a main coronary vessel[8]. Mild CAD was defined as luminal stenosis < 50% in a main coronary vessel. No significant CAD was defined as absence of luminal irregularity on cardiac catheterization or negative cardiac stress testing without a history of MI, PCI or CABG. We reviewed right heart catheterization reports to identify Fick's cardiac index, Fick's cardiac output, pulmonary capillary wedge pressure and right ventricular systolic pressure. We identified the presence of heart failure, hypertension, obstructive sleep apnea and need for renal replacement therapy by manual review of diagnosis codes and physician documentation.

Statistical analysis

The statistical methods of this study were reviewed by Ruishen Lyu, a biostatistician in the Department

of Quantitative Health Sciences at the author's institution. Patient characteristics were described using means and standard deviations for normally distributed continuous variables, medians and quartiles for non-normally distributed continuous variables, and frequency (percentage) for categorical variables. Analysis of variance or the non-parametric Kruskal-Wallis tests were used to assess differences in continuous variables. The chi-square test and Fisher's exact test were used to compare categorical variables as appropriate.

Time to event was defined by the number of months from the date of listing to the date of transplant or the date of removal due to death or clinical deterioration. Unadjusted Cox proportional hazards regression models were used to assess the association between each risk factor and time to development of the competing event (death or clinical deterioration). Multivariable Cox proportional hazards regression model was performed to build a model to assess the association between the outcome, time to event, and risk factors collected at baseline, including confounding variables of age, sex, and MELD score. In the multivariable model development, the multivariate imputation by chained equation was performed to impute missing values to conduct a complete dataset for variable selection. The stepwise variable selection method based on Akaike information criterion was used to choose the final model. The variables that had a large portion of missing values, were unbalanced between levels with small number of events, or were highly correlated to others were excluded from the model. Analyses were performed using R software (version 3.6.2; Vienna, Austria) and P value < 0.05 was considered statistically significant.

RESULTS

Between January 2015 and January 2021, 265 patients with NAFLD were listed for LT at our institution. Table 1 shows baseline patient characteristics at the time of listing for LT. Our patient sample had a median age of 63.1 [57.4, 67.2], median MELD score of 17 and median BMI of 31.6; 48.3% (n = 128) of patients were male and 51.7% (n = 137) female.

Of these 265 patients, 197 (74.3%) survived to transplant and 68 (25.7%) died or clinically deteriorated prior to transplant. Table 1 shows that patient characteristics were similar between groups except for the presence of obstructive sleep apnea (32.4% *vs* 20.3%, P value = 0.043) and median elevation in estimated right ventricular systolic pressure (34.0 *vs* 30.0, P value = 0.012) in the group not transplanted because of death or clinical deterioration.

Table 2 describes the univariate analysis of factors' impact on death or clinical deterioration prior to transplant, and expressed in hazard ratios with 95% confidence intervals. MELD and renal replacement therapy had increased hazard ratios of 1.18 (95%CI 1.14-1.23, P < 0.001) and 3.20 (95%CI 1.49-6.88, P = 0.003), respectively. Tricuspid regurgitation had a hazard ratio of 3.50 (95%CI 1.26-9.72, P = 0.016) whereas hazard ratios were insignificant for aortic stenosis, aortic insufficiency and mitral regurgitation. Compared to no CAD, mild or moderate CAD represented a hazard ratio of 2.06 (95%CI 1.14-3.74, P = 0.017) and severe CAD represented a hazard ratio of 2.43 (95%CI 1.17-5.05, P = 0.017).

Table 3 describes results of the multivariable Cox proportional hazards regression model on survival failure to transplant after adjustment for possible confounders and statistically significant variables that were included in the regression model. Variables included in the model were age, sex, MELD score and coronary artery disease. When adjusted for other variables in the multivariable model, the presence of mild or moderate CAD independently represented a hazard ratio of 2.013 (95%CI 1.078-3.759, P = 0.029) for death or clinical deterioration. Severe CAD lost statistical significance after adjustment for other variables with 95%CI 0.968-4.538. MELD score represented a hazard ratio of 1.188 (95%CI 1.139-1.239, P < 0.001).

DISCUSSION

This retrospective cohort analysis aimed to identify cardiovascular disease that limit survival to liver transplant in patients with nonalcoholic fatty liver disease while on the LT waitlist. In our study, we found that the presence of mild or moderate coronary artery disease at the time listed for LT significantly increased the risk for patient death or clinical deterioration prior to receiving a transplanted organ when adjusted for potential confounders.

Contrary to our expectations, severe coronary artery disease did not represent a significant hazard for death prior to LT in our study. Patients with severe CAD met one of the following criteria: coronary artery occlusion of 70% or greater, history of myocardial infarction, history of PCI or history of CABG. Patients with severe CAD were, therefore, more likely to have received procedural or surgical intervention for CAD, and this may explain the lack of increased hazard on waitlist mortality.

It is established in numerous studies that patients with NAFLD are at an increased risk for cardiovascular disease including coronary artery disease, heart failure, and arrhythmia[2-6]. Despite this, few data exist that analyze the impact of cardiovascular disease on survival outcomes prior to LT in patients with NAFLD.

Table 1 Patient characteristics with nonalcoholic fatty liver disease listed for liver transplant

Variable	Total (*n* = 265)	Transplanted (*n* = 197)		Not transplanted due to death or clinical deterioration (*n* = 68)		*P* value
Demographics						
Age (yr)	63.1 [57.4, 67.2]	197	62.8 [56.6, 66.7]	68	64.1 [59.6, 68.5]	0.060[a]
Sex		197		68		0.42[b]
Female	137 (51.7)		99 (50.3)		38 (55.9)	
Male	128 (48.3)		98 (49.7)		30 (44.1)	
Model for End-Stage Liver Disease	17.0 [13.0, 24.0]	197	17.0 [13.0, 24.0]	68	18 [12.0, 24.5]	0.44[a]
Body mass index (kg/m^2)	31.6 [28.3, 37.2]	197	32.2 [28.3, 36.8]	68	31.3 [28.2, 38.6]	0.79[a]
Comorbid Conditions						
Renal replacement therapy	32 (12.1)	197	24 (12.2)	68	8 (11.8)	0.93[b]
Hypertension	152 (57.4)	197	113 (57.4)	68	39 (57.4)	0.99[b]
Obstructive sleep apnea	62 (23.4)	197	40 (20.3)	68	22 (32.4)	0.043[b]
Cardiac disease						
Atrial fibrillation/Atrial flutter	8 (3.0)	197	5 (2.5)	67	3 (4.5)	0.42[c]
Heart failure	18 (6.9)	194	10 (5.2)	68	8 (11.8)	0.091[c]
Left ventricular ejection fraction	65.0 [61.0, 70.0]	196	66.0 [61.5, 70.5]	67	65.0 [61.0, 70.0]	0.80[a]
Estimated right ventricular systolic pressure	31.0 [25.0, 36.0]	142	30.0 [25.0, 35.0]	48	34.0 [26.5, 38.5]	0.012[a]
Aortic stenosis	8 (3.0)	197	7 (3.6)	68	1 (1.5)	0.68[c]
Aortic insufficiency	2 (0.75)	197	1 (0.51)	68	1 (1.5)	0.45[c]
Mitral regurgitation	4 (1.5)	197	3 (1.5)	68	1 (1.5)	0.99[c]
Tricuspid regurgitation	8 (3.0)	197	4 (2.0)	68	4 (5.9)	0.21[b]
CAD		197		68		0.12[b]
No significant CAD	196 (74.0)		152 (77.2)		44 (64.7)	
Mild or Moderate CAD	45 (17.0)		30 (15.2)		15 (22.1)	
Severe CAD	24 (9.1)		15 (7.6)		9 (13.2)	
History of myocardial infarction	12 (4.5)	197	7 (3.6)	68	5 (7.4)	0.19[c]
History of coronary artery stenting	13 (4.9)	197	8 (4.1)	68	5 (7.4)	0.33[c]
History of coronary artery bypass grafting	11 (4.2)	197	7 (3.6)	68	4 (5.9)	0.48[c]
Lab values						
Hemoglobin A1c		125		46		0.37[b]
< 5.6	59 (34.5)		47 (37.6)		12 (26.1)	
5.6-6.5	62 (36.3)		43 (34.4)		19 (41.3)	
> 6.5	50 (29.2)		35 (28.0)		15 (32.6)	
High-density lipoprotein		188		63		0.31[b]
≥ 50	75 (29.9)		53 (28.2)		22 (34.9)	
< 50	176 (70.1)		135 (71.8)		41 (65.1)	
Triglycerides		190		65		0.37[c]
≤ 150	240 (94.1)		177 (93.2)		63 (96.9)	

> 150	15 (5.9)	13 (6.8)	2 (3.1)

[a]Wilcoxon Rank Sum test.
[b]Pearson's chi-square test.
[c]Fisher's Exact test. Statistics presented as Median [P25, P75] and *n* (column %). CAD: Coronary artery disease.

Table 2 Univariate analysis on time to development of death/clinical deterioration prior to liver transplant

Variable	*n*	Events	Cox univariate hazard ratio (95%CI)	*P* value
Age	265	68 (26%)	1.018 (0.985, 1.053)	0.28
Sex				
Female	137	38 (28%)	-	
Male	128	30 (23%)	0.84 (0.52, 1.37)	0.49
Model for End-Stage Liver Disease	265	68 (26%)	1.18 (1.14, 1.23)	< 0.001
Body mass index	265	68 (26%)	0.975 (0.941, 1.011)	0.17
Renal replacement therapy				
No	233	60 (26%)	-	
Yes	32	8 (25%)	3.20 (1.49, 6.88)	0.003
Hypertension				
No	113	29 (26%)	-	
Yes	152	39 (26%)	1.15 (0.71, 1.88)	0.57
Obstructive sleep apnea				
No	203	46 (23%)	-	
Yes	62	22 (35%)	1.10 (0.66, 1.85)	0.72
Atrial fibrillation/Atrial flutter				
No	256	64 (25%)	-	
Yes	8	3 (38%)	2.97 (0.92, 9.61)	0.069
Heart failure				.
No	244	60 (25%)	-	
Yes	18	8 (44%)	1.81 (0.86, 3.82)	0.12
Left ventricular ejection fraction	263	67 (25%)	0.99 (0.96, 1.03)	0.69
Estimated right ventricular systolic pressure	190	48 (25%)	1.026 (0.997, 1.055)	0.075
Aortic stenosis				
No	257	67 (26%)	-	
Yes	8	1 (13%)	0.95 (0.13, 6.86)	0.96
Aortic insufficiency				
No	263	67 (25%)	-	
Yes	2	1 (50%)	1.38 (0.19, 10.04)	0.75
Mitral regurgitation				
No	261	67 (26%)	-	
Yes	4	1 (25%)	2.92 (0.40, 21.48)	0.29
Tricuspid regurgitation				
No	257	64 (25%)	-	
Yes	8	4 (50%)	3.50 (1.26, 9.72)	0.016

CAD				
No significant CAD	196	44 (22%)	-	
Mild or Moderate CAD	45	15 (33%)	2.06 (1.14, 3.74)	0.017
Severe CAD	24	9 (38%)	2.43 (1.17, 5.05)	0.017
History of myocardial infarction				
No	253	63 (25%)	-	
Yes	12	5 (42%)	2.29 (0.92, 5.74)	0.076
History of coronary artery stenting				
No	252	63 (25%)	-	
Yes	13	5 (38%)	1.86 (0.74, 4.66)	0.19
History of coronary artery bypass grafting				
No	254	64 (25%)	-	
Yes	11	4 (36%)	2.03 (0.73, 5.65)	0.17
Hemoglobin A1c				
< 5.6	59	12 (20%)	-	-
5.6-6.5	62	19 (31%)	1.11 (0.53, 2.32)	0.79
> 6.5	50	15 (30%)	0.80 (0.37, 1.76)	0.58
High-density lipoprotein				
≥ 50	75	22 (29%)	-	
< 50	176	41 (23%)	1.18 (0.69, 2.01)	0.54
Triglycerides				
≤ 150	240	63 (26%)	-	
> 150	15	2 (13%)	0.49 (0.12, 2.00)	0.32

CAD: Coronary artery disease.

Our study expands previous knowledge of the associations of NAFLD and cardiac disease[2-6], as well as the impact of cardiac disease on LT outcomes[7], by specifically evaluating the impact of these known cardiovascular associations of NAFLD on waitlist mortality**.** Our study identifies the negative impact of even mild or moderate coronary artery disease on patient outcomes prior to LT independent of severity of liver disease. This finding compels a better identification of CAD and treatment of less severe forms in patients who are undergoing liver transplant, especially in patients who otherwise are not candidates for coronary reperfusion therapy.

There is a large body of evidence showing that a comprehensive cardiovascular risk management strategy reduces risk of a variety of outcomes including cardiac events and death. These include weight loss in obesity[9,10], glycemic control in diabetes mellitus[11], intensive lipid-lowering therapy[12,13], management of hypertension[14], and smoking cessation[15]. In an effort to improve patient survival to LT, it may be beneficial to follow practice guidelines published by the American Heart Association and American College of Cardiology Foundation on secondary prevention and risk reduction therapy for patients with NAFLD and non-obstructive coronary disease who are listed for LT. Current guidelines recommend smoking cessation, use of beta-blockers and/or ACE inhibitors for blood pressure control, statin therapy to achieve an LDL-C of < 100 mg/dL or non-HDL-C of < 130 mg/dL in patients with triglycerides > 200 mg/dL, and weight management to maintain a BMI between 18.5 and 24.9 kg/m^2[16].

This study has several strengths. The identification of patients, NAFLD diagnosis and MELD score were collected from the United Network of Organ Sharing national database. We manually reviewed patient charts to ensure accuracy of diagnoses, lab values and reason not transplanted. We utilized rigorous methods in our statistical analysis to account for potential confounding variables.

A number of questions remain unanswered, such as the impact of mild CAD and moderate CAD independently on survival to LT. Our ability to analyze these variables independently was limited by a small number of events with patients with moderate CAD. Further prospective study with a larger sample of patients will help address this question. An important, but unanswered, question is how medical and lifestyle interventions for coronary artery disease will impact survival to transplant in patients with NAFLD. In our study, we did not identify the use of medications for risk reduction in

Table 3 Multivariable model on failure to survive to liver transplant

Variable	Hazard ratio	95%CI	*P* value
Age	1.008	0.973-1.044	0.655
Sex: Male *vs* Female	1.026	0.592-1.777	0.927
Model for End-Stage Liver Disease	1.188	1.139-1.239	< 0.001
Mild or Moderate CAD *vs* No significant CAD	2.013	1.078-3.759	0.029
Severe CAD *vs* No significant CAD	2.096	0.968-4.538	0.060
Observations	265		

CAD: Coronary artery disease.

CAD. We, therefore, did not analyze the influence of lifestyle intervention and risk-lowering medications on patient outcomes during the study period, and were not able to assess the duration of such intervention being a tertiary referral center. This represents a meaningful opportunity for future studies to evaluate the impact of lifestyle intervention and medical therapy on waitlist mortality.

One inherent limitation of our study is the observational methodology utilized. While we performed a multivariable analysis to minimize confounding variables, observational studies are prone to bias and confounding, and cannot be used to demonstrate causality. Additionally, inclusion of patients listed for transplant at a single tertiary academic medical center in the Midwest United States limited the generalizability of our findings to the broader population of patients with NAFLD.

CONCLUSION

Mild or moderate coronary artery disease in patients with NAFLD who are listed for liver transplant is associated with a significant risk of death or clinical deterioration leading to removal from the transplant list. Our findings suggest that management of mild or moderate CAD may be needed to improve patient outcomes in the pre-transplant period.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is rising in prevalence and is a leading cause of liver transplant. Patients with NAFLD are at increased risk for cardiac disease, which is a known contributor to post-transplant mortality. We aimed to identify cardiac disease that limits survival while on the transplant waitlist.

Research motivation

To identify cardiac disease that limits survival while on the transplant waitlist. This would lead to further insights into how we may need to improve testing and optimization of cardiac disease for patients being considered to liver transplant.

Research objectives

To identify cardiac disease that limits survival while on the transplant waitlist. We found that non-obstructive coronary artery disease (CAD) is associated with failure to survive to liver transplant in patients with NAFLD. Further study is needed to assess impact on pre-transplant outcomes after improvement in medical management of patients with non-obstructive CAD.

Research methods

We performed a retrospective cohort study of patients with NAFLD listed for liver transplant. We analyzed the presence of various cardiac disease states and their association with failure to survive to transplant.

Research results

Mild or moderate coronary artery disease represented a hazard for death or clinical deterioration prior to liver transplant in patients with NAFLD.

Research conclusions

Mild or moderate coronary artery disease represented a hazard for death or clinical deterioration prior to liver transplant. Improvement in identification and management of non-obstructive coronary artery disease may be needed to improved patient outcomes in the pre-transplant period.

Research perspectives

Further study is needed to assess impact on pre-transplant outcomes after improvement in medical management of patients with NAFLD and non-obstructive coronary artery disease who are listed for liver transplant.

FOOTNOTES

Author contributions: Delicce M, Wakim-Fleming J, Lyu R and Fares M designed the research study; Delicce M, Mauch J, Joseph A, Lyu R, Bartow R, Ferchill D and Kren H performed the research; Lyu R performed statistical analysis; Delicce M, Mauch J and Lyu R analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript.

Institutional review board statement: This study was reviewed and approved by the Cleveland Clinic Institutional Review Board [IRB#21-372].

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

Conflict-of-interest statement: All authors have no conflicts of interest to disclose.

Data sharing statement: The consent was not obtained but the presented data are anonymized and risk of identification is low.

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ORIGINAL ARTICLE

Retrospective Study

Differential distribution of gene polymorphisms associated with hypercholesterolemia, hypertriglyceridemia, and hypoalphalipoproteinemia among Native American and Mestizo Mexicans

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Abstract

BACKGROUND

Dyslipidemias are metabolic abnormalities associated with chronic diseases caused by genetic and environmental factors. The Mexican population displays regional differences according to ethnicity with an impact on the type of dyslipidemia.

AIM

To define the main dyslipidemias, the frequency of lipid-related risk alleles, and their association with hyperlipidemic states among different ethnic groups in West Mexico.

METHODS

In a retrospective study, 1324 adults were selected to compare dyslipidemias and lipid-related gene polymorphisms. Demographic, clinical, and laboratory data were collected. A subgroup of 196 normal weight subjects without impaired glucose was selected for the association analyses. Genotyping was determined by allelic discrimination assay.

RESULTS

Hypercholesterolemia was the most prevalent dyslipidemia (42.3%). The frequency of the risk alleles associated with hypoalphalipoproteinemia (*ABCA1*) and hypercholesterolemia (*APOE*, *LDLR*) was higher in the Native Americans (P = 0.047). In contrast, the Mestizos with European ancestry showed a higher frequency of the risk alleles for hypertriglyceridemia (*APOE2, MTTP*) (P = 0.045). In normal weight Mestizo subjects, the *APOB* TT and *LDLR* GG genotypes were associated risk factors for hypercholesterolemia (OR = 5.33, 95%CI: 1.537-18.502, P = 0.008 and OR = 3.90, 95%CI: 1.042-14.583, P = 0.043, respectively), and displayed an increase in low-density lipoprotein cholesterol levels (*APOB*: β = 40.39, 95%CI: 14.415-66.366, P = 0.004; *LDLR*: β = 20.77, 95%CI: 5.763-35.784, P = 0.007).

CONCLUSION

Gene polymorphisms and dyslipidemias showed a differential distribution. Regional primary health care strategies are required to mitigate their prevalence considering the genetic and environmental features which could have important implications for personalized medicine within the new era of precision medicine.

Key Words: Dyslipidemia; Ethnicity; Genes; Obesity; Lipids; Liver disease; Diet

Core Tip: Dyslipidemia is a metabolic alteration caused by gene-environmental interactions influenced by ethnicity. Genetic polymorphisms can modify the frequency and outcome of the hyperlipidemic state. Our results showed a differential distribution of gene polymorphisms associated with hypercholesterolemia (*APOE4*, *LDLR*), hypertriglyceridemia (*APOE2, MTTP*), and hypoalphalipoproteinemia (*ABCA1*) among Native Americans and Mestizo Mexicans of West Mexico. Hypercholesterolemia was the predominant dyslipidemia. In normal weight subjects, the *APOB* TT and *LDLR* GG genotypes increased the risk for hypercholesterolemia in the context of the Mestizo ethnicity. Regional personalized-medicine prevention strategies based on the host's genetic and environmental factors are required to decrease the prevalence of dyslipidemias.

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INTRODUCTION

Obesity is a leading health problem worldwide of epidemic proportions affecting the health of many societies regardless of socioeconomic status[1]. Currently, 75.2% of the Mexican adult population has excess weight (39.1% overweight and 36.1% obesity), and in recent years, Mexico has ranked first and second in the worldwide list of obesity[2,3]. Globalization is one of the main drivers of the national nutrition transition occurring in the last four decades[4]. It has shifted the consumption of the staple traditional Mexican diet towards high-calorie processed food products and sugary beverages, leading to unhealthy body weight and type 2 diabetes mellitus (T2DM) in the general population[5,6]. The leading causes of mortality in Mexico are T2DM, cardiovascular disease (CVD), and liver cirrhosis due to different etiologies; however, excess weight plays an important role in the development of these pathologies[7,8].

Dyslipidemia is one of the main metabolic alterations involved in these obesity-related co-morbidities [9]. Commonly, hypertriglyceridemia (HTG) is associated with insulin resistance which in turn causes both T2DM and liver fibrosis/cirrhosis, while hypercholesterolemia (HChol) is associated with CVD [10]. However, up to 30% of obese people do not have lipid abnormalities, while normal weight patients can present dyslipidemia[11,12]. It is also feasible that lean patients may present with nonalcoholic steatohepatitis (NASH), while some obese patients show no fatty liver or NASH[12]. These contrasting findings suggest that genetic and environmental factors are involved.

In terms of population genetics, 85% of Mexico's inhabitants are denoted Mestizos (MTZ) due to the admixture of Native American (NA), European, and African ancestral source populations that were

initiated 500 years ago after the Spanish conquest. In comparison, 10% and 5% are exclusively descendants of NA and African forefathers, respectively[13]. Concomitantly, with foodstuffs and food cuisine, a cultural syncretism between the eastern hemisphere (Spain, Africa, France, England) and the west (the Americas) took place, including the different geographic and ecological regions of Mexico[14]. Therefore, Mexico's population genetics and food culture are widely heterogenic, and the impact of these determinants can vary by region.

In this sense, the association of several single nucleotide polymorphisms (SNPs) located at different loci with dyslipidemias and their impact on non-communicable chronic diseases among the Mexicans has been acknowledged[15]. Distinctively, *APOE4*, *APOB* -516 C/T, as well as the *LDLR* A1413G and C52T are known to modulate the low-density lipoprotein cholesterol (LDL-c) levels and the susceptibility for HChol and CVD[16]. In the case of high-density lipoprotein cholesterol (HDL-c), the *ABCA1* R230C variant has been strongly associated with hypoalphalipoproteinemia (HALP), particularly in NA [17]. Additionally, the *MTTP*-943 G/T and the *MTHFR* C677T variants, as well as the *APOE2* allele, have been associated with increased triglycerides levels[18,19,20].

West Mexico´s population is characterized by NA inhabitants living in the rural areas, while the geographically dispersed MTZ populations have a variable degree of European and NA ancestries[21]. Previously, we documented that the *APOE4* allele is widespread among the NA but decreases significantly among the MTZ population with marked European ancestry, while conversely, the *APOE2* allele is predominant among this group[21,22]. However, studies jointly accessing these lipid-related gene polymorphisms have not been carried out among West Mexican populations. Thus, this study aimed to define the main dyslipidemias, the frequency of lipid-related risk alleles, and their association with hyperlipidemic states among different subpopulations.

MATERIALS AND METHODS

Study population and design

In this comparative cross-sectional study, a total of 1324 un-related adult individuals were retrospectively evaluated from January 2015 to December 2019 at the Department of Genomic Medicine in Hepatology, Civil Hospital of Guadalajara, "Fray Antonio Alcalde" in Guadalajara, Jalisco, Mexico. Each subject was interviewed, and a standardized questionnaire was used to register demographics, medical history, and laboratory data. The main exclusion criteria were the presence of any type of cancer, autoimmune and thyroid diseases, drug use in the last six months of recruitment, pregnant women, and use of hypolipidemic drugs.

In this study, populations of West Mexico with evidence of a representative NA ancestral component [22] were included, Nahua (NAH) (n = 84) and Wixárika (WXK) or "Huicholes" (n = 106) are indigenous ethnic groups, and five Mestizo populations: Guadalajara (GDL), Jalisco (n = 754), Tepic (TPC), Nayarit (n = 184), Cuquio (CUQ), Jalisco (n = 131), Villa Purificación (VP), Jalisco (n = 32), and San Miguel el Alto (SMA), Jalisco (n = 33). NA were identified according to the ethnic group, native language spoken, use of traditional attire, parents belonging to the ethnic group, and residence in a rural community. The Mestizo populations were defined as those born in Mexico, spoke Spanish, had Mexican parents, and did not belong to any native ethnicity.

For the association analyses between HChol and the related SNPs, 193 Mestizo subjects from GDL, Jalisco with normal weight determined by a body mass index (BMI) of 18.5-24.9 kg/m^2 and a body fat percentage of < 20% for men and < 30% for women, as well as without impaired glucose defined by fasting serum glucose of < 100 mg/dL and homoeostasis model assessment for insulin resistance (HOMA-IR) index < 2.5 were selected. This study subgroup was established as a reference population to decipher the influence of these genetic polymorphisms on dyslipidemia, since it is mestizo group with a more balanced genetic ancestry between NA and Europeans.

Definition for dyslipidemias

Dyslipidemias were defined according to the National Cholesterol Education Program expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (ATP III) and Mexican Official Norm 037 for the prevention, treatment, and control of dyslipidemias (NOM-037-SSA-2012): HChol was total cholesterol (TC) ≥ 200 mg/dL; HTG as triglycerides (TG) ≥ 150 mg/dL; HALP as HDL-c ≤ 40 mg/dL for men and ≤ 50 mg/dL for women; and high LDL-c as LDL-c ≥ 130 mg/dL[23,24].

Body composition

Body composition and BMI were assessed by bioelectrical impedance (InBody 3.0, Analyzer Body Composition, Biospace, South Korea) or a Tanita TBF_300A instrument (Tanita Corporation, Japan). Normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), and obesity (≥ 30 kg/m^2) were defined according to World Health Organization criteria[25].

Laboratory tests

Blood samples (10 mL) were obtained by venipuncture after a 12-h overnight fast. Biochemical tests included glucose, insulin, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, TC, TG, and HDL-c. All biochemical tests were determined with the AU5800 Clinical Chemistry System (Beckman Coulter's Inc. United States). The concentration of LDL-c was calculated using the Friedewald equation[26]. The very low-density lipoprotein-cholesterol (VLDL-c) was estimated by the formula of TC-(LDL-c + HDL-c). The HOMA-IR index was calculated with fasting plasma glucose (mg/dL) × fasting serum insulin (mU/L)/405. IR was defined as a HOMA-IR index of 2.5 or above to assess IR as a metabolic alteration.

DNA extraction and genotyping characterization

As previously described, genomic DNA (gDNA) was extracted from leukocytes using a modified salting-out method[27]. The genotypes of each SNPs were determined by a real-time PCR system using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster, CA, United States). The characteristics of context sequence of these probes correspond to the following catalog numbers: C_11720861_10 for *ABCA1* (rs9282541), C_7615488_10 for *APOB* (rs934197), C_8726910_10 and C_8726960_10 for *LDLR* (rs5930 & rs14158), C_1202883_20 for *MTHFR* (rs1801133), C_ 8934089_10 for *MTTP* (rs1800591), and C_3084793_20 and C_904973_10 for *APOE* (rs429358 & rs7412) (ThermoFisher Scientific). gDNA was used at a final concentration of 20 ng. PCR conditions were initial enzyme activation for 10 min at 95 °C, followed by 40 cycles of denaturalization for 15 s at 95 °C and alignment/extension for 1 min at 60 °C in a StepOnePlus thermocycler (Applied Biosystems, Foster, CA, United States). For genotype error checking, three positive controls corresponding to the possible genotypes for each SNP and a blank were included in every 96-well plate. A 20% of randomly selected samples were re-genotyped, of which 100% were concordant. Genotypic and allelic frequencies were obtained by the direct counting method. The Hardy-Weinberg equilibrium expectation was assessed by Arlequin version 3.1.

Statistical analysis

Kolmogorov-Smirnov test was used to analyze the normal distribution of all quantitative variables. Continuous variables were expressed as mean ± SD and categorical variables were reported as frequencies and percentages. Data with normal distribution was analyzed with parametric statistical tests (student's *t*-test and one-way ANOVA with the respective post-hoc analyses) and non-normal data through non-parametric statistical tests (Kruskal-Wallis and Mann-Whitney U). The chi-square was used when variables were categorical. Univariate and multivariate logistic and linear regression tests were performed to analyze the association of *APOB* -516C/T and *LDLR* A1413G SNPs as a risk factor for HChol. The results were expressed as odds ratio with 95%CI and R^2. All the tests with significant *P* value were corrected by the Bonferroni method. Statistical analyses were performed in the statistical program IBM SPSS Statistics version 21.0 for Windows (IBM Corp, Inc., Chicago, IL, United States). Statistical significance was set at $P < 0.05$ to two-tailed.

Ethical guidelines

The study protocol complied with the last updated ethical guidelines of the 2013 Declaration of Helsinki from Fortaleza, Brazil. This study was revised and approved by the Institutional Review Board. All patients signed a written informed consent before enrollment, and anonymized data was employed to continue the statistical analysis.

RESULTS

Clinical and lipidic characteristics of the study populations

The clinical characteristics and the lipid profile of study populations from West Mexico are depicted in Table 1. The average age and gender frequencies were similar among the seven groups, except for the higher frequency of men in the MTZ from TPC compared with the other groups ($P = 0.001$). All the groups had excess weight, but the MTZ group from GDL had the highest BMI (33.8 ± 10.3 kg/m^2, $P = 8 \times 10^{-27}$). The lipid profile showed differences by study group. MTZ from TPC had higher serum levels of TC, TG, and LDL-c compared to the rest of the study groups ($P < 0.05$). On the other hand, the NAH group showed lower levels of HDL-c than those from CUQ and WXK groups ($P = 0.011$).

Prevalence of dyslipidemias in West Mexico populations

Table 2 shows the prevalence of dyslipidemias in the populations from West Mexico. The most prevalent dyslipidemia was HChol, with 42.3%. HTG was detected in 40.4%, HALP in 37.8%, and high LDL-c in 35.8% of all study subjects. Among study populations, heterogeneity in the frequency of dyslipidemias was observed. The MTZ from TPC and VP had the highest frequency of HChol and HTG (75.5%, 65.6%, and 51.1%, 46.9% respectively, $P = 0.001$). The NAH group showed a lower frequency of HChol (7.1%), as well as MTZ from VP and WXK group a lower prevalence of HALP compared to the

Table 1 Clinical characteristics and lipid profile of West Mexico populations

Variables	Native American ancestry		Mestizos (low-to-high European ancestry)					Total WMX	*P* value
	NAH	WXK	TPC	GDL	CUQ	VP	SMA		
n (%)	84 (9.5)	106 (12.0)	184 (20.8)	321 (36.4)	131 (14.8)	32 (3.6)	26 (2.9)	884 (100)	
Age (yr)	29.5 ± 11	43.5 ± 15	52.5 ± 8.3	36.4 ± 12.6	48 ± 15.4	40.4 ± 21.1	44 ± 15	43.7 ± 14.8	0.022[a]
Male *n* (%)	24 (29)	41 (39)	77 (42)	91 (28)	34 (26)	13 (39)	9 (35)	289 (32.7)	0.001[d]
Female *n* (%)	60 (71)	65 (61)	107 (58)	230 (72)	97 (74)	19 (61)	17 (65)	595 (67.3)	0.001[d]
BMI (kg/m^2)	26.3 ± 4.3	ND	28.3 ± 4.7	33.8 ± 10.3	28.6 ± 5.8	26.4 ± 5.1	25.5 ± 3.8	29.9 ± 7.7	8×10^{-27}[c]
TC (mg/dL)	164.3 ± 39.8	190.4 ± 37.1	228.1 ± 49.2	187.7 ± 42.4	182.1 ± 34.4	210 ± 52.6	179.7 ± 37.2	199.3 ± 48.8	1×10^{-35}[a]
TG (mg/dL)	151.5 ± 86.2	150.6 ± 98.1	197.3 ± 123.6	161.6 ± 148.3	150.6 ± 95	171.7 ± 93.1	148.9 ± 86.4	169.7 ± 122.5	0.023[a]
LDL-c (mg/dL)	95.6 ± 30.3	120.2 ± 31.7	158.4 ± 46.4	114.5 ± 36.9	107.3 ± 9.1	141.5 ± 38.3	111.3 ± 30.3	128.8 ± 44.7	1×10^{-30}[a]
VLDL-c (mg/dL)	29.1 ± 28.6	24.8 ± 10.6	29 ± 16	32.6 ± 30.4	30.3 ± 19.2	36.2 ± 23.3	29.8 ± 17.3	30.4 ± 23.0	0.350
HDL-c (mg/dL)	39.5 ± 6.8	46.2 ± 10.6	41.1 ± 10.8	42.5 ± 14.4	44.0 ± 9.6	43 ± 3.5	39.9 ± 8.1	42.3 ± 11.2	0.010[b]

[a]Tepic (TPC) *vs* the other groups by post hoc tests, $P = 0.002$.
[b]Nahua (NAH) *vs* Cuquio (CUQ) & Wixárika group by post hoc tests, $P = 0.015$.
[c]Guadalajara (GDL) & NAH *vs* the other groups by post hoc tests, $P = 0.001$.
[d]TPC *vs* GDL, NAH & CUQ by post hoc tests, $P = 0.035$.
Values are presented as mean ± SD. Gender is expressed as number of cases and percentage. The one-way ANOVA for quantitative variables and Chi-square test for qualitative variables were the statistical approach. NAH: Nahua indigenous group; WXK: Wixárika indigenous group; ND: No data; TPC: Tepic; GDL: Guadalajara; CUQ: Cuquio; VP: Villa Purificación; SMA: San Miguel el Alto; WMX: West Mexico; BMI: Body mass index; TC: Total cholesterol; TG: Triglycerides; LDL-c: Low-density lipoprotein cholesterol; VLDL: Very low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol.

Table 2 Prevalence of the type of dyslipidemias in West Mexico populations

Dyslipidemia	Native American ancestry		Mestizos (low-to-high European ancestry)					Total WMX (*n* = 884)	*P* value
	NAH (*n* = 84)	WXK (*n* = 106)	TPC (*n* = 184)	GDL (*n* = 321)	CUQ (*n* = 131)	VP (*n* = 32)	SMA (*n* = 26)		
HChol	6 (7.1)	41 (38.7)	139 (75.5)	113 (35.2)	43 (32.8)	21 (65.6)	11 (42.3)	374 (42.3)	0.001[a]
HTG	35 (41.7)	36 (34.0)	94 (51.1)	116 (36.1)	49 (37.4)	15 (46.9)	12 (46.2)	357 (40.4)	0.001[b]
High LDL-c	13 (15.5)	43 (40.6)	137 (74.5)	72 (22.4)	24 (18.3)	21 (65.6)	7 (26.9)	317 (35.8)	0.003[a]
HALP	42 (50.0)	28 (26.4)	87 (47.3)	112 (34.9)	45 (34.3)	5 (15.6)	15 (57.7)	334 (37.8)	0.002[c]

[a]Tepic (TPC) *vs* the other groups.
[b]TPC *vs* Wixárika (WXK) & Cuquio (CUQ) group.
[c]Villa Purificación & WXK *vs* the other groups.
The Chi-square test was the statistical approach. Values are presented as number of cases and percentage. NAH: Nahua indigenous group; WXK: Wixárika indigenous group; TPC: Tepic; GDL: Guadalajara; CUQ: Cuquio; VP: Villa Purificación; SMA: San Miguel el Alto; WMX: West Mexico; HChol: Hypercholesterolemia; HTG: Hypertriglyceridemia; HALP: Hypoalphalipoproteinemia.

other study groups (15.6% and 26.4%, respectively, $P = 0.002$) (Table 2).

Frequency of risk alleles of SNPs associated with dyslipidemias in West Mexican populations

The genetic risk alleles associated with HALP (*ABCA1* R230C, RC + CC genotypes) and HChol (*APOE4* allele and *LDLR* 1413G allele) were more prevalent in the NAH and WXK groups compared to the other study groups ($P = 0.047$) (Table 3). The MTZ from VP and SMA showed a higher frequency of the risk alleles that have been associated with HTG (*APOE2* allele and *MTTP* -943G/T, T allele) compared with the other groups ($P = 0.045$) (Table 3).

Table 3 Frequency of risk allele of polymorphisms associated with lipid disorders in West Mexican populations

Lipid abnormality	SNPs (risk allele)	Native American ancestry		Mestizos (low-to-high European ancestry)					Total WMX (*n* = 1324)	*P* value
		NAH (*n* = 84)	WXK (*n* = 106)	TPC (*n* = 184)	GDL (*n* = 754)	CUQ (*n* = 131)	VP (*n* = 32)	SMA (*n* = 33)		
Low HDL-c	*ABCA1* R230C (RC + CC genotypes)	15 (17.9)	43 (40.6)	24 (13.0)	53 (7.0)	18 (13.7)	4 (12.5)	2 (6.1)	159 (12.0)	0.010[c]
High TC[e]	*APOE* (E4 allele)	21 (12.5)	53 (25.0)	ND	145 (9.6)	ND	2 (3.1)	2 (3.0)	223 (8.4)	2×10^{-12}[a]
	APOB-516C/T (T allele)	49 (29.2)	58 (27.4)	ND	433 (28.7)	ND	24 (37.5)	18 (27.3)	582 (22.0)	0.129
	LDLR A1413G (G allele)	121 (72.0)	161 (75.9)	ND	1045 (69.3)	ND	42 (65.6)	44 (66.7)	1413 (53.5)	0.047[a]
	LDLR C*52T (C allele)	124 (73.8)	145 (68.4)	ND	1068 (70.8)	ND	55 (85.9)	52 (78.8)	1444 (54.7)	0.045[b]
High TG[e]	*APOE* (E2 allele)	0 (0)	0 (0)	ND	51 (3.4)	ND	1 (1.6)	7 (10.6)	59 (2.2)	0.028[d]
	MTTP-493G/T (T allele)	17 (10.1)	2 (0.9)	ND	253 (16.8)	ND	11 (17.2)	10 (15.2)	293 (11.1)	2×10^{-6}[b]
	MTHFR C677T (T allele)	103 (61.3)	111 (52.4)	172 (46.7)	670 (44.4)	117 (44.6)	25 (39.1)	21 (31.8)	930 (35.2)	0.038[b]

[a]Nahua & Wixárika (WXK) *vs* the other groups.
[b]Villa Purificación & San Miguel el Alto (SMA) *vs* the other groups.
[c]WXK *vs* the other groups.
[d]SMA *vs* the other groups. The Chi-square test was the statistical approach.
[e]The allelic frequencies were obtained considering the diploid number of chromosomes (2n).
Values are expressed as *n* (%). NAH: Nahua indigenous group; WXK: Wixárika indigenous group; TPC: Tepic; GDL: Guadalajara; VP: Villa Purificación; SMA: San Miguel el Alto; WMX: West Mexico; ND: No data; HDL-c: High-density Lipoprotein cholesterol; TC: Total Cholesterol; TG: Triglycerides.

Association of APOB -516C/T and LDLR A1413G polymorphisms with hypercholesterolemia in normal-weight MTZ individuals

The clinical and biochemical characteristics of the 193 MTZ subjects selected to evaluate the possible effect of these Hchol-related polymorphisms are shown in Table 4. In this study subgroup, 38.9% (*n* = 75) had any type of dyslipidemia and HChol was the most prevalent with 27.9% (*n* = 54) (Table 4).

Table 5 depicts the lipid profile and frequency of dyslipidemias according to the SNPs *APOB* -516C/T and *LDLR* A1413G genotypes. *APOB* homozygous TT genotype carriers had significantly higher levels of TC (*P* = 0.033) and LDL-c (*P* = 0.017), as well as a higher frequency of HChol (*P* = 0.012) (Table 5). Besides, the carriers of the homozygous GG genotype of *LDLR* had significantly higher levels of LDL-c (*P* = 0.042) and higher frequency of HChol (*P* = 0.034) (Table 5).

As shown in Table 6, the frequency of subjects with HChol was greater among carriers of the homozygous genotypes TT of *APOB* and GG of *LDLR* than the non-HChol (26.1% *vs* 6.9%, *P* = 0.005; 60.9% *vs*. 38.6%, *P* = 0.043, respectively). Also, both genotypes, TT of *APOB* and GG of *LDLR* were associated with HChol (OR = 5.33, 95%CI: 1.537-18.502, *P* = 0.008; OR = 3.90 95%CI: 1.042-14.583, *P* = 0.043, respectively) (Table 6).

Finally, through a linear regression test, an increase of 30% higher LDL-c was associated with the homozygous TT genotype of *APOB* (R^2 = 0.30, β = 40.39, 95%CI: 14.415-66.366, *P* = 0.004), and an increase of 11% higher LDL-c was associated with the GG genotype of *LDLR* (R^2 = 0.11 β = 20.77, 95%CI: 5.763-35.784, *P* = 0.007) (Table 7).

DISCUSSION

Dyslipidemias are severe abnormalities commonly associated with excessive body fat, a pathogenic factor contributing to the development of co-morbidities such as T2DM, fatty liver disease, and CVD [28]. However, genetic and environmental factors cause differences across the country in the incidence of these pathologies. Previously, we have documented the admixed genetic architecture of West Mexico [21,22]. In this region, NAH and WXK are representative of the NA genetic component, while the inhabitants of TPC, GDL, CUQ, VP, and SMA are historically known to carry a significant European genetic component. Therefore, we hypothesized that the distribution of dyslipidemias and the lipid-related alleles could be variable according to the ancestral inheritance. Herein, we present the first study

Table 4 Clinical and biochemical characteristics and frequency of dyslipidemias in normal weight Mestizos individuals

Variable	Reference values	Study group
n		193
Age (yr)		32.8 ± 12.3
Male		55 (28.5%)
Female		138 (71.5%)
BMI (kg/m^2)	18.5-24.9	22.3 ± 1.1
Total body fat (%)	< 24%	21.3 ± 6.1
Glucose (mg/dL)	< 100	84.4 ± 7.9
HOMA-IR	< 2.5	1.7 ± 0.5
TC (mg/dL)	< 200	180.1 ± 33.1
TG (mg/dL)	< 150	112.2 ± 61.3
LDL-c (mg/dL)	< 130	109.2 ± 27.6
VLDL-c (mg/dL)	< 25	22.5 ± 12.3
HDL-c (mg/dL)	> 40	49.4 ± 13.7
AST (UI/L)	< 54	26.3 ± 10.5
ALT (UI/L)	< 42	25.1 ± 14.1
GGT (UI/L)	< 35	20.2 ± 5.3
Dyslipidemia		75 (38.9%)
HChol	(TC > 200 mg/dL)	54 (27.9%)
HTG	(TG > 150 mg/dL)	35 (18.1%)
High LDL-c	(LDL-c ≥ 130 mg/dL)	39 (20.2%)
HALP	(HDL-c < 40 mg/dL)	40 (20.7%)

Values are presented as mean ± SD; *n*: Number of cases and percentage. MTZ: Mestizo; BMI: Body mass index; HOMA-IR: Homeostasis model assessment insulin resistance; TC: Total cholesterol; TG: Triglycerides; LDL-c: Low-density lipoprotein cholesterol; VLDL-c: Very low-density lipoprotein cholesterol; HDL: High-density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl-transferase; HChol: Hypercholesterolemia; HTG: Hypertriglyceridemia; HALP: Hypoalphalipoproteinemia.

jointly detecting several lipid-related risk alleles that confer dyslipidemia among the West Mexican population. An evident heterogeneity in the type of dyslipidemia and lipid-related risk alleles was observed between the study groups consistent with their genetic and environmental background.

Overall, the most prevalent dyslipidemia was HChol (42.3%). These data were discrepant with the National Health and Nutrition Survey 2006 and 2018, with HALP in nearly 60% nationwide[29,30]. A plausible explanation is that national surveys tend to focus on central regions of the country in which the NA component is predominant compared to West Mexico, in which the European ancestry is more prevalent. Furthermore, the breakdown analysis of the type of dyslipidemias adjusted by study group revealed that the NA had lower HChol (7.1%) while the MTZ from TPC and VP had higher rates of HChol (75.5% and 65.6%) and HTG (51.1% and 46.9%), respectively.

Given this panorama of dyslipidemias, we explored the frequency of several SNPs associated with these lipid abnormalities finding that the NA groups showed genetic susceptibility for HChol and HALP (*ABCA1* 230C allele, *APOE4* allele and *LDLR* 1413G allele); while the frequency of the risk alleles associated with HTG (*APOE2* allele and *MTTP* -943T allele) were higher in MTZ groups with a significant European ancestry. Notably, the *MTHFR* 677T risk allele prevalence revealed a high to low gradient (from NA to MTZ) which may have implications for fatty liver disease[31]. Thus, in conjunction, these findings highlight the importance of considering the ancestral components regarding the genetic susceptibility for lipid-related chronic diseases.

Furthermore, in this study, the TT genotype of *APOB* and GG genotype of *LDLR* were associated as risk factors for HChol. APOB is the main structural protein of LDL lipoprotein, essential for the assembly and secretion of chylomicrons and VLDL lipoprotein, and it is the primary ligand for LDLr mediated internalization of LDL-c in target tissues[33]. An imbalance between the production and degradation of APOB-containing lipoproteins leads to the development of HChol and, potentially, atherosclerosis[32]. In this context, *in vitro* studies have documented that the "T" allele of the *APOB*

Table 5 Association of *APOB* -516C/T and *LDLR* A1413G polymorphism with lipid levels in normal weight Mestizos individuals

Variable	*APOB* -516 C/T genotypes			*P* value	*LDLR* A1413G genotypes			*P* value
	CC (*n* = 85)	CT (*n* = 54)	TT (*n* = 15)		AA (*n* = 22)	AG (*n* = 65)	GG (*n* = 63)	
TC (mg/dL)	177.8 ± 31	178.2 ± 37.4	196.2 ± 27.6	0.033[a]	177.2 ± 25.7	175.4 ± 33.1	182.9 ± 34.7	0.366
TG (mg/dL)	110.4 ± 68	119.9 ± 71.3	108.7 ± 25.9	0.574	106.9 ± 47.5	98.7 ± 44.6	124.3 ± 79.8	0.094
LDL-c (mg/dL)	106.3 ± 24	107.3 ± 30.6	129.5 ± 31.6	0.017[a]	103.3 ± 23.9	106.2 ± 26.8	111.8 ± 29.5	0.042[b]
VLDL-c (mg/dL)	22 ± 13.6	24.3 ± 14.3	21.8 ± 5.1	0.451	21.3 ± 9.5	19.7 ± 9.0	25.1 ± 16.0	0.075
HDL-c (mg/dL)	49.3 ± 13.9	48.5 ± 10.7	50.3 ± 16.9	0.908	51.1 ± 15.3	51.6 ± 11.8	46.8 ± 13.3	0.143
Dyslipidemia, *n* (%)								
HChol	9 (11)	8 (15)	6 (40)	0.012[a]	1 (5)	8 (12)	14 (22)	0.034[b]
HTG	15 (18)	14 (26)	0 (0)	0.076	3 (14)	7 (11)	15 (24)	0.315
High LDL-c	13 (15)	12 (22)	7 (47)	0.007[a]	3 (14)	10 (15)	17 (27)	0.046[b]
HALP	19 (22)	10 (18)	3 (20)	0.760	3 (14)	8 (12)	18 (13)	0.178

[a]TT *vs* CC.
[b]GG *vs* AA.
The Kruskall Wallis test and U Mann-Whitney test for quantitative variables and Chi-square test for qualitative variables were the statistical approach. Values are expressed as mean ± SD, number of cases and percentage. MTZ: Mestizos; TC: Total cholesterol; TG: Triglycerides; LDL-c: Low-density lipoprotein cholesterol; VLDL-c: Very low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol; HChol: Hypercholesterolemia; HTG: Hypertriglyceridemia; HALP: Hypoalphalipoproteinemia.

Table 6 Association of *APOB* and *LDLR* genotypes with hypercholesterolemia in normal weight Mestizos individuals

Genotype	Non-HChol	HChol	*P* value	Genotype comparison	Odds ratio (95%CI)	*P* value
APOB -516C/T genotypes						
CC	76 (58.0%)	9 (39.1%)	0.120	TT *vs* CC	5.33 (1.537-18.502)	0.008
CT	46 (35.1%)	8 (34.8%)	0.895	TT *vs* CC + CT	4.63 (1.463-14.634)	0.009
TT	9 (6.9%)	6 (26.1%)	0.005	TT *vs* CT	3.83 (1.069-13.746)	0.039
LDLR A1413G genotypes						
AA	21 (16.5%)	1 (4.3%)	0.135	GG *vs* AA	3.90 (1.042-14.583)	0.043
AG	57 (44.9%)	8 (34.8%)	0.340	GG *vs* AA + AG	2.53 (1.216-5.282)	0.013
GG	49 (38.6%)	14 (60.9%)	0.043	GG *vs* AG	2.24 (1.028-4.890)	0.042

Values are expressed as number of cases and percentage. MTZ: Mestizos; HChol: Hypercholesterolemia. The Chi-square test and logistic regression test were the statistical approach.

-516C/T polymorphism increases the transcription of the *APOB* gene by more than 40%. Consequently, this causes a substantial increase in plasma LDL-c concentration[34].

Moreover, it was reported that in a healthy Swedish population, the -516T allele of this SNP increased the plasma LDL-c concentration by 12%, and in a French population was associated with a high plasma LDL-c concentration and the presence of carotid atherosclerotic disease[34,35]. In this study, the TT genotype of *APOB* -516C/T polymorphism increased the plasma LDL-c concentration by 30% in lean subjects. This is the highest percentage of LDL-c increase associated with the TT genotype of *APOB* reported so far. This information highlights that despite a lower frequency of -516T allele of *APOB* compared to other populations, the genetic effect on the plasma LDL-c concentration is more remarkable.

The most common genetic causes of HChol are mutations in the gene that codes the LDLr. These mutations drastically alter the functional activity of this surface receptor, thereby delaying the clearance of LDL particles[36]. Several studies have documented the relation of *LDLR* A1413G polymorphism with pathologies involving lipid disorders. For example, this genetic variant was found in 17% of patients with familial hypercholesterolemia from Iran[37], and in the United States, this same polymorphism was associated with Alzheimer's disease[38]. In this study, the GG genotype of *LDLR*

Table 7 Increased serum level of low-density lipoprotein cholesterol associated with *APOB* and *LDLR* genotypes in individuals with normal weight from West Mexico

Genotype comparison	R^2	β	95%CI	*P* value
APOB -516C/T				
TT *vs* CC	0.30	40.39	14.415-66.366	0.004
TT *vs* CC + CT	0.23	39.79	16.226-63.363	0.001
TT *vs* CT	0.31	39.01	0.996-67.029	0.091
LDLR A1413G				
GG *vs* AA	0.11	23.29	1.640-44.946	0.036
GG *vs* AA + AG	0.11	20.77	5.763-35.784	0.007
GG *vs* AG	0.08	19.74	0.915-37.270	0.082

Linear regression test was the statistical approach.

A1413G polymorphism was associated with HChol and increased plasma LDL-c concentration by 11%. This study is the first to establish a direct association between the GG genotype of *LDLR* with the levels of LDL-c and the presence of hypercholesterolemia in a healthy population from Mexico and Latin America.

The implications of these findings require addressing the role of the interrelationship between diet-related adaptive alleles and the current diet of the population. In this sense, NA groups have followed a frugal lifestyle for millennia in which lipid-related alleles may have been positively selected to cope with the Paleolithic and Neolithic Mesoamerican environments[39]. Their traditional diets mainly contained low amounts of saturated fats and were high in mono- and polyunsaturated vegetal fats and high complex carbohydrates which are protective against lipid-related chronic diseases despite the host's "risk alleles"[40,41]. However, lifestyle changes caused by the current nutrition transition place at risk both the NA population and MTZ, regardless of the degree of European ancestry. Likewise, the MTZ may be at higher risk for HTG particularity if they are carriers of the European risk alleles if changes in the dietary pattern occur. In this sense, the current dietary patterns in Mexico are notably unhealthy, characterized as obesogenic and hepatopathogenic leading to considerable increase in the prevalence of non-communicable chronic diseases such as T2DM, CVD, and nonalcoholic fatty liver disease[12,20,41,42].

Furthermore, dietary patterns are different by region nationwide. In West Mexico, the intake of pork meat is higher throughout the entire year. A traditional practice is eating pork rind "carnitas," cracklings, and doing barbecues almost every weekend. On the other hand, the fast-paced lifestyle in the central region of the country led to the consumption of processed food, which is rich in saturated fatty acids, trans fat, and simple carbohydrates[42]. These elements have been associated with the presence of dyslipidemias, particularly HTG and HALP[43]. These results reflect that the epidemiological pattern of dyslipidemias is not homogeneous throughout the country and the necessity to perform comparatively specific studies per region in Mexico and other countries.

This study has some limitations. First, despite that several representative populations of West Mexico with different ancestral compositions were included, it was not possible to complete the genetic profile of all populations. Nonetheless, the frequencies of risk alleles reported in this study are sufficient to demonstrate a differential distribution of gene polymorphisms associated with dyslipidemias among Native Americans and Mestizo Mexicans (Table 3). Next, the cross-sectional design may limit a complete extrapolation of the results obtained. Finally, the data was recorded through standardized questionnaires that provide sufficient and detailed information; information bias may be present. Thus, further prospective and longitudinal studies involving lipid-related genetic variants and lifestyle factors (physical activity, behavior, and mental health) are required.

In summary, the frequency of dyslipidemias in West Mexico differed from the national reports. The NA groups (WXK and NAH) showed a greater genetic susceptibility for developing HChol and HALP. The TT genotype of *APOB* -516C/T and GG genotype of *LDLR* A1413G were associated as risk factors for HChol and increased LDL-c levels in Mestizo healthy population.

CONCLUSION

Given the differential distribution of gene polymorphisms and rate of dyslipidemias found in this study, primary health care strategies are required to establish preventive actions to mitigate their prevalence

considering the regional genetic and cultural differences, which could have important implications for personalized medicine within the new era of precision medicine.

ARTICLE HIGHLIGHTS

Research background

Further investigations are needed to provide medical and nutritional therapies based on the genetic background of the population and the role of lifestyle changes including diet, exercise and mental health.

Research motivation

Given the differential distribution of gene polymorphisms and rate of dyslipidemias found in this study, primary health care strategies are required to establish preventive actions to mitigate their prevalence considering the regional genetic and cultural differences, which could have important implications for personalized medicine within the new era of precision medicine.

Research objectives

We aimed to describe if there are important differences between Native American and Mestizo Mexicans in regard to the type of dyslipidemias and lipid-related genetic polymorphisms.

Research methods

In this retrospective study, 1324 adults were selected to compare dyslipidemias and lipid-related gene polymorphisms. Demographic, clinical, and laboratory data were collected. A subgroup of 196 normal weight Mestizo subjects without impaired glucose was selected for the association analyses. Genotyping was determined by allelic discrimination assay.

Research results

The Native Americans showed a greater genetic susceptibility for developing hypercholesterolemia (HChol) (*APOE4*, *LDLR*) and hypoalphalipoproteinemia (*ABCA1*). The TT genotype of *APOB* -516C/T and GG genotype of *LDLR* A1413G were associated risk factors for HChol and increased low-density lipoprotein cholesterol levels in Mestizo healthy population.

Research conclusions

Deciphering the role of ethnicity in the type of dyslipidemia and defining the prevalence of lipid-related gene polymorphisms.

Research perspectives

Genetic and environmental factors are involved in the onset and progression of dyslipidemias among the Mexican population.

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FOOTNOTES

Author contributions: Panduro A conceived and designed the study; Torres-Valadez R, Ojeda-Granados C and Gonzalez-Aldaco K carried out experimentation, and data collection; Panduro A, Torres-Valadez R, Roman S, Ojeda-Granados C, Gonzalez-Aldaco K did analyses and interpretation of data; Torres-Valadez R drafted the manuscript. All authors critically revised the manuscript for intellectual content. All authors revised and approved the final version of the manuscript.

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Informed consent statement: All patients signed a written informed consent before enrollment, and anonymized data was employed to continue the statistical analysis.

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ORIGINAL ARTICLE

Retrospective Study

Effect of thrombocytopenia and platelet transfusion on outcomes of acute variceal bleeding in patients with chronic liver disease

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Abstract

BACKGROUND

Platelet transfusion in acute variceal bleeding (AVB) is recommended by few guidelines and is common in routine clinical practice, even though the effect of thrombocytopenia and platelet transfusion on the outcomes of AVB is unclear.

AIM

To determine how platelet counts, platelets transfusions, and fresh frozen plasma transfusions affect the outcomes of AVB in cirrhosis patients in terms of bleeding control, rebleeding, and mortality.

METHODS

Prospectively maintained database was used to analyze the outcomes of cirrhosis patients who presented with AVB. The outcomes were assessed as the risk of rebleeding at days 5 and 42, and risk of death at day 42, considering the platelet counts and platelet transfusion. Propensity score matching (PSM) was used to compare the outcomes in those who received platelet transfusion. Statistical comparisons were done using Kaplan-Meier curves with log-rank tests and Cox-proportional hazard model for rebleeding and for 42-d mortality.

RESULTS

The study included 913 patients, with 83.5% men, median age 45 years, and Model for End-stage Liver Disease score 14.7. Platelet count < 20 × 10^9/L, 20-50 ×

10^9/L, and > 50 × 10^9/L were found in 23 (2.5%), 168 (18.4%), and 722 (79.1%) patients, respectively. Rebleeding rates were similar between the three platelet groups on days 5 and 42 (13%, 6.5%, and 4.7%, respectively, on days 5, P = 0.150; and 21.7%, 17.3%, and 14.4%, respectively, on days 42, P = 0.433). At day 42, the mortality rates for the three platelet groups were also similar (13.0%, 23.2%, and 17.2%, respectively, P = 0.153). On PSM analysis patients receiving platelets transfusions (n = 89) had significantly higher rebleeding rates on day 5 (14.6% *vs* 4.5%; P = 0.039) and day 42 (32.6% *vs* 15.7%; P = 0.014), compared to those who didn't. The mortality rates were also higher among patients receiving platelets (25.8% *vs* 23.6%; P = 0.862), although the difference was not significant. On multivariate analysis, platelet transfusion and not platelet count, was independently associated with 42-d rebleeding. Hepatic encephalopathy was independently associated with 42-d mortality.

CONCLUSION

Thrombocytopenia had no effect on rebleeding rates or mortality in cirrhosis patients with AVB; however, platelet transfusion increased rebleeding on days 5 and 42, with a higher but non-significant effect on mortality.

Key Words: Gastrointestinal Hemorrhage; Platelet transfusion; Thrombocytopenia; Fresh frozen plasma; Portal hypertension; Mortality

Core Tip: This is a retrospective study to assess the impact of thrombocytopenia at presentation and that of platelet transfusion in the management of acute variceal bleeding in patients with chronic liver disease. Ten percent of patients received platelet transfusions and were found to have significantly higher rebleed rates on day 5 and 42 after the index bleeding episode but did not result in significantly higher mortality rates in these patients. On multivariate analysis, platelet transfusion was an independent risk factor for 42-d rebleeding, while hepatic encephalopathy was a significant risk factor for 42-d mortality.

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INTRODUCTION

Patients with cirrhosis are conventionally considered to be at a greater risk of bleeding than healthy controls due to "cirrhotic coagulopathy", characterized by thrombocytopenia and deranged prothrombin time (PT)[1]. Barring Factor VIII and von Willebrand Factor (vWF), which are produced by the vascular endothelium, the liver produces both pro- and anti-coagulant factors. The conventional tests of coagulation, namely PT, international normalized ratio (INR), activated partial thromboplastin time (aPTT) and platelet count, assess only specific components of the coagulation system (intrinsic or extrinsic pathway) and therefore do not provide a complete overview of the hemostatic derangements in cirrhotics. Thromboelastography (TEG) and rotational thromboelastometry provide a more accurate "global assessment" of the coagulation system[2]. However, they have important caveats of not being able to assess Protein C and von Willebrand factor levels, which play an important role in the coagulation pathway in cirrhotics[3].

Up to 15% of patients with cirrhosis experience an episode of variceal bleeding each year[4]. Thrombocytopenia is common in patients with cirrhosis. Severe thrombocytopenia (defined as platelets < 50 × 10^9/L) may be associated with an increased risk of procedural bleeding[5,6]. Several studies have demonstrated a lack of predictive value of platelet count for procedure-related bleeding in cirrhotics[7,8]. The impact of thrombocytopenia on the severity of acute variceal bleeding (AVB) is unclear. Prior studies have demonstrated that platelet counts greater than 56 × 10^9/L are required to control variceal bleeding, resulting in several clinical guidelines to advocate platelet transfusion for the control of bleeding[9,10]. However, neither of these studies were prospective controlled clinical trials, and the fact that patients undergoing liver transplantation (which is arguably one of the most invasive procedures a cirrhotic can undergo) show higher rates of hepatic arterial or venous thrombosis with increased use of platelet or fresh frozen plasma (FFP), casts doubt over the guiding principles advocating platelet

transfusion[7,8]. Despite several major guidelines advocating against the use of platelets, the decision is largely empirical and based on local practices in a real-world clinical setting. Transfusion practices regarding the use of FFP are clearer, with a recent retrospective cohort study demonstrating the potential harm of FFP transfusion in patients with AVB[11]. Prophylactic blood product transfusion is common in clinical practice, as reported in various studies[12,13]. The current study aimed to determine how platelet counts, platelets transfusions, and FFP transfusions affect the outcomes of AVB in cirrhosis patients in terms of bleeding control, rebleeding, and mortality.

MATERIALS AND METHODS

Patients and methods

The study comprised cirrhosis patients with AVB who presented to the All India Institute of Medical Sciences, New Delhi, India, a tertiary care center. A prospectively managed database was used to include patients diagnosed with bleeding from esophageal or fundal varices on esophagogastroduodenoscopy (EGD) between October 2017 and October 2021. AVB was defined on EGD by visible spurt, white nipple, or signs of recent hemorrhage. Patients with variceal bleeding not associated with liver cirrhosis, such as non-cirrhotic portal fibrosis, extrahepatic portal venous obstruction, splenic vein thrombosis with chronic pancreatitis *etc.*, were excluded, as were patients with non-variceal hematemesis and those who did not give consent. Cirrhosis was defined based on imaging, histology or fibroscan (liver stiffness measurement > 12 kPa).

Ethical clearance was obtained from the institutional ethics committee (IECPG). Some of the patients were also part of a TEG-based transfusion trial (CTRI/2017/02/007864)[14] and secondary prophylaxis of gastric varices (CTRI/2021/02/031396).

Management of patients with AVB

Baseline treatment included resuscitation and airway management. Following resuscitation, patients were transfused packed red blood cells (based on existing guidelines) targeting a hemoglobin level of 7 gm/dL in cirrhotics without cardiac dysfunction and 10 gm/dL in patients with cardiac comorbidities. Inotropes were initiated in patients with shock to maintain a mean arterial pressure of 65-70 mmHg. Mechanical ventilation indications included respiratory failure or airway protection prior to EGD. All patients received prophylactic antibiotics and vasoactive therapy with somatostatin/terlipressin prior to EGD, which was performed within 12 h of presentation to the hospital. The vasoactive agents were continued until day 3 of admission. The patients were initiated on non-selective beta-blockers, such as carvedilol or propranolol, with doses titrated according to heart rate/or blood pressure. The decision for transfusion of blood products (FFP, platelets) was taken by the treating team in the emergency department or as part of the randomized controlled trial[14]. The decision for repeat endoscopy, balloon-occluded retrograde transvenous obliteration (BRTO) or rescue transjugular intrahepatic portosystemic shunt (TIPS) was taken by the treating team based on the patient's clinical condition.

Data collection

Baseline demographic, hematologic, and biochemical parameters were collected. Child-Turcotte-Pugh (CTP) and Model for End-stage Liver Disease (MELD) scores were calculated on admission. The details of type and units of blood products transfused (FFP/platelet and PRBCs) were noted from the patient's chart. Requirements of rescue therapies: TIPS, Sengstaken-Blakemore tube (SB tube), self-expanding Ella Danis stent (SX-Ella Danis) or BRTO were noted.

Rebleeding or failure of therapy was defined as per the Baveno V consensus as follows[15]: (1) Death within 120 h; (2) Fresh hematemesis or nasogastric aspiration of 100 mL of fresh blood 2 h after starting a specific drug treatment or therapeutic endoscopy; (3) Development of hypovolemic shock; and (4) A 3g drop in hemoglobin (equivalent to a 9% drop in hematocrit) within any 24 h if no transfusion is administered

Assessment of outcomes

The primary outcome of the study was the rebleeding at days 5 and 42, and death at day 42 after an episode of AVB in the 3 platelet groups. We also analyzed the differences in the rebleeding and death rates between those who received platelet transfusions and those who did not. Propensity score matching was done to compare the outcomes in those who received and did not receive platelet transfusion. The secondary outcomes were rebleeding at days 5 and 42, and death at day 42, after an episode of AVB in patients receiving FFP alone or in combination with platelet transfusion. In addition, we assessed the risk factors for rebleeding and death on day 42.

Statistical analysis

The normality of the data was assessed using the Shapiro-Wilk test. Skewed continuous variables were expressed as median [interquartile range (IQR)], and non-skewed as mean (sd). The qualitative data

were expressed as numbers (%). Kruskal–Wallis test was used to compare more than two groups with non-parametric data. Comparison of categorical variables was made using the Fisher's exact test or Pearson's chi-squared test. For statistical evaluation, patients were further classified into three groups based on platelet counts of < 20 × 10^9/L, 20 × 10^9-50 × 10^9/L, and > 50 × 10^9/L. Survival analysis and rebleeding at 5 and 42 d stratified as per the platelet counts and transfusion of blood products were performed using Kaplan-Meier and compared with the log-rank test. Mortality and rebleeding were used as endpoints, and patients were censored at last patient contact. Univariate and multivariate Cox-proportional model regression analysis was done to assess the predictors of rebleeding and mortality at 42 d. Effect sizes for the identified predictors were reported as hazard ratio with 95% confidence interval. A *P* value of 0.05 was considered statistically significant. The data were analyzed using IBM SPSS Statistics software (version 20.0, Chicago, IL, United States) and Medcalc software (version 15.11.4, MedCalc Software, Ostend, Belgium)

RESULTS

A total of 913 cirrhosis patients with AVB comprising 762 males (83.5%) and 151 females (16.5%) were enrolled (Figure 1). The median age of the patients' cohort was 45 years (35-54), and their median MELD and CTP score were 14.7 (11.1-20.3) and 7 (6-9), respectively. At the time of presentation, the median hemoglobin level was 7.6 gm/dL (6.1-9.4 gm/dL), and platelet counts were 96 × 10^9/L (55 × 10^9-135 × 10^9/L). The number of patients in each of the three groups based on platelet counts < 20 × 10^9L, 20 × 10^9 -50 × 10^9/L and > 50 × 10^9/L were 23 (2.5%), 168 (18.4%), and 722 (79.1%), respectively. The most common feature of decompensation was ascites in 456 patients (49.9%), followed by hepatic encephalopathy (HE) in 93 patients (10.2%). The most common etiology of cirrhosis was chronic alcohol use in 393 cases (43%). Endotherapy was offered to 711 patients (77.9%), and rebleeding was observed in 48 patients (5.3%) at 5 d and 138 patients (15.1%) at 42 d. Radiological interventions for management of rebleed were done in 17 (1.9%) patients and included TIPS in 8, BRTO in 3, SB tube in 2 and SX-Ella Danis stent placement in 4 patients (Table 1). The overall 42-d mortality rate was found to be 18.2% (*n* = 166).

Comparison of baseline parameters and outcomes in three platelets groups

Demographic and vital parameters were well matched across the three groups. All groups had similar values of hemoglobin and INR. Patients with platelet counts < 20 × 10^9/L had significantly higher creatinine values at baseline as compared to the group with platelet count between 20-50 × 10^9/L (1.1 mg/dL *vs* 0.8 mg/dL, *P* < 0.001), however, there were no significant differences with the other two groups in terms of etiology of cirrhosis, liver related parameters, hepatocellular carcinoma at presentation, or features of decompensation (Ascites, HE). There were no differences in baseline MELD scores; however, the median CTP score was lower in the group with platelet counts > 50 × 10^9/L than those with platelet count < 20 × 10^9/L (7 *vs* 8, *P* = 0.044) (Table 1).

Among patients with platelet counts less than 20 x 10^9/L, 20-50 × 10^9/L and greater than 50 × 10^9/L, 10 (43.5%), 53 (31.5%) and 28 (3.9%) patients received platelet transfusion, respectively (*P* < 0.001). There were no significant differences in the source of bleeding, which was most commonly from high-grade esophageal varices, the requirement of PRBC or FFP transfusion, endotherapy offered, rebleeding rates at 5 and 42 d, or mortality at 42 d among the three groups when analyzed for baseline platelet counts (Table 2, Figure 2A and B).

On comparison of patients who underwent endotherapy *vs* no endotherapy, there was no difference in the rebleed at 5 d [36/711 (5.1%) *vs* 12/202 (5.9%), *P* = 0.595] and 42 d [102/711 (14.3%) *vs* 36/202 (17.8%), *P* = 0.223].

Analysis of results based on platelet transfusion

Ninety-one (10%) patients received platelet transfusions as a part of management, while 822 patients did not. There was a significant difference in age between the groups receiving platelets compared to those who did not (median age 42 *vs* 45 years, *P* = 0.012). As expected, platelet counts were significantly lower in the group receiving platelets than the non-receiving group with the median value 40 × 10^9/L *vs* 100 × 10^9/L, (*P* < 0.001). These patients also had lower median heart rate (90/min *vs* 96/min, *P* = 0.016), total leucocyte counts (5.6 × 10^9/L *vs* 6.6 × 10^9/L, *P* = 0.012) and serum creatinine (0.7 mg/dL *vs* 0.8 mg/dL, *P* = 0.003) than their counterparts (Table 3). There were no significant differences noted in the etiology of cirrhosis, alcohol use, liver-related parameters, CTP scores and MELD score, although patients who received platelets were more likely to present with ascites (64.8% *vs* 48.3%, *P* = 0.003) and HE (16.5% *vs* 9.5%, *P* = 0.044) than those who did not.

The most common bleeding source in either group was high-grade esophageal varices (84.6% and 86.6%, respectively). There was no difference in endotherapy rates offered to patients in either group. Patients receiving platelets had significantly higher rebleeding rates at day 5, 13/91 (14.3%) as compared to those who did not 35/822 (4.3%) (*P* < 0.001). The rate of rebleeding among those receiving platelets was even higher 29/91 (31.9%) at day 42 as compared to those who did not 109/822 (13.3%) (*P*

Table 1 Comparison of baseline characteristics of cirrhosis patients with platelet counts < 20 × 10^9/L, 20-50 × 10^9/L and > 50 × 10^9/L

Characteristics	Total (*n* = 913)	Platelet count < 20 × 10^9/L (*n* = 23)	Platelet count 20-50 × 10^9/L (*n* = 168)	Platelet count > 50 × 10^9/L (*n* = 722)	*P* value
Age (years)	45 (35-54)	42.0 (33-46)	43 (34-53)	45 (36-54)	0.068
Sex (Males:Female)	762 (83.5): 151 (16.5)	20 (87.0): 3 (13.0)	136 (81.0): 32 (19.0)	606 (83.9): 116 (16.1)	0.581
Heart rate (per minute)	96 (86-110)	94 (86-100)	94 (85-110)	96 (86-110)	0.397
MAP (mm of Hg)	82 (74-89)	81 (74-84)	81 (75-88)	82 (73-89)	0.771
Hemoglobin (g/dl)	7.6 (6.1-9.4)	8.3 (5.7-9.6)	7.4 (6.0-8.7)	7.8 (6.1-9.5)	0.168
TLC (×10^9/L)	6.5 (3.8-9.2)	6.9 (3.7-9.6)	5.1 (3.1-7.9)	6.8 (4.2-9.7)	< 0.001[b]
Platelet count (×10^9/L)	96 (55-135)	12.0 (10.0-15.0)	40.0 (34.0-46.0)	118.0 (80.0-150.0)	< 0.001[b,c]
INR	1.5 (1.3-1.9)	1.7 (1.3-2.0)	1.6 (1.3-1.9)	1.5 (1.3-1.9)	0.337
Serum urea (mg/dL)	37 (24-64)	45 (22-101)	36 (23-55)	37 (25-66)	0.298
Creatinine (mg/dL)	0.8 (0.6-1.2)	1.1 (0.7-2.0)	0.8 (0.6-1.1)	0.8 (0.6-1.3)	0.010[a]
Sodium (meq/L)	139 (135-142)	137 (131-141)	140 (136-143)	139 (135-142)	0.065
Bilirubin (mg/dL)	1.6 (0.9-3.1)	2.1 (1.0-7.7)	1.7 (0.9-3.4)	1.6 (0.9-2.9)	0.317
AST (IU/L)	51 (34-86)	49.0 (35.0-103.0)	56.0 (38.0-82.0)	50.0 (33.0-87.0)	0.410
ALT (IU/L)	35 (23-55)	36.0 (23.0-120.0)	37.0 (24.0-56.0)	34.0 (22.0-54.0)	0.500
Albumin (g/dL)	3.2 (2.7-3.8)	2.8 (2.1-3.7)	3.1 (2.7-3.8)	3.2 (2.7-3.8)	0.146
CTP	7 (6-9)	8.0 (7.0-10.0)	7.0 (6.0-10.0)	7.0 (6.0-9.0)	0.044[c]
MELD scores	14.7 (11.1-20.3)	17.2 (10.0-28.3)	14.4 (11.3-19.6)	14.8 (11.1-20.3)	0.551
Ascites	456 (49.9)	12 (52.2)	97 (57.7)	347 (48.1)	0.076
HCC	35 (3.8)	0	8 (4.8)	27 (3.7)	0.515
HE	93 (10.2)	2 (8.7%)	22 (13.1)	69 (9.6)	0.382
Endotherapy					0.815
No therapy	202 (22.1)	7 (30.4)	34 (20.2)	161 (22.3)	
Glue	105 (11.5)	1 (4.3)	19 (11.2)	85 (11.8)	
Ethoxysclerol	43 (4.7)	2 (8.7)	6 (3.6)	35 (4.8)	
EVL	537 (58.8)	12 (52.2)	102 (60.7)	423 (58.6)	
APC	2 (0.2)	0	1 (0.6)	1 (0.1)	
Glue and EVL	24 (2.6)	1 (4.3)	6 (3.6)	17 (2.4)	
Child Class					0.047
A	374 (41.0)	5 (21.7)	65 (38.7)	304 (42.1)	
B	361 (39.5)	10 (43.5)	61 (36.3)	290 (40.2)	
C	178 (19.5)	8 (34.8)	42 (25.0)	128 (17.7)	
Etiology					0.772
Alcohol	393 (43.0)	9 (39.1)	76 (45.2)	308 (42.7)	
Others	520 (57.0)	14 (60.9)	92 (54.8)	414 (57.3)	
RBC					0.548
0	542 (59.4)	12 (52.2)	91 (54.2)	439 (60.8)	
1	143(15.7)	4 (17.4)	29 (17.3)	110 (15.2)	
≥2	228 (25.0)	7 (30.4)	48 (28.6)	173 (24.0)	
FFP transfusion	108 (11.8)	3 (13.0)	23 (13.7)	82 (11.4)	0.689
Number of FFP transfusion	3 (3-4)	3 (3-3)	3 (3-4)	3 (3-4)	0.728

Platelets transfusion	91 (10.0)	10 (43.5)	53 (31.5)	28 (3.9)	< 0.001[a]
Number of platelet transfusion	3 (3-3)	3 (2.7-3.2)	3 (3-3)	3 (3-3.7)	0.728
Rescue therapy (Radiological intervention)	17 (1.9)	2 (8.7)	7 (4.2)	8 (1.1)	0.001[a]
Grade of varices low:high	128 (14.0): 785 (86.0)	4 (17.4): 19 (82.6)	24 (14.3): 144 (85.7)	100 (13.9): 622 (86.1)	0.885
Cause of bleed variceal					0.898
Esophageal	789 (86.4)	21 (91.3)	148 (88.1)	620 (85.9)	
Fundal	55 (6.0)	1 (4.3)	9 (5.4)	45 (6.2)	
Esophageal and Fundal	69 (7.6)	1 (4.3)	11 (6.5)	57 (7.9)	

[a]20 × 10^9/L *vs* 20-50 × 10^9/L.
[b]20-50 × 10^9/L *vs* > 50 × 10^9/L.
[c]< 20 × 10^9/L *vs* > 50 × 10^9/L. All values are represented as *n* (%) or median (IQR).
APC: Argon plasma Coagulation, AST: Aspartate Transaminase, ALT: Alanine Transaminase, CTP: Child-Turcotte-Pugh score, INR: Internationalized Normalized Ratio, EVL: Endoscopic Variceal Ligation, FFP: Fresh Frozen Plasma, HE: Hepatic Encephalopathy, HCC: Hepatocellular carcinoma, MAP: Mean Arterial Pressure, MELD: Model for End Stage Liver Disease, RBC: Red blood cells, TLC: Total Leucocyte Count

Table 2 Rebleed rates at 5 days and 42 days, and mortality at 42 days in cirrhosis patients with platelet counts < 20 × 10^9/L, 20-50 × 10^9/L and > 50 × 10^9/L

Characteristics	Total (*n* = 913)	Platelet count < 20 × 10^9/L (*n* = 23)	Platelet count 20-50 × 10^9/L (*n* = 168)	Platelet count > 50 × 10^9/L (*n* = 722)	*P* value
Rebleed at 5 d	48 (5.3)	3 (13.0)	11 (6.5)	34 (4.7)	0.150
Rebleed at 42 d	138 (15.1)	5 (21.7)	29 (17.3)	104 (14.4)	0.433
Death at 42 d	166 (18.2)	3 (13.0)	39 (23.2)	124 (17.2)	0.153

All values are represented as *n* (%).

< 0.001) (Figure 3A). Patients who received transfusions had a significantly greater rate of rebleeding in the groups with platelet counts between 20 × 10^9/L and 50 × 10^9/L (log-rank *P* < 0.001) and > 50 × 10^9/L (log-rank *P* = 0.038), but not in the group with platelet count < 20 × 10^9/L (log-rank *P* = 0.303) (Figure 3B-D). Patients receiving platelets had higher mortality rates overall 23/91 (25.3%) as compared to those who did not 143/822 (17.4%), although the difference was not significant (*P* = 0.074) (Figure 4A). There were no significant differences in mortality rates when assessed for group-wise outcomes (Figure 4B-D).

Propensity score matching

To compare the outcomes in those who received and those who did not receive platelet transfusion, we matched the 2 groups for variables such as age, heart rate, creatinine, sodium, presence of ascites, HE, and transfusion of FFP. The comparison of the 2 groups is shown in Table 3.

In the matched cohort (*n* = 89), patients receiving platelets had significantly higher rebleeding rates at day 5, 13/89 (14.6%) as compared to those who did not 4/89 (4.5%) (*P* = 0.039). The rate of rebleeding among those receiving platelets was even higher 29/89 (32.6%) at day 42 as compared to those who did not 14/89 (15.7%) (*P* = 0.014) (Figure 5A). Patients receiving platelets had higher mortality rates overall 23/89 (25.8%) as compared to those who did not 21/89 (23.6%), although the difference was not significant (*P* = 0.862) (Figure 5B).

Factors associated with 42-d rebleeding

In the pre-matched group, univariate Cox-proportional hazard analysis identified lower mean arterial pressure (MAP) at presentation, elevated levels of INR, serum urea, serum bilirubin, and AST to be associated with a significantly higher risk of rebleeding at 42 d. Patients with higher CTP and MELD scores, those presenting with decompensation in the form of ascites and HE, and those receiving PRBCs, FFP or platelets transfusions were at a higher risk of experiencing a rebleed within 42 d of the index event. Platelet count at presentation was not associated with rebleeding at 42 d. The Hazard ratio of the relevant risk factors is provided in Table 4.

On PSM-analysis, the factors significant on univariate Cox-proportional hazard analysis are shown in Table 3. On multivariate analysis, platelet transfusion was independently associated with 42-d rebleeding (HR, 2.924, 95%CI, 1.448-5.903, *P* = 0.003) after adjusting for MAP, INR, AST, albumin, HE,

Table 3 Comparison of baseline characteristics of cirrhosis patients who received platelet and those who did not

	Before PSM analysis			After PSM analysis		
Characteristics	**Platelets transfusion (*n* = 91)**	**No platelets transfusion (*n* = 822)**	***P* value**	**Platelet transfusion (*n* = 89)**	**No platelet transfusion (*n* = 89)**	***P* value**
Age (yr)	42 (34-50)	45 (35-54)	0.012	42 (34-50)	40 (30-50)	0.716
Sex (Male:Female)	77 (84.6): 14 (15.4)	685 (83.3): 137 (16.7)	0.882	75 (84.3): 14 (15.7)	80 (89.9): 9 (10.1)	0.372
Heart rate (per minute)	90 (84-100)	96 (86-110)	0.016	90 (85-100)	89 (82-100)	0.546
MAP (mm of Hg)	81 (75-87)	82 (74-90)	0.341	81 (75-87)	81 (73-88)	0.968
Hemoglobin (g/dL)	7.7 (6.1-9.4)	7.6 (6.1-9.4)	0.890	7.7 (6.1-9.4)	7.5 (6.3-9.0)	0.720
TLC ($\times 10^9$/L)	5.6 (3.1-8.3)	6.6 (4.0-9.4)	0.012	5.6 (3.1-8.3)	7.0 (4.4-12.0)	0.002
Platelet count ($\times 10^9$/L)	40.0 (32.0-58.0)	100.0 (63.0-139.0)	< 0.001	40.0 (32.0-58.0)	81 (57-126)	< 0.001
INR	1.6 (1.3-2.0)	1.5 (1.3-1.9)	0.266	1.6 (1.3-2.0)	1.7 (1.4-2.2)	0.402
Serum urea (mg/dL)	41 (28-60)	36 (24-64)	0.864	41 (28-61)	34 (24-69)	0.369
Creatinine (mg/dL)	0.7 (0.5-1.0)	0.8 (0.6-1.3)	0.003	0.7 (0.5-1.0)	0.8 (0.6-1.4)	0.040
Sodium (meq/L)	140.2 (137.0-143.0)	139.0 (135.0-142.0)	0.023	140 (137-143)	140 (135-143)	0.529
Bilirubin (mg/dL)	1.7 (0.9-3.8)	1.6 (0.9-3.0)	0.771	1.7 (0.9-3.8)	2.4 (1.3-4.8)	0.071
AST (IU/L)	49 (34-79)	51 (34-88)	0.570	49 (34-79)	67 (38-119)	0.019
ALT (IU/L)	32 (22-58)	35 (23-54)	0.905	32 (22-58)	41 (30-67)	0.029
Albumin (g/dL)	3.2 (2.7-3.8)	3.2 (2.7-3.8)	0.897	3.2 (2.7-3.8)	3.1 (2.6-3.6)	0.355
CTP	7 (6-10)	7 (6-9)	0.119	8 (6-10)	8 (6-10)	0.186
MELD	14.6 (10.9-20.2)	14.7 (11.1-20.3)	0.878	14.6 (10.9-20.2)	16.1 (12.5-24.1)	0.079
Ascites	59 (64.8)	397 (48.3)	0.003	59 (66.3)	58 (65.2)	1.000
HCC	6 (6.6)	29 (3.5)	0.150	6 (6.7)	4 (4.5)	0.747
HE	15 (16.5)	78 (9.5)	0.044	15 (16.9)	22 (24.7)	0.268
Endotherapy (yes)	71 (78.0)	640 (77.9)	1.000	72 (80.9)	71 (79.8)	1.000
Child class			0.210			0.313
A	33 (36.3)	341 (41.5)		31 (34.8)	22 (24.7)	
B	34 (37.4)	327 (39.8)		34 (38.2)	37 (41.6)	
C	24 (26.4)	154 (18.7)		24 (27.0)	30 (33.7)	
Etiology			0.824			0.176
Alcohol	38 (41.8)	355 (43.2)		37 (41.6)	47 (52.8)	
Other	53 (58.2)	467 (56.8)		52 (58.4)	42 (47.2)	
RBC			0.483			0.294
0	49 (53.8)	493 (60.0)		48 (53.9)	56 (62.9)	
1	15 (16.5)	128 (15.6)		14 (15.7)	15 (16.9)	
≥ 2	27 (29.7)	201 (24.5)		27 (30.3)	18 (20.2)	
FFP transfusion	22 (24.2)	86 (10.5)	< 0.001	22 (24.7)	22 (24.7)	1.000
Grade of varices low:high	71 (78.0)	714 (86.9)	0.026	69 (77.5)	84 (94.4)	0.002

All values are represented as *n* (%) or median (IQR). AST: Aspartate transaminase; ALT: Alanine transaminase; CTP: Child-Turcotte-Pugh score; INR: Internationalized normalized ratio; EVL: Endoscopic variceal ligation; FFP: Fresh frozen plasma; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; MAP: Mean arterial pressure; MELD: Model for end stage liver disease; RBC: Red blood cells; TLC: Total leucocyte count.

Table 4 Cox-proportional analysis of variables associated with 42-days rebleeding in the whole cohort and after propensity score matching

	Whole cohort		After propensity score matching					
Characteristics	**Univariate analysis HR (95%CI)**	***P* value**	**Univariate analysis HR (95%CI)**	***P* value**	**Model 1 (Excluding CTP)**		**Model 2 (Including CTP)**	
					Adjusted HR (95%CI)	***P* value**	**Adjusted HR (95%CI)**	***P* value**
Age (yr)	1.000 (0.987-1.013)	0.973	1.007 (0.984-1.031)	0.584				
Sex								
Male	1		1					
Female	0.662 (0.393-1.114)	0.120	1.330 (0.560-3.158)	0.517				
Heart rate (per minute)	0.995 (0.986-1.004)	0.298	1.011 (0.996-1.026)	0.157				
MAP (mm of Hg)	0.979 (0.965-0.992)	0.002	0.966 (0.938-0.996)	0.024	9.972 (0.938-1.008)	0.131	0.968 (0.936-1.001)	0.057
Hemoglobin (g/dL)	0.969 (0.906-1.036)	0.349	0.914 (0.800-1.045)	0.189				
TLC ($\times 10^9$/L)	1.023 (0.995-1.053)	0.111	0.991 (0.933-1.052)	0.761				
Platelet count ($\times 10^9$/L)	0.999 (0.997-1.001)	0.360	0.995 (0.989-1.001)	0.129				
INR	1.528 (1.296-1.802)	< 0.001	1.427 (0.976-2.086)	0.067	1.341 (0.784-2.295)	0.284		
Serum urea (mg/dL)	1.005 (1.001-1.008)	0.012	1.004 (0.997-1.011)	0.237				
Creatinine (mg/dL)	1.158 (1.021-1.314)	0.023	1.031 (0.805-1.319)	0.811				
Sodium (meq/L)	0.993 (0.966-1.022)	0.647	0.980 (0.929-1.033)	0.451				
Bilirubin (mg/dL)	1.065 (1.045-1.086)	< 0.001	1.016 (0.976-1.058)	0.434				
AST (IU/L)	1.001 (1.000-1.001)	0.005	1.001 (1.000-1.002)	0.002	1.001 (1.000-1.002)	0.284	1.001 (1.000-1.002)	0.021
ALT (IU/L)	1.000 (0.998-1.002)	0.920	1.000 (0.998-1.003)	0.761				
Albumin (g/dL)	0.833 (0.672-1.031)	0.093	0.543 (0.363-0.811)	0.003	0.690 (0.431-1.105)	0.122		
CTP	1.255 (1.177-1.337)	< 0.001	1.169 (1.048-1.303)	0.005	-		1.081 (0.959-1.220)	0.203
MELD	1.057 (1.038-1.077)	< 0.001	1.028 (0.995-1.063)	0.102	-			
Ascites, yes	2.525 (1.757-3.630)	< 0.001	1.906 (0.939-3.870)	0.074	0.857 (0.376-1.953)	0.713		
HCC, yes	2.532 (1.367-4.690)	0.003	0.370 (0.051-2.687)	0.326				
HE, yes	3.969 (2.700-5.836)	< 0.001	2.489 (1.324-4.679)	0.005	1.791 (0.836-3.836)	0.134		
Endotherapy (yes)	0.702 (0.480-1.027)	0.069	0.999 (0.463-2.155)	0.998				
Child class								
A	1		1					
B	1.849 (1.187-2.879)	0.007	1.811 (0.738-4.444)	0.195				
C	4.653 (2.988-7.245)	< 0.001	3.695 (1.567-8.715)	0.003				
Etiology								
Alcohol	1	0.095	1	0.124				
Other	0.753 (0.539-1.051)		0.622 (0.339-1.140)					
RBC (units)								

0	1		1		1		1	
1	1.482 (0.944-2.327)	0.087	1.162 (0.464-2.910)	0.748	1.173 (0.460-2.992)	0.738	1.253 (0.493-3.180)	0.636
≥ 2	1.434 (0.979-2.098)	0.064	2.571 (1.349-4.902)	0.004	1.998 (0.962-4.152)	0.064	1.900 (0.942-3.831)	0.073
FFP transfusion	3.078 (2.096-4.518)	< 0.001	1.490 (0.777-2.858)	0.220				
Platelet transfusion	2.613 (1.735-3.936)	< 0.001	2.204 (1.165-4.172)	0.015	2.924 (1.448-5.903)	0.003	2.702 (1.345-5.429)	0.005
Grade of varices (high)	0.829 (0.526-1.308)	0.421	0.671 (0.311-1.446)	0.308				

AST: Aspartate transaminase; ALT: Alanine transaminase; CTP: Child-Turcotte-Pugh score; INR: Internationalized normalized ratio; EVL: Endoscopic variceal ligation; FFP: Fresh frozen plasma; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; MAP: Mean arterial pressure; MELD: Model for end stage liver disease; RBC: Red blood cells; TLC: Total leucocyte count.

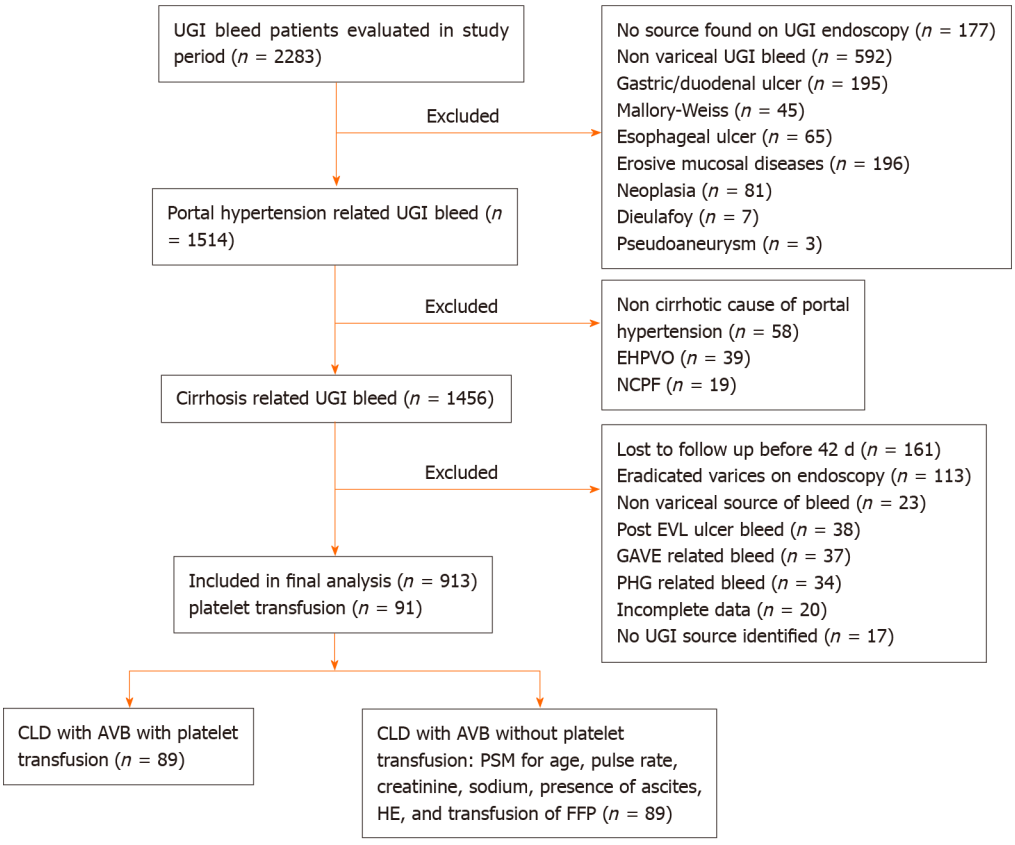

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Figure 1 CONSORT chart for inclusion of patients. AVB: Acute variceal bleeding; HE: Hepatic encephalopathy; PSM: Propensity score matching; FFP: Fresh frozen plasma; EHPVO: Extrahepatic portal vein obstruction; NCPF: Non-cirrhotic portal fibrosis; UGI: upper gastrointestinal; EVL: Endoscopic variceal ligation; GAVE: Gastric antral vascular ectasia; PHG: Phenylethanoid glycosides.

and PRBC transfusion. In another multivariate model, platelet transfusion was also independently associated with 42-d rebleeding after adjusting for CTP score and other significant variables (Table 4).

Factors associated with 42-d mortality

The factors associated with 42-d mortality on univariate Cox-proportional hazard analysis are shown in Table 5. Platelet count/platelet transfusion was not associated with 42-d mortality in the PSM cohort. Presence of HE was independently associated with mortality after adjusting for INR, creatinine, bilirubin, AST, albumin, presence of ascites, endotherapy, etiology of chronic liver disease, and FFP transfusion.

Table 5 Cox-proportional analysis of variables associated with 42-days mortality in the whole cohort and after propensity score matching

Characteristics	Whole cohort		After propensity score matching			
	Univariate analysis HR (95%CI)	*P* value	Univariate analysis HR (95%CI)	*P* value	Adjusted HR (95%CI)	*P* value
Age (yr)	1.010 (0.998-1.022)	0.118	1.016 (0.993-1.039)	0.174		
Sex						
Male	1		1			
Female	0.682 (0.427-1.088)	0.108	0.889 (0.350-2.256)	0.804		
Heart rate (per minute)	0.999 (0.990-1.007)	0.759	1.004 (0.989-1.021)	0.581		
MAP (mm of Hg)	0.988 (0.976-1.000)	0.046	0.996 (0.967-1.027)	0.816		
Hemoglobin (g/dL)	0.949 (0.892-1.010)	0.100	0.992 (0.872-1.128)	0.903		
TLC ($\times 10^9$/L)	1.047 (1.023-1.071)	< 0.001	1.034 (0.986-1.084)	0.172		
Platelet count ($\times 10^9$/L)	0.998 (0.995-1.000)	0.053	0.998 (0.992-1.003)	0.422		
INR	1.903 (1.689-2.143)	< 0.001	1.656 (1.246-2.201)	0.001	1.361 (0.825-2.244)	0.228
Serum urea (mg/dL)	1.009 (1.006-1.012)	< 0.001	1.005 (0.998-1.012)	0.142		
Creatinine (mg/dL)	1.374 (1.263-1.495)	< 0.001	1.205 (1.004-1.446)	0.046	0.985 (0.771-1.258)	0.901
Sodium (meq/L)	0.991 (0.966-1.017)	0.494	0.996 (0.943-1.052)	0.876		
Bilirubin (mg/dL)	1.077 (1.061-1.094)	< 0.001	1.040 (1.010-1.072)	0.010	1.013 (0.967-1.061)	0.588
AST (IU/L)	1.001 (1.001-1.002)	< 0.001	1.001 (1.001-1.002)	< 0.001	1.001 (1.000-1.002)	0.113
ALT (IU/L)	1.002 (1.001-1.002)	< 0.001	1.001 (0.999-1.003)	0.249		
Albumin (g/dL)	0.671 (0.548-0.821)	< 0.001	0.641 (0.433-0.948)	0.026	0.964 (0.619-1.501)	0.871
CTP	1.369 (1.294-1.448)	< 0.001	1.239 (1.114-1.378)	< 0.001		
MELD	1.097 (1.080-1.115)	< 0.001	1.060 (1.029-1.091)	< 0.001		
Ascites, yes	2.673 (1.911-3.739)	< 0.001	1.876 (0.926-3.799)	0.080	1.043 (0.431-2.525)	0.925
HCC, yes	1.637 (0.836-3.206)	0.150	1.258 (0.390-4.063)	0.701		
HE, yes	5.686 (4.102-7.881)	< 0.001	3.825 (2.014-6.953)	< 0.001	2.586 (1.260-5.307)	0.010
Endotherapy, yes	0.548 (0.394-0.760)	< 0.001	0.423 (0.226-0.790)	0.007	0.589 (0.296-1.169)	0.130
Child class						
A	1		1			
B	1.771 (1.142-2.747)	0.011	1.002 (0.395-2.538)	0.997		
C	6.785 (4.502-10.227)	< 0.001	3.759 (1.698-8.321)	0.001		
Etiology						
Alcohol	1		1		1	
Other	0.641 (0.473-0.870)	0.004	0.600 (0.329-1.094)	0.096	0.920 (0.470-1.799)	0.808
RBC						
0	1		1			
1	1 0.966 (0.629-1.484)	0.874	1.158 (0.522-2.567)	0.718		
≥ 2	0.741 (0.505-1.086	0.125	1.024 (0.504-2.081)	0.948		
FFP transfusion	2.532 (1.762-3.637)	< 0.001	1.923 (1.040-3.555)	0.037	1.066 (0.510-2.230)	0.865
Platelet transfusion	1.489 (0.958-2.312)	0.077	1.098 (0.608-1.984)	0.757		
Grade of varices (high)	1.348 (0.826-2.198)	0.232	0.880 (0.392-1.975)	0.757		

AST: Aspartate transaminase; ALT: Alanine transaminase; CTP: Child-Turcotte-Pugh score; INR: Internationalized normalized ratio; EVL: Endoscopic variceal ligation; FFP: Fresh frozen plasma; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; MAP: Mean arterial pressure; MELD: Model for end stage liver disease; RBC: Red blood cells; TLC: Total leucocyte count.

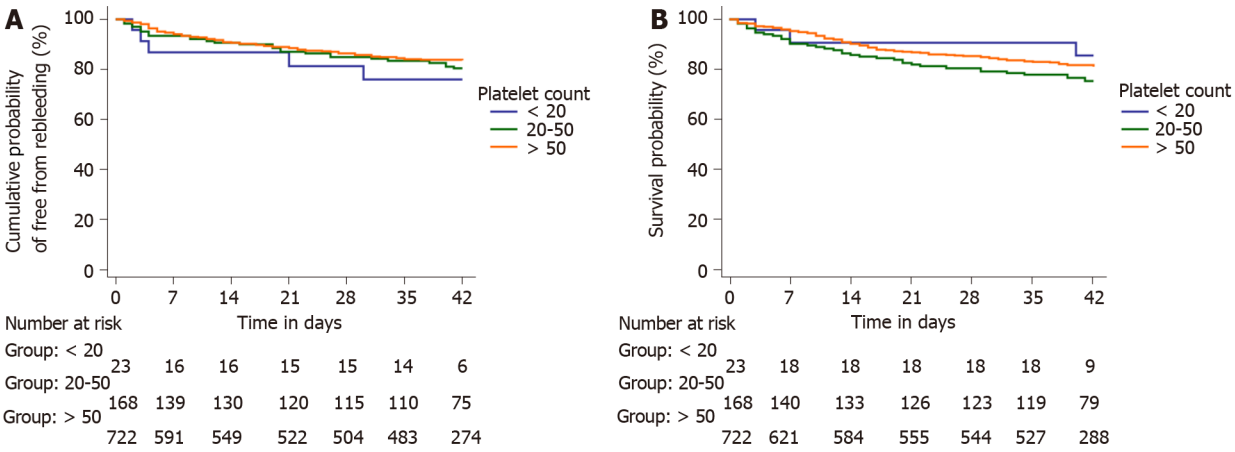

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Figure 2 Kaplan Meier curves for the entire cohort of patients based on baseline platelet counts demonstrating cumulative probability. A: Free from rebleed (log-rank $P = 0.396$); B: Survival (log-rank $P = 0.176$).

Analysis of results based on FFP transfusion

Patients were also assessed for FFP transfusions received as part of management (details appended as Supplementary data). Patients who received FFP had significantly higher PRBC requirements (61.1% *vs* 37.9%; $P < 0.001$), with significantly more patients experiencing rebleed on day 5 (16.7% *vs* 3.7%; $P < 0.001$) and day 42 (32.4% *vs* 12.8%; $P < 0.001$) with higher mortality rates within 42 d of index bleeding (35.2% *vs* 15.9%; $P < 0.001$), as compared to those who did not receive transfusion (Supplementary Table 1).

Kaplan Meier estimates revealed significantly higher rebleed rates at days 5 and 42 and higher 42-d mortality from index bleeding episode ($P < 0.001$) among patients who received FFP transfusions compared to those who did not (Supplementary Figure 1A and B).

Analysis based on any transfusion- either FFP or platelets

A further subgroup analysis was done to assess outcomes of 177 patients who received either blood product (FFP or platelet) compared to 736 patients who received no transfusions (Supplementary Table 2). A significantly higher proportion of these patients were decompensated at presentation with ascites in 67.2% *vs* 45.8%; $P < 0.001$ and HE in 20.9% *vs* 7.6%; $P < 0.001$ compared to those not receiving transfusions. The severity of illness scores was significantly higher in those receiving transfusions (CTP: 9 *vs* 7; $P < 0.001$ and MELD 18.7 *vs* 14.1; $P < 0.001$). Patients receiving transfusions had higher rebleeding rates at day 5 (14.1% *vs* 3.1%; $P < 0.001$) and 42 (31.6% *vs* 11.1%; $P < 0.001$) with higher PRBC requirements (53.1% *vs* 37.6%; $P = 0.001$). The overall 42-d mortality was also higher in those receiving transfusions (30.5% *vs* 15.2%; $P < 0.001$) (Supplementary Figure 2A and B).

DISCUSSION

Cirrhosis-related coagulopathy is a topic of long-standing debate. Clinically, some patients demonstrate increased bleeding rates with invasive procedures. In contrast, others may develop spontaneous thrombosis of the main portal vein or its tributaries, indicating that the coagulation system in cirrhotics behaves differently in individual patients, demonstrating both pro- and anticoagulant tendencies[16-18]. Thus, coagulopathy in cirrhosis exists as a spectrum ("rebalanced hemostasis") with anticoagulant and procoagulant nature being the two extreme endpoints. Recent evidence supports this approach to the management of bleeding risks in such patients[19].

Transfusion of blood products in cirrhotics is associated with several risks despite the apparent clinical benefits of correcting thrombocytopenia and deranged INR[20]. Prior studies have demonstrated rise portal pressures by 1.4 ± 0.7 mm of Hg for every 100 mL of blood product transfusion[21,22]. Overzealous resuscitative measures may predispose patients to a vicious cycle of rebleeding with higher transfusion requirements, extended hospital stays and poorer outcomes. This was demonstrated in the

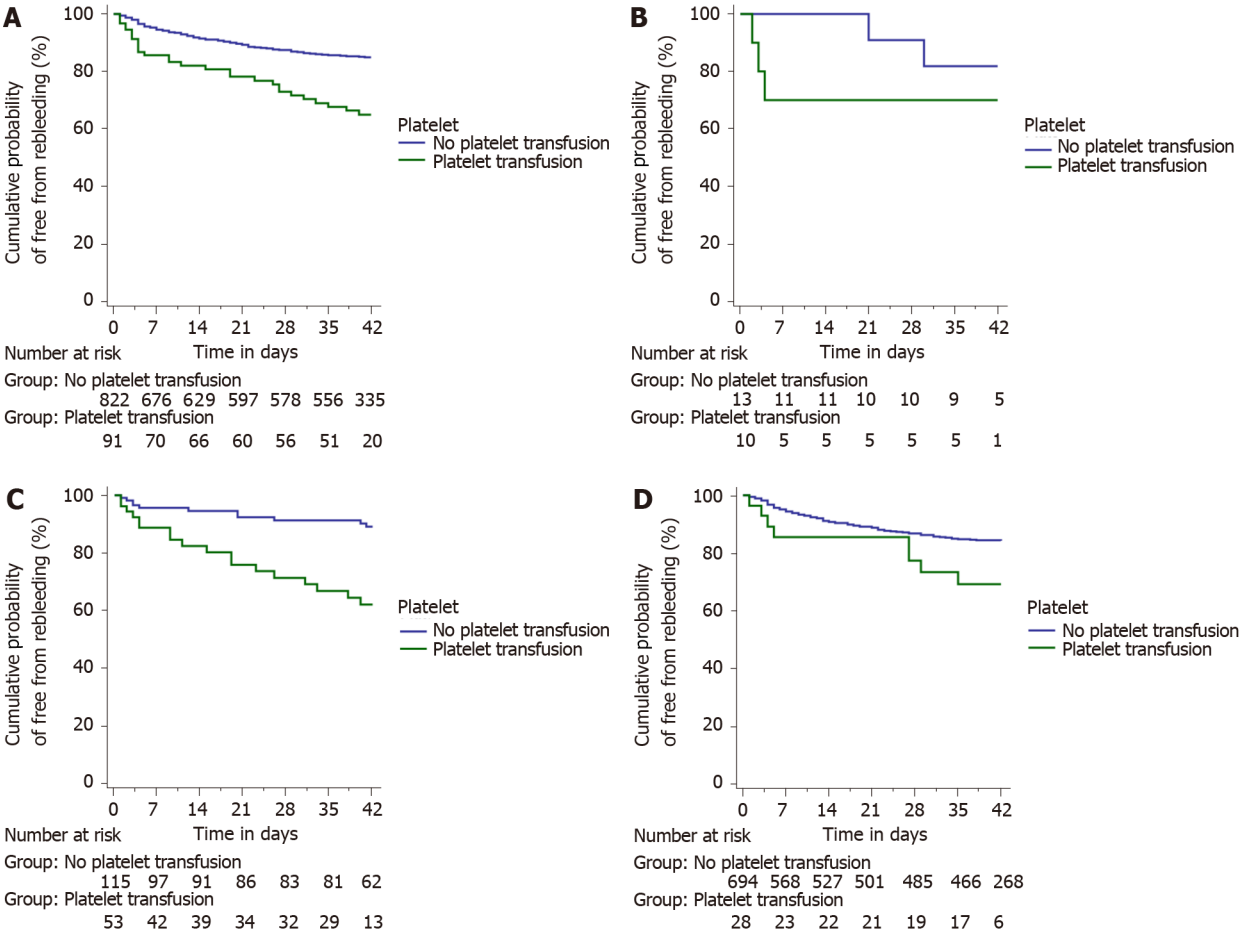

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Figure 3 Kaplan Meier curves of cumulative probability of free from rebleed in patients receiving platelets compared to those who did not. A: Overall cohort (log-rank $P < 0.001$); B: Platelet counts < 20 × 10^9/L (log-rank $P = 0.303$); C: Platelet counts 20 × 10^9/L-50 × 10^9/L (log-rank $P < 0.001$); D: Platelet counts > 50 × 10^9/L (log-rank $P = 0.038$).

study by Villanueva *et al*[23], who reported that a restrictive transfusion strategy is beneficial in cirrhotics as compared to a more liberal transfusion strategy.

There is a significant discrepancy between recommendations of major societies and actual clinical practice regarding transfusions in cirrhotics. A recent study from a tertiary healthcare center in India revealed that 40.5% of cirrhotics admitted over a 6 mo period for various indications received transfusions, 82.8% of which were prophylactic[13]. The American Gastroenterology Association (AGA, 2019), European Association for the Study of the Liver (EASL, 2018, 2022) and the American Association for the Study of Liver Diseases (AASLD, 2016) recommend against the use of FFP for prophylactic correction of deranged PT/INR levels during AVB[24-28]. The AGA 2019 guidelines suggest that platelets may be transfused to a target of 50 × 10^9/L based on low level of evidence while the other major societies (including the recent Baveno VII guidelines) cite insufficient evidence for recommending for or against transfusion of platelets in cirrhotics with AVB[24,28]. Studies have shown that platelet and FFP transfusion may increase procoagulant factor levels, endogenous thrombin potential and platelet counts in hemodynamically stable patients. However, the actual need for these transfusions and the clinical benefit during an episode of AVB remains uncertain[29]. Evidence for transfusion to correct thrombocytopenia is drawn from studies of prophylactic platelet transfusion to limit elective procedure related bleeding in CLD patients[30-32]. There is also a lot of scepticism associated with FFP transfusion in these patients based on the results of the retrospective study of 244 patients by Mohanty *et al*[11] which reported more severe episodes of bleeding along with higher rebleed rates at day 5, longer hospital stay and higher mortality at 42 d among 100 patients with AVB who received FFP. Even for patients undergoing prophylactic EVL of varices, higher rates of post EVL bleed were associated with advanced liver disease and not baseline INR or platelets as reported by Blasi *et al*[33] Thus baseline thrombocytopenia or deranged INR do not lead to higher post EVL bleeding rates in a prophylactic or emergent setting and attempting to correct it with transfusions may lead to more harm than good.

In our study, we identified 913 patients with cirrhosis experiencing AVB. Eighty percent of the study population were either Child-Pugh class A (374) or B (361). At baseline, 191 patients (20.9%) had a platelet count below 50 × 10^9/L, with 23 patients (2.5%) having platelets less than 20 × 10^9/L. There were

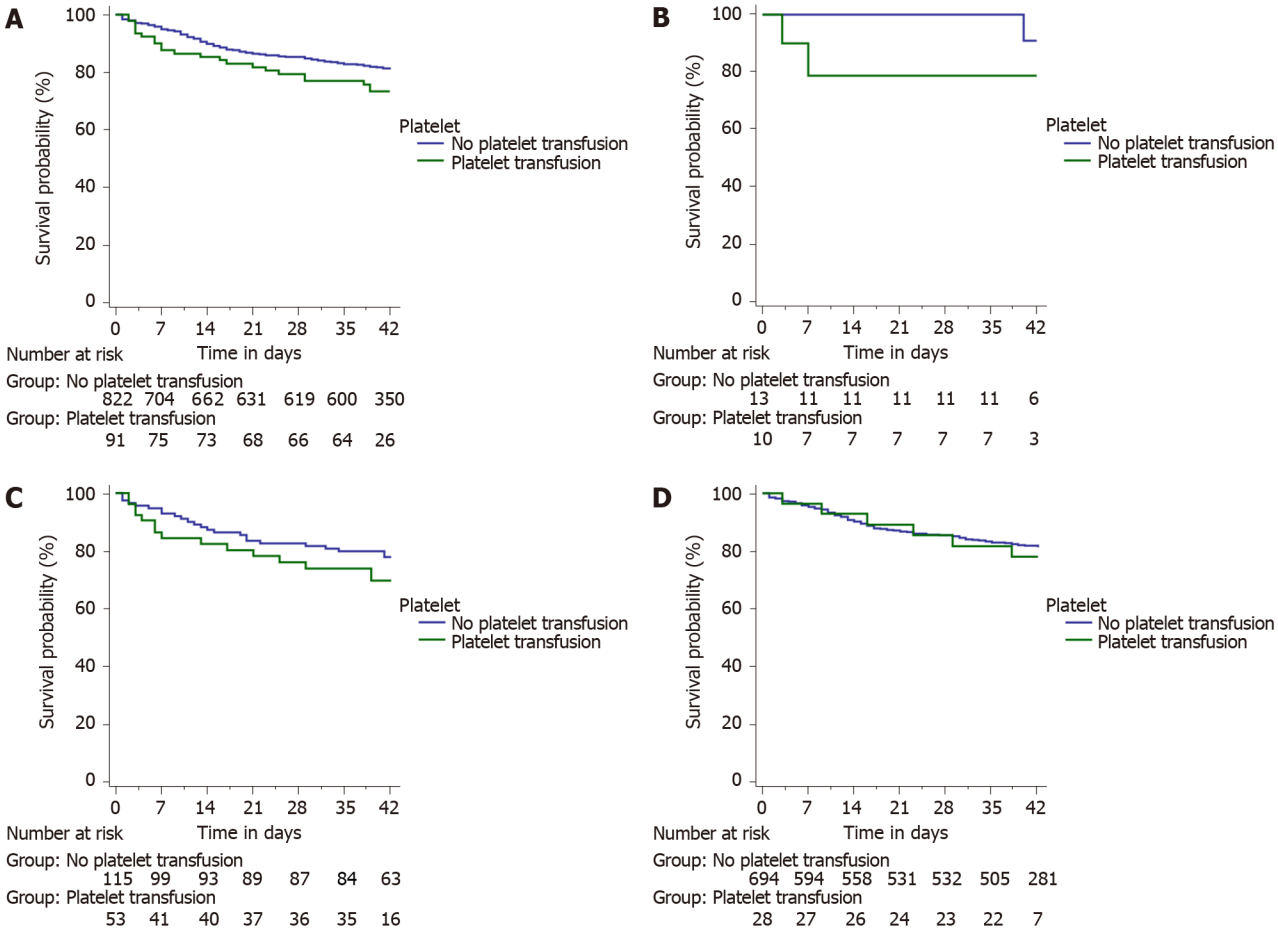

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Figure 4 Kaplan Meier curves of survival probability in patients based on whether they received platelet transfusions or not. A: Overall cohort (log rank $P = 0.074$), B: Platelet counts < 20 × 10^9/L (log-rank $P = 0.375$); C: Platelet counts 20 × 10^9/L-50 × 10^9/L (log-rank $P = 0.250$); D: Platelet counts > 50 × 10^9/L (log-rank $P = 0.716$).

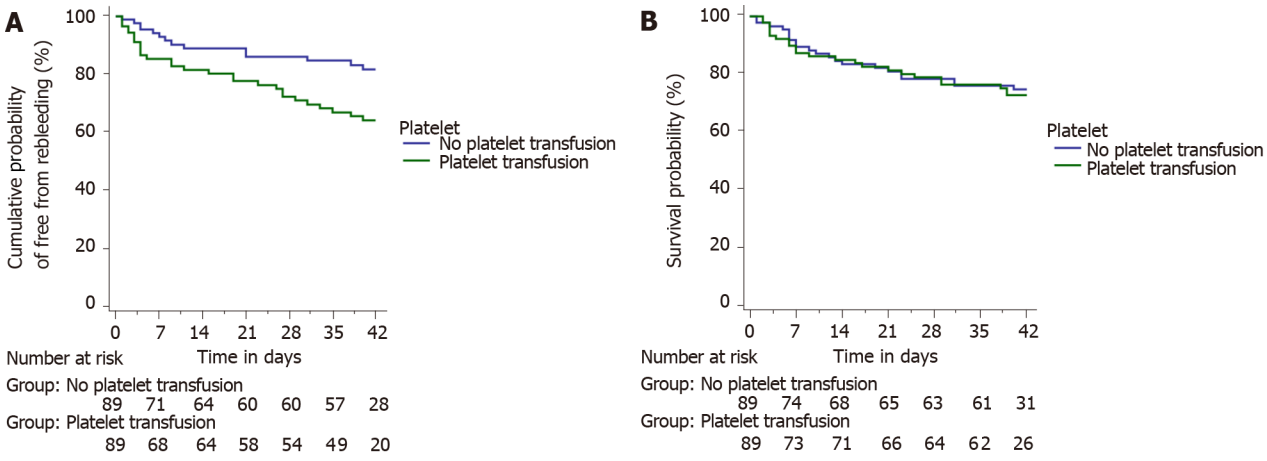

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Figure 5 Kaplan Meier curves of patients receiving platelets compared to those who did not in the PSM matched cohort demonstrating cumulative probability. A: Free from rebleed (log-rank $P = 0.012$); B: Survival probability (log-rank $P = 0.755$).

no major statistically significant differences in clinical and biochemical parameters, CTP, or MELD score among the three groups. Patients with thrombocytopenia did not have higher PRBC requirements, rebleed rates or mortality post endotherapy. A point of clinical concern is the feasibility of endotherapy at platelet counts < 20 × 10^9/L, but our data (although limited by absolute numbers) demonstrates no increased risk of therapy failure in these patients[34]. Similar results were reported by Thinrungroj *et al*

[35] in their cohort of 116 patients in which they demonstrated endotherapy to be safe at platelet counts as low as 30 × 10^9/L.

Overall, 91 patients (10%) received platelet transfusions. We used PSM analysis to adjust the baseline differences between the groups who received and did not receive platelet transfusion. Those receiving platelet transfusions had significantly higher rebleed rates within day 5 of transfusion (14.6%), which rose to 32.6% at day 42. Rebleeding rates were higher among patients with platelet counts > 20-50 × 10^9/L and > 50 × 10^9/L who received transfusions. Despite the higher rebleeding rates, there were no difference in PRBC requirements, indicating that the episodes did not result in a significant loss of blood volume. The mortality rates in those receiving transfusions were higher (25.8% *vs* 23.6%) but not statistically significant. Thus, patients with baseline platelets > 20 × 10^9/L are more likely to experience a rebleed if transfused platelets, but this does not translate to higher mortality rates at day 42. Hepatic encephalopathy was associated with poor outcomes in patients with cirrhosis and AVB[36].

Patients receiving FFP transfusion had significantly higher CTP and MELD scores than those who did not, indicating a sicker cohort. This is clinically expected as deranged INR occurs directly because of hepatic dysfunction. Significantly higher 5 and 42 d rebleed rates with higher 42-d mortality rates was noted among those receiving FFP. These patients also experienced higher blood volume loss with significantly higher PRBC requirement, lower hemoglobin level, and mean arterial pressures in this group. These results are in agreement with the recent study by Mohanty *et al*[11], who reported that bleeding in patients receiving FFP was more difficult to control and resulted in more extended hospital stays.

Comparing patients who receive any transfusion (FFP or platelets or both) vs. those who received none demonstrated the same trend of results, with those receiving transfusions being more likely to be decompensated clinically (elevated bilirubin, ascites and HE) with significantly higher rebleed rates on day 5 and 42 with higher 42-d mortality.

Our findings support the current evidence that both FFP and platelet transfusions lead to greater rebleed rates at 5 d, with FFP transfusions also adding to the mortality at 42 d. This highlights the fact that correction of coagulopathy in an attempt to control variceal bleeding is a futile target in the management of AVB. Thrombin generation assays may be helpful to guide transfusion practices and prevent unnecessary transfusions[37-39]. In recent times, two RCTs have demonstrated that TEG based transfusions have a role in restricting transfusions both in cirrhotics with AVB as well as those undergoing invasive procedures without compromising hemostasis[36,40].

Our study has certain limitations. The number of patients with platelet counts less than 20 × 10^9/L were few; hence our conclusions on endotherapy in this group are statistically underpowered. Being a tertiary care centre, we receive more sick patients with a poorer hemodynamic profile than other centres. The decision to transfuse blood products and the number of units was subjective and based on the treating physician's discretion. Being a high-volume centre, we are not able to admit all patients and some patients are sent to other centres for admission post-endotherapy. We do not have data regarding the length of the hospital stay and intensive care unit requirement in these patients. However, despite these limitations, a key strength of our study is that we had several patients with varying severity of illness as graded by the CTP and MELD scores, which is reflective of a real-world scenario. Adding to the pragmatism of the study was that the patients were initially stabilized in the casualty by a team of physicians which included specialists and trainees in emergency medicine and internists prior to review by gastroenterologists. Thus, the transfusion practices reflect both the permeation and dissemination of clinical recommendations by the major societies in gastroenterology among physicians involved in patient management and its acceptability and adoption in general practice.

CONCLUSION

In conclusion, platelet and FFP transfusions do not lead to improved hemostasis in patients with cirrhosis experiencing an AVB and are associated with higher rebleed rates at 5 and 42 d. Platelet transfusions lead to higher rebleed rates at day 5 and 42 but do not contribute to higher mortality rates, while FFP transfusions are associated with higher rebleed rates at 5 and 42 d and are also associated with higher mortality rates at 42 d from index bleeding episodes.

ARTICLE HIGHLIGHTS

Research background

The most important question answered by this study is that platelet transfusions are not beneficial but harmful to chronic liver disease patients presenting with variceal bleeding. We clearly have shown that thrombocytopenia at baseline did not impact the rebleed rates or mortality. Higher rebleed rates were seen only in those receiving platelets and FFP while those receiving FFP also demonstrated higher mortality rates. Moving further a prospective study to compare the impact of transfusions may be

contemplated, but considering the potential of harm to patients, it may not be ethically feasible.

Research motivation

Platelet transfusions increase the rebleed rate at days 5 and 42 but do not contribute to higher mortality rates at day 42. FFP transfusions lead to more severe rebleeds on days 5 and 42 with higher mortality among recipients on day 42.

Research objectives

The study included 913 patients. Rebleeding rates were similar between the three platelet groups (< 20 × 10^9/L, 20-50 × 10^9/L, and > 50 × 10^9/L) on days 5 and 42. On day 42, the mortality rates for the three platelet groups were also similar. On PSM analysis, patients receiving platelets transfusions (n = 89) had significantly higher rebleeding rates on day 5 and day 42 than those who didn't. The mortality rates were also higher among patients receiving platelets, although the difference was insignificant. However, patients who received FFP had higher rebleed rates on days 5 and 42, along with higher mortality rates on day 42, with higher packed red blood cell requirements, indicating a more severe bleed with greater blood loss. On multivariate analysis, platelet transfusion and not platelet count, was independently associated with 42-d rebleeding. Hepatic encephalopathy was independently associated with 42-d mortality.

Research methods

All patients with chronic liver disease presenting with acute variceal bleed over 4 years period from 2017 to 2021 and giving consent were enrolled for the study. Demographic and clinical data were collected at baseline and the patients followed up till death or 42 days whichever was later. Patients were divided into 3 groups based on platelet counts- < 20 × 10^9/L, 20-50 × 10^9/L, and > 50 × 10^9/L for analysis. A subgroup analysis was done for those receiving fresh frozen plasma (FFP) and platelets and FFP.

Research results

Our objectives were to identify the impact of platelet count and platelet transfusions in patients with chronic liver disease presenting with an acute variceal bleed in terms of rebleed rates on days 5 and 42 and mortality rates on day 42.

Research conclusions

The lack of data on platelet transfusion often leads to unnecessary transfusions of high volumes of platelets or fresh frozen plasma to chronic liver disease patients with acute variceal bleeding. Transfusions lead to a rise in portal pressure and may precipitate a rebleed, leading to further transfusions and a vicious cycle. Thus patient outcomes may be potentially worsened by unnecessary and empiric transfusions.

Research perspectives

There is a paucity of data on the impact of platelet transfusion on outcomes of patients of chronic liver disease presenting with acute variceal bleed. None of the major clinical guidelines provides definitive recommendations on transfusion of platelets during a variceal bleed to correct thrombocytopenia. Thus clinical management of such patients is guided by local policies rather than evidence-based.

FOOTNOTES

Author contributions: The study was designed by Shalimar; Biswas S, Vaishnav M, Pathak P, Gunjan D, Mahapatra SJ, Kedia S, Rout G, Nayak B, Kumar R, Shalimar were all involved in the clinical management of the enrolled patients as well as the collection of data; The analysis of the collected data was done by Shalimar, Thakur B, Vaishnav M, and Biswas S; The manuscript was drafted by Biswas S and Vaishnav M under the guidance of Shalimar and was reviewed and approved by all of the authors.

Institutional review board statement: This study was reviewed and approved by the Institutional Ethics Committee for Post Graduate research (IECPG) of the All India Institute of Medical Sciences, New Delhi, India.

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ORIGINAL ARTICLE

Observational Study

Polymorphism AGT2 (rs4762) is involved in the development of dermatologic events: Proof-of-concept in hepatocellular carcinoma patients treated with sorafenib

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Abstract

BACKGROUND

Dermatologic adverse events (DAEs) are associated with a better outcome in patients with hepatocellular carcinoma (HCC) irrespective of the therapeutic agent received. The exact mechanisms associated with the development of DAEs are unknown although several studies point to direct toxicity of tyrosine kinase inhibitors (TKIs) to the skin or an immune-mediated reaction triggered by the oncologic treatment. As is the case in other conditions, individual genetic variants may partially explain a higher risk of DAEs.

AIM

To evaluate the contribution of several gene variants to the risk of developing DAEs in HCC patients treated with TKIs.

METHODS

We first analyzed 27 single-nucleotide polymorphisms (SNPs) from 12 genes selected as potential predictors of adverse event (AE) development in HCC patients treated with sorafenib [Barcelona Clinic Liver Cancer 1 (BCLC1) cohort]. Three additional cohorts were analyzed for *AGT1* (rs699) and *AGT2* (rs4762) polymorphisms-initially identified as predictors of DAEs: BCLC2 (n = 79), Northern Italy (n = 221) and Naples (n = 69) cohorts, respectively. The relation between SNPs and DAEs and death were assessed by univariate and multivariate Cox regression models, and presented with hazard ratios and their 95% confidence intervals (95%CI).

RESULTS

The BCLC1 cohort showed that patients with arterial hypertension (AHT) (HR = 1.61; P value = 0.007) and/or *AGT* SNPs had an increased risk of DAEs. Thereafter, *AGT2* (rs4762) AA genotype was found to be linked to a statistically significant increased probability of DAEs (HR = 5.97; P value = 0.0201, AA *vs* GG) in the Northern Italy cohort by multivariate analysis adjusted for BCLC stage, ECOG-PS, diabetes and AHT. The value of this genetic marker was externally validated in the cohort combining the BCLC1, BCLC2 and Naples cohorts [HR = 3.12 (95%CI: 1.2-8.14), P value = 0.0199, *AGT2* (rs4762) AA *vs* AG genotype and HR = 2.73 (95%CI: 1.18-6.32) P value = 0.0188, *AGT2* (rs4762) AA *vs* GG genotype]. None of the other gene variants tested were found to be associated with the risk of DAE development.

CONCLUSION

DAE development in HCC patients receiving TKIs could be explained by the *AGT2* (rs4762) gene variant. If validated in other anti-oncogenic treatments, it might be considered a good prognosis

marker.

Key Words: HCC; Early DAE; Single-nucleotide polymorphisms; *AGT1* (rs699); *AGT2* (rs4762), Tyrosine kinase inhibitors

Core Tip: Dermatologic adverse events (DAEs) are associated with a better outcome in patients with hepatocellular carcinoma (HCC) irrespective of the therapeutic agent received. Our study shows that DAE development in these patients can be explained by individual genetic variants in the *AGT2* gene. *AGT2* (rs4762) AA genotype was associated with DAE risk in the Northern Italy cohort and was externally validated in a cohort combining the BCLC1, BCLC2 and Naples cohorts. Therefore, DAE development in HCC patients receiving TKIs can be explained by the *AGT2* (rs4762) gene variant. If validated in other anti-oncogenic treatments, it might be considered a good prognosis marker.

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INTRODUCTION

Treatment-related dermatologic adverse events (DAEs) are reported in a great number of oncological therapies. The profile and timing of on-target skin adverse events (AEs) varies across treatments and cancer types. In this regard, hand-foot skin reaction (HFSR) reported in patients receiving tyrosine kinase inhibitor (TKI) therapy resembles the already described hand-foot syndrome (HFS) described in patients treated with cytotoxic chemotherapies[1,2]. Moreover, several studies have described the association between DAE development and better patient outcome, and this association has been reported for different therapies [TKI, monoclonal antibody directed against EGFR[3] or immunotherapy [4,5] and different cancer types such as colorectal, renal, prostate, non-small cell lung and breast cancer as well as melanoma and hepatocellular carcinoma (HCC)][6]. Therefore, it appears that the association between DAE development and better patient outcome is observed regardless of cancer type or oncological treatment.

Although there are several hypotheses explaining the potential mechanisms of DAE development, the exact mechanisms remain unknown. Previous studies postulated that direct toxicity of TKIs to the skin could depend on drug secretion into eccrine glands[7] somehow copying the already described detection of doxorubicin in the sweat of treated patients[8]. Apart from other speculative explanations, inhibition of proangiogenic pathways could potentially prevent vascular repair mechanisms from functioning correctly and causing HFSR in high pressure areas that may be repeatedly exposed to subclinical trauma[9]. This would be applicable mainly to anti-angiogenic treatments but would leave other therapies out. Considering other drug treatments, a study on immune checkpoint inhibitors (ICIs) therapy in non–small cell lung cancer patients suggested that T cells would recognize antigens shared by both lung tumors and skin[10]. Consequently, treatment would target both organs thus leading to tumor regression associated with autoimmune skin toxic effects. However, the low frequency of tumors harboring potent neoantigens clearly compromises the rationale of this hypothesis. More recently, a study published by Ruiz-Pinto and colleagues[11] described the association between *CDH4* genetic variants with the risk of developing capecitabine-induced HFS. In that study, *CDH4* gene downregulation negatively impacted skin barrier function.

In 2018, we demonstrated that 91.6% of HCC patients who received sorafenib and achieved complete radiological response also developed DAEs within the first 2 mo of treatment[12,13]. Recently published data obtained in our group allowed us to identify the potential role of TKI in peripheral immune cell population profile modification towards a more pro-inflammatory behavior and phenotype[14]. Thus, we envision skin toxicity as a consequence of an immune-mediated reaction triggered by the oncologic treatment in patients prone to developing this side effect.

In order to uncover potential mechanisms underlying individual genetic susceptibility to AEs with clinical implications for risk prediction, we first analyzed 27 Single-Nucleotide Polymorphisms (SNPs) in 12 different genes as potential predictors of AE development in a Barcelona Clinic Liver Cancer 1 (BCLC1) cohort of 82 HCC patients treated with sorafenib. Upon identification of the potential relevance

of the angiotensin genes, which include *AGT1* (rs699) and *AGT2* (rs4762), as predictors of DAEs, we further explored the association in three additional cohorts: a second BCLC cohort (n = 79), a Northern Italy cohort (n = 221) and a Naples cohort (n = 69).

MATERIALS AND METHODS

Four cohorts of patients were analyzed in this study, two prospective cohorts from BCLC1 and BCLC2, and two additional cohorts from Northern Italy [Milan, Bologna, Meldola (FC) and Cagliari Hospitals] and Naples (Figure 1).

The study was approved by the institutional review board of each center (HCB/2009/4755, HCB/2015/0352, Ethical Board 2 480_2018 and CE/2014/193) and complied with the provisions of the Good Clinical Practice guidelines and the Declaration of Helsinki. A Data Transfer Protocol (DTP) was written according to the European regulation [General Data Protection Regulation (GDPR) 2016/679] and approved by each cohort responsible.

Patient eligibility

BCLC1 cohort: This cohort included patients referred to BCLC between February 2009 and March 2015 for sorafenib treatment.

Inclusion criteria were: (1) HCC diagnosed according to EASL guidelines[15]; (2) advanced HCC following the BCLC staging system or patients with earlier stages who could not benefit from treatments of higher priority; (3) normal liver or compensated cirrhosis with preserved liver function (Child-Pugh score ≤ 7 points without clinical ascites and/or encephalopathy; (4) performance status 0-1; (5) controlled arterial hypertension (AHT) and/or stable peripheral vascular disease; (6) adequate hematologic profile (platelet count > 60 × 10^9/L; hemoglobin > 8.5 g/dL; and prothrombin time > 50%); (7) adequate hepatic function (albumin > 2.8 g/dL; total bilirubin ≤ 3 mg/dL; and alanine and aspartate aminotransferases ≤ 5 times the upper limit of the normal range); and (8) adequate renal function (serum creatinine ≤ 1.5 times the upper limit of the normal range).

Exclusion criteria were: (1) Myocardial infarction in the last year or active ischemic heart disease; (2) acute variceal bleeding in the last month; (3) severe peripheral arterial disease; (4) arrhythmia under treatment with drugs different from beta-blockers or digoxin; (5) uncontrolled ascites; and (6) encephalopathy. All patients provided written informed consent before enrolment.

Follow-up: Clinical and laboratory assessments were performed monthly and radiologic tumor evaluation at week 4 and every 8 wk thereafter. Unscheduled visits due to AEs occurred according to patients' needs.

DAEs were graded according to version 3.0 of the CTCAE of the National Cancer Institute, during treatment and 30 days after the last dose. We focused on DAEs within the first 60 days (eDAE) +/-7 days of treatment, which determined dose modification.

BCLC2 cohort: This cohort included patients referred to BCLC between June 2015 and August 2018 for sorafenib treatment.

The inclusion and exclusion criteria as well as the follow-up of this cohort were the same as for the BCLC1 cohort.

Northern Italy cohort: The Northern Italy cohort included patients with HCC treated with sorafenib prospectively enrolled between July 2008 and June 2018 in four tertiary centers in Italy whose data have already been published in several multicenter studies on sorafenib treatment[16,17]. Briefly, all patients with advanced HCC or intermediate-stage HCC refractory to or unsuitable for locoregional therapies, either histologically proven or diagnosed according to the AASLD guidelines (American Association for the Study of Liver Diseases 2005) and receiving sorafenib were eligible for analysis. Exclusion criteria were those established by the Italian Medicines Agency (AIFA), *i.e.*, a performance status score > 2 and clinical decompensation. All patients received sorafenib with the standard schedule (400 mg bid continuously) with dose reduction applied as clinically indicated.

Follow-up: Follow-up consisted of a physical examination and complete blood count every 3 wk and Computed Tomography (CT) /Magnetic Resonance Imaging (MRI) scanning every 8 wk or as clinically indicated. Each visit included the recording of AEs, clinical laboratory tests, physical examination, and assessment of vital signs. At any time during treatment, the patient could have direct access to physicians for AE management. Safety was assessed in all patients who received at least one dose of sorafenib; AEs were graded according to the National Cancer Institute's Common Terminology Criteria (version 3.0 CTCAE). Hepatic function deterioration was defined as a Child-Pugh score increase ≥ 2 points, which was evaluated at each visit and at predefined time points of week 12 and 24 of therapy. In line with the aim of the study, independently of clinical practice, we focused on the AEs which determined dose modification within the first 30 and 60 days of treatment, respectively. Treatment with sorafenib was continued until disease progression, unacceptable toxicity, or death. In each patient, the

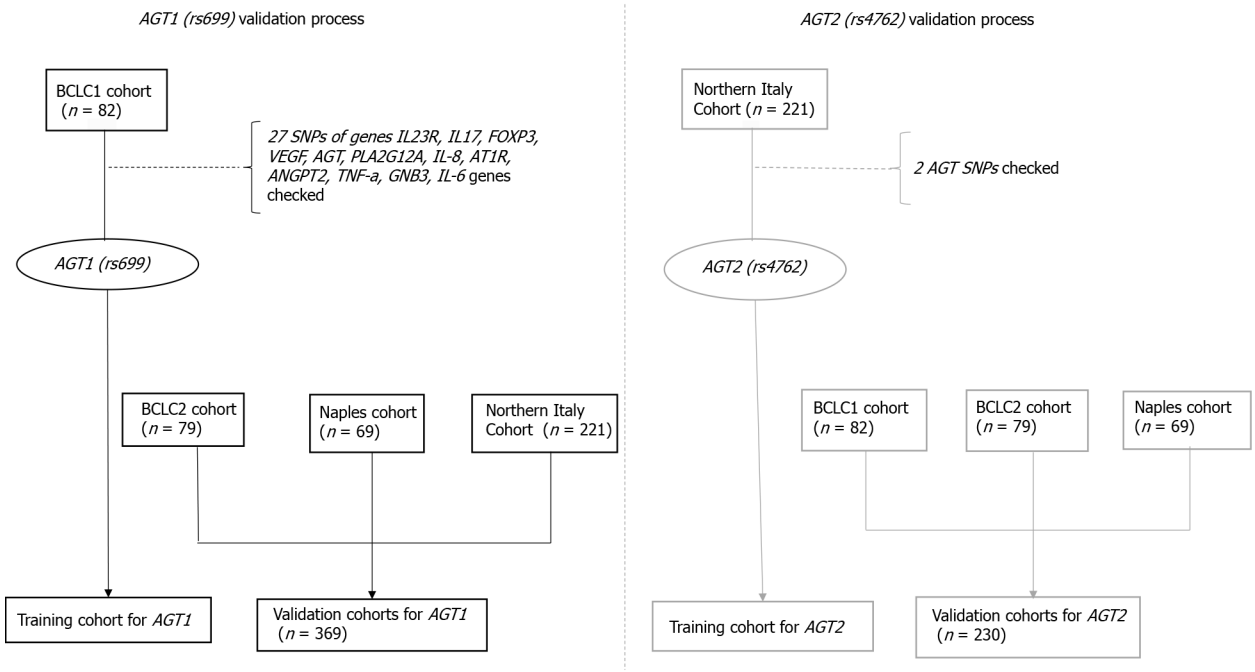

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Figure 1 Study flowchart.

medical history, physical examination, blood cell count, serum chemistry, coagulation and alpha-fetoprotein levels were obtained at baseline and every 4 wk thereafter.

Naples cohort: This cohort included patients referred to the Gastroenterology Unit of the University Hospital Federico II of Naples between January 2014 and December 2019 for sorafenib treatment.

Inclusion criteria were: (1) HCC diagnosed according to EASL guidelines[15]; (2) advanced HCC following the BCLC staging system or patients with earlier stages who could not benefit from treatments of higher priority; (3) normal liver or compensated cirrhosis with preserved liver function (Child-Pugh score ≤ 7 points without clinical ascites and/or encephalopathy; (4) performance status 0-1; (5) controlled AHT and/or stable peripheral vascular disease; (6) adequate hematologic profile (platelet count > 30 × 10^3/L; hemoglobin > 8.5g/dL; and INR < 1.7; (7) adequate hepatic function (albumin > 2.8 g/dL; total bilirubin < 3 mg/dL; and alanine and aspartate aminotransferases < 5 times the upper limit of the normal range); and (8) adequate renal function (serum creatinine < 1.5 times the upper limit of the normal range).

Exclusion criteria were: (1) Myocardial infarction in the last year or active ischemic heart disease; (2) acute variceal bleeding in the last month; (3) severe peripheral arterial disease; (4) arrhythmia under treatment with drugs different from beta-blockers or digoxin; (5) uncontrolled ascites; and (6) encephalopathy. All patients provided written informed consent before enrolment.

Follow-up: Clinical and laboratory assessments were performed monthly and radiologic tumor evaluation at week 8 and every 8 wk thereafter. Unscheduled visits due to AEs occurred according to patients' needs.

DAEs were graded according to version 3.0 of the CTCAE of the National Cancer Institute, during treatment and 30 days after the last dose. We focused on DAEs within the first 60 days (eDAE)+/-7 days of treatment, which determined dose modification.

Genomic DNA (gDNA) purification: gDNA was purified from isolated peripheral blood mononuclear cells (PBMCs) in the BCLC cohorts of patients and from 500 mL of whole frozen blood in the Naples cohort. gDNA purification was performed using the PureLink gDNA mini kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions.

Patient genotyping

BCLC1 cohort: Patients were genotyped for a series of SNPs in *IL23R, IL17, FOXP3, VEGF, AGT, PLA2G12A, IL-8, AT1R, ANGPT2, TNF-a, GNB3,* and *IL-6* genes. SNPs were selected according to reported associations with susceptibility to cardiovascular disease, hypertension, stroke, inflammatory pathways or even cancer development. The genes and SNPs analyzed are detailed in Supplementary Table 1.

Twenty ng of gDNA were used for each SNP reaction. All SNPs were evaluated by means of TaqMan predesigned genotyping assays (Applied Biosystems, Thermo Fisher Scientific) and the procedure was performed following the manufacturer's instructions. A list of assays used is specified in Supplementary Table 2.

Briefly, TaqMan® MGB probes from the genotyping assay provide a fluorescent signal for the amplification of each allele. SNP genotyping uses a 60 s extension time at 60ºC for 40 cycles. Real-time PCR software plots the results of the allelic discrimination data as a scatter plot of Allele 1 (VIC® dye) *vs* Allele 2 (FAM™ dye). Each well of the 96-well reaction plate is represented as an individual point on the allelic discrimination plot. Positive controls were used for each homozygote and heterozygote genotype.

Patients from the BCLC2, Northern Italy and Naples cohorts were genotyped for 2 SNPs of the AGT-gene [*AGT1* (rs699) and *AGT2* (rs4762)] using the TaqMan endpoint-genotyping assay, following the same techniques as previously described.

Statistical analysis

The statistical methods and analysis of this study were performed by Víctor Sapena and reviewed by Ferran Torres from the Hospital Clínic de Barcelona.

Quantitative variables are expressed as median and interquartile range [IQR 25th-75th percentiles]. Categorical variables are described as absolute frequencies and percentages (%).

Time to event variables are expressed as median and 95% confidence intervals (95%CI) using the Kaplan-Meier method. The log-rank test was used to compare Kaplan-Meier curves. Univariate and multivariate Cox regression models were used to estimate Hazard Ratios (HR) and 95%CI to evaluate the increased probability of developing grade II or early dermatologic events (eDAEs), dermatologic events (DAEs) or death according to each SNP. The multivariate adjusting factors were previously selected according to their clinical relevance, and these were BCLC stage (A or B *vs* C), ECOG-PS (0 *vs* ≥ 1), history of AHT (No *vs* Yes) and history of diabetes (No *vs* Yes). An analysis using 67 days as the landmark timepoint was used to calculate overall survival (OS) according to eDAE.

The level of significance was set at the two-tailed 5% level and all analyses and data base integration structure were performed with SAS 9.4 software (SAS Institute, Cary, NC, United States).

RESULTS

This study included 82 patients from the BCLC1 cohort, 79 from the second BCLC2 cohort, 221 from the Northern Italy cohort, and 69 from the Naples cohort.

Baseline characteristics

Tables1, 2 and 3 describe the characteristics, OS and follow-up at the time of locking the database (December 2019) and the AE rates of all patients included in the study.

BCLC1 cohort: All but 2 (2.4%) patients were cirrhotic. A total of 54 (65.9%) patients had Hepatitis C Virus (HCV) and 10 (12.2%) had Hepatitis B Virus (HBV). Ninety-three percent of patients were asymptomatic (ECOG-PS 0) and 40 (48.8%) were BCLC B that failed or had a contraindication to loco-regional treatment, 70 (85.4%) were Child-Pugh class A. Twenty-two (26.8%) had vascular invasion, and 24 (29.3%) had extra-hepatic spread. AHT was present in 45.1% of patients and diabetes in 26.8%. Seventy-seven patients (93.9%) started sorafenib treatment at 800 mg.

BCLC2 cohort: All but 5 (6.3%) patients were cirrhotic. A total of 38 (48.1%) patients had HCV and 6 (7.6%) had HBV. Ninety-three percent of patients were asymptomatic (ECOG-PS 0) and 36 (45.6%) were BCLC B that failed or had a contraindication to loco-regional treatment, 63 (79.8%) were Child-Pugh class A. Twenty-six (32.9%) had vascular invasion, and 27 (34.2%) had extra-hepatic spread. AHT was present in 45.6% of patients and diabetes in 35.4%. Seventy-seven patients (97.4%) started sorafenib treatment at 800 mg.

Northern Italy cohort: All patients were cirrhotic. A total of 111 (50.2%) patients had HCV and 46 (20.8%) had HBV. Seventy percent of patients were asymptomatic (ECOG-PS 0) and 76 (34.4%) were BCLC B that failed or had a contraindication to loco-regional treatment, 207 (93.7%) were Child-Pugh class A. Sixty-one (27.6%) had vascular invasion, and 79 (35.8%) had extra-hepatic spread. AHT was present in 29.4% of patients and diabetes in 27.6%. One hundred ninety-seven patients (89.1%) started sorafenib treatment at 800 mg.

Naples cohort: All but 1 (1.5%) patient were cirrhotic. A total of 44 (63.7%) patients had HCV and 12 (17.4%) had HBV. All patients were asymptomatic (ECOG-PS 0) and 20 (29%) were BCLC B that failed or had a contraindication to loco-regional treatment, 58 (84.1%) were Child-Pugh class A. Thirty-one (44.9%) had vascular invasion, and 23 (33.3%) had extra-hepatic spread. AHT was present in 65.2% of patients and diabetes in 33.3%. All patients started sorafenib treatment at 800 mg.

Table 1 Baseline characteristics of patients included in each cohort

	BCLC1 cohort	BCLC2 cohort	Northern Italy cohort	Naples cohort
Patients, *n*	82	79	221	69
Gender (Male)	73 (89.02)	67 (84.81)	184 (83.26)	60 (86.96)
Age (Years)	63 (56-71)	63 (56-72)	69 (60-74)	70 (60-74)
AGT1 (rs699)				
AA	26 (31.71)	25 (31.65)	72 (32.58)	22 (31.88)
AG	34 (41.46)	35 (44.3)	101 (45.7)	38 (55.07)
GG	22 (26.83)	19 (24.05)	47 (21.27)	9 (13.04)
NA	0 (0)	0 (0)	1 (0.45)	0 (0)
AGT2 (rs4762)				
AA	5 (6.1)	3 (3.8)	5 (2.26)	0 (0)
AG	16 (19.51)	10 (12.66)	44 (19.91)	15 (21.74)
GG	61 (74.39)	66 (83.54)	172 (77.83)	54 (78.26)
AHT (Yes)	37 (45.12)	36 (45.57)	65 (29.41)	45 (65.22)
Diabetes (Yes)	22 (26.83)	28 (35.44)	61 (27.6)	23 (33.33)
HBV (Yes)	10 (12.2)	6 (7.59)	46 (20.81)	12 (17.39)
HCV (Yes)	54 (65.85)	38 (48.1)	111 (50.23)	44 (63.77)
HIV (Yes)	2 (2.44)	1 (1.27)	3 (1.36)	0 (0)
Child-Pugh				
A: 5-6	70 (85.37)	63 (79.75)	207 (93.67)	58 (84.06)
B: 7-9	10 (12.2)	11 (13.93)	14 (6.33)	10 (14.49)
Not applicable	2 (2.44)	5 (6.33)	0 (0)	1 (1.45)
ECOG-PS (0)	77 (93.9)	74 (93.67)	155 (70.14)	69 (100)
Ascites (Yes)	11 (13.41)	9 (11.39)	25 (11.31)	14 (20.29)
Encephalopathy (Yes)	0 (0)	0 (0)	11 (4.98)	0 (0)
Extrahepatic spread (Yes)	24 (29.27)	27 (34.18)	79 (35.75)	23 (33.33)
Vascular Invasion (Yes)	22 (26.83)	26 (32.91)	61 (27.6)	31 (44.93)
BCLC (A[1] or B / C)	42 (51.22) / 40 (48.78)	36 (45.57) / 43 (54.43)	76 (34.39) / 145 (65.61)	20 (28.99) / 49 (71.01)
Alpha-fetoprotein (ng/mL)	20.5 (7-212.5)	25 (8-228)	100.5 (10-869)	98 (5-1903)
Hemoglobin basal (g/dL)	13.8 (12.95-14.95)	13.1 (11.9-14.5)	12.5 (11.2-14)	13 (11.9-13.9)
Prothrombin time (%)	88.3 (76.5-95.6)	76 (65-88)	NA	84.5 (76-100)
International normalized ratio	NA	NA	1.1 (1-1.22)	1.13 (1.03-1.24)
Total bilirubin (mg/dL)	1 (0.8-1.6)	1.1 (0.6-1.7)	0.9 (0.72-1.3)	0.95 (0.7-1.4)
AST (UI/L)	78 (46-119)	54 (34-84)	NA	52 (35-80)
ALT (UI/L)	72 (35-106.5)	44 (25-65)	43 (23-56)	42 (32-55)
GGT (UI/L)	134.5 (93.5-285.5)	143 (83-264)	NA	96 (48-204)
Albumin (mg/L)	38.5 (35-43)	40 (35-43)	38 (35-40)	3.6 (3.3-4)
Initial dosage of sorafenib (mg)				
400	5 (6.1)	2 (2.6)	19 (8.6)	0 (0)
600	0 (0)	0 (0)	5 (2.26)	0 (0)
800	77 (93.9)	77 (97.4)	197 (89.14)	69 (100)

Descriptive statistics are frequencies (%) or median (IQR: Interquartile range), as appropriate. AHT: Arterial Hypertension; HCV: Hepatitis C Virus; HBV: Hepatitis B Virus; HIV: Human immunodeficiency virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transpeptidase; IQR: Interquartile range; ECOG-PS: Eastern Cooperative Oncology Group Performance Status; BCLC: Barcelona Clinic Liver Cancer; INR: International normalized ratio; NA: Not available.
[1]5 BCLC A patients.

Adverse events

The rate of DAEs at any time point in the BCLC1, BCLC2, Northern Italy and Naples cohorts were 51.2 %, 35.4%, 14.5% and 39.1%, respectively (Table 3). The incidence of eDAEs in the BCLC1 cohort was 40.2% and was 27.8%, 12.7% and 36.2% in the BCLC2, Northern Italy and Naples cohorts, respectively.

The distribution of patients with a history of diabetes and AHT who did or did not develop eDAEs or DAEs in each cohort and the association between DAEs and AHT are summarized in Supplementary Tables 4 and 5, respectively.

The association between DAEs and a history of AHT was statistically significant in the BCLC1 cohort, with a HR = 1.96 (95%CI: 1.05-3.65; *P* value = 0.04) and confirmed when all patients were analyzed as a unique cohort with a HR = 1.61 (95%CI: 1.14-2.28; *P* value = 0.007).

Follow-up and Overall survival

BCLC1 cohort: The median follow-up was 18.6 mo (IQR: 10.3-34.2) and 75 (91.5%) patients died. Ninety-eight percent of deaths were due to HCC-related causes. The median treatment duration and OS were 9.1 (IQR: 4.1-17.5) and 18.8 mo (95%CI: 14.7-23.6), respectively.

BCLC2 cohort: The median follow-up was 13.1 mo (IQR: 6.6-22.4) and 47 (59.5%) patients died. Ninety-seven percent of deaths were due to HCC-related causes. The median treatment duration and OS were 5.9 (IQR: 2.1-13.5) and 18.3 mo (95%CI: 13.1-26.4), respectively.

Northern Italy cohort: The median follow-up was 12.7 mo (IQR: 6.1-25.9) and 180 (81.4%) patients died. Sixty-five percent of deaths were due to HCC-related causes. The median treatment duration and OS were 8.5 (IQR: 2.6-20.8) and 14.3 mo (95%CI: 11.8-18), respectively.

Naples cohort: The median follow-up was 9.9 mo (IQR: 4.5-18.3) and 57 (82.6%) patients died. Eighty-four percent of deaths were due to HCC-related causes. The median treatment duration and OS were 8.1 (IQR: 3.7-17) and 9.9 mo (95%CI: 7.7-12.8), respectively.

Overall survival according to eDAE

Using a landmark timepoint of 60 (+7) days and excluding 17 patients with less than 60 (+7) days of follow-up, the median OS in eDAE and in non-eDAE patients was 21.6 mo (95%CI: 12.7-28.2) and 14.8 mo (95%CI: 9.9-17.6) in BCLC1, 19.5 mo (95%CI: 8-24.2) and 14.2 mo (95%CI: 8.9-30.5) in BCLC2, 15.9 mo (95%CI: 8.3-40.6) and 12.1 mo (95%CI: 9.6-16.6) in the Northern Italy cohort, 12.4 mo (95%CI: 7.86-21.14) and 6.8 mo (95%CI: 2.7-8.7) in the Naples cohort, respectively.

Single-nucleotide polymorphisms (SNPs)

BCLC1 cohort: Supplementary Table 1 describes the assessed SNPs in this cohort. Of all SNPs analyzed, only the *AGT1* (rs699) AA genotype had a significant estimated increase in the probability of eDAE with a HR = 2.31 (95%CI: 1.03-5.14; *P* value = 0.04; AA *vs* AG) in the univariate model and a HR = 2.3 (95%CI: 1.02-5.16; *P* value = 0.04; AA *vs* AG) in the multivariate model (Table 4). For DAEs at any time point, *AGT1* (rs699) AA genotype showed a significant estimated increase in the probability of DAEs with a HR = 2.7 (95%CI: 1.27-5.75; *P* value = 0.01; AA *vs* AG) in the univariate model and a HR = 2.68 (95%CI: 1.25-5.77; *P* value = 0.01; AA *vs* AG) in the multivariate model. No other polymorphism showed a significant association with general AEs or specifically DAE or eDAE development in the BCLC1 cohort.

Allele distribution of Single-nucleotide polymorphisms (SNPs) AGT1 (rs699) and AGT2 (rs4762)

Allele distributions of *AGT1* (rs699) and *AGT2* (rs4762) are summarized in Table 1. There were no significant differences between the included cohorts (*P* value 0.5 and 0.2 for *AGT1* rs699 and *AGT2* rs4762, respectively). Thus, the present cohorts are comparable in terms of genetic variants.

AGT1 (rs699) and AGT2 (rs4762) influence in the development of DAE and eDAE

Tables 4 and 5 describe the Cox regression models for eDAE and DAE development by *AGT1* (rs699) and *AGT2* (rs4762), respectively. The results of the BCLC1 cohort are mentioned above.

BCLC2 cohort: The *AGT1 (rs699)* did not show a significant association with DAEs. By contrast, the *AGT2* (rs4762) AA genotype was associated with a significant increased risk of eDAE with a HR = 4.43 (95%CI: 1.01-19.39; *P* value = 0.048; AA *vs* GG) in the univariate analysis, and showed a trend in the multivariate model with a HR = 4.24 (95%CI: 0.95-19.06]; *P* value = 0.06; AA *vs* GG), Table 5.

Table 2 Overall survival of each cohort by single-nucleotide polymorphisms

		SNP alleles (A/G)	Patients at risk	Events	Median OS (95%CI), months	*P* value (log-rank)
BCLC1cohort			82	75	18.81 (14.76-23.58)	
BCLC2 cohort			79	47	18.32 (13.05-26.44)	
Northern Italy cohort			221	180	14.3 (11.84-17.99)	
Naples cohort			69	57	9.9 (7.69-12.82)	
BCLC1 cohort	*AGT1* (rs699)		82	75		0.16
		AA	26	23	18.73 (11.84-41.4)	
		AG	34	33	18.43 (10.75-22.76)	
		GG	22	19	18.81 (9.67-30.42)	
	AGT2 (rs4762)		82	75		0.4
		AA	5	5	41.34 (0.39-74.12)	
		AG	16	15	13.95 (7.3-23.87)	
		GG	61	55	19.11 (14.86-24.47)	
BCLC2 cohort	*AGT1* (rs699)		79	47		0.15
		AA	25	15	23.74 (7.46-26.5)	
		AG	35	19	21.74 (11.15-33.77)	
		GG	19	13	6.64 (3.42-30.29)	
	AGT2 (rs4762)		79	47		0.3
		AA	3	1	NE (13.61-NE)	
		AG	10	5	30.29 (3.88-32.69)	
		GG	66	41	16.41 (8.78-23.74)	
Northern Italy cohort	*AGT1* (rs699)		220	179		0.5
		AA	72	58	13.58 (10.92-19.2)	
		AG	101	83	17.59 (10.85-20.68)	
		GG	47	38	12.43 (8.81-20.68)	
	AGT2 (rs4762)		221	180		0.7
		AA	5	2	NE (1.94-NE)	
		AG	44	36	14.3 (7.46-20.68)	
		GG	172	142	14.9 (11.25-18.09)	
Naples cohort	*AGT1* (rs699)		69	57		0.7
		AA	22	19	12.66 (6.15-18.25)	
		AG	38	31	8.32 (4.9-11.71)	
		GG	9	7	10.95 (2.6-21.83)	
	AGT2 (rs4762)		69	57		0.6
		AG	15	11	9.8 (2.89-24.93)	
		GG	54	46	10.1 (7.14-12.82)	

NE: Not estimable; OS: Overall survival; 95%CI: 95% confidence interval; SNP: Single-nucleotide polymorphisms; BCLC: Barcelona clinic liver cancer.

Northern Italy cohort: In this cohort, the *AGT2* (rs4762) AA genotype showed a statistically significant increased probability of eDAE both in the univariate analysis (HR = 4.54 [95%CI: 1.05-19.64]; *P* value = 0.04; AA *vs* GG) and in the multivariate analysis (HR = 5.15 [95%CI: 1.17-22.63]; *P* value = 0.03; AA *vs* GG).

Table 3 Follow-up and evolutionary events in the included patients of each cohort

	BCLC1cohort	BCLC2cohort	Northern Italy cohort	Naples cohort
Patients, *n*	82	79	221	69
Follow-up (mo)	18.58 (10.33-34.17)	13.05 (6.64-22.36)	12.73 (6.05-25.88)	9.87 (4.51-18.25)
Treatment duration (mo)	9.06 (4.11-17.46)	5.95 (2.14-13.52)	8.52 (2.56-20.78)	8.06 (3.72-16.97)
Adverse Events				
Gastrointestinal (Yes)	35 (42.68)	27 (34.18)	23 (10.41)	38 (55.07)
Dermatologic (Yes)	42 (51.22)	28 (35.44)	32 (14.48)	27 (39.13)
Early Dermatologic (Yes)	33 (40.24)	22 (27.85)	28 (12.67)	25 (36.23)
Performance status deterioration (Yes)	44 (53.66)	46 (58.23)	53 (23.98)	0 (0)
Cardiovascular (Yes)	18 (21.95)	14 (17.72)	16 (7.24)	16 (23.19)
Dermatologic and Cardiovascular simultaneously (Yes)	7 (8.54)	5 (6.33)	0 (0)	10 (14.49)
Other (Yes)	48 (58.54)	34 (43.04)	45 (20.36)	65 (94.2)
Death (Yes)	75 (91.46)	47 (59.49)	180 (81.44)	57 (82.61)
Cause of death				
HCC	74 (98.67)	46 (97.87)	118 (65.56)	48 (84.21)
Not HCC related	0 (0)	1 (2.13)	58 (32.22)	9 (15.79)
Others[1]	1 (1.33)	0 (0)	4 (2.22)	0 (0)

Descriptive statistics are frequencies (%) or median (IQR: Interquartile range), as appropriate. AE: Adverse events; DAE: Dermatological adverse events; eDAE: early Dermatological adverse events.

[1]Other causes of Exitus are: 1 Sudden death, 4 unknown.

Naples cohort: In the Naples cohort, none of the SNPs showed a significant effect on DAE or eDAE development.

Validation of the AGT2 (rs4762) value identified in the Northern Italy cohort in the large cohort combining all cohorts but the Northern Italy one

The results in the individual cohorts suggested that the inconclusive results obtained in the BCLC and Naples cohorts could be due to a limited sample size. Thus, we combined these cohorts into a single cohort that would match the Northern Italy sample size.

This analysis showed that *AGT2* (rs4762) was significantly associated with DAE development with a HR = 2.94 (95%CI: 1.14-7.6; *P* value = 0.03; AA *vs* AG) and HR = 2.49 (95%CI: 1.08-5.73; *P* value = 0.03; AA *vs* GG) in univariate models, and HR = 2.85 (95%CI: 1.1-7.39; *P* value = 0.03; AA *vs* AG) and HR = 2.48 (95%CI: 1.08-5.72; *P* value = 0.03; AA *vs* GG) in multivariate models (Table 5).

Influence ofAGT2 (rs4762) in DAE and eDAE development after adjusting for baseline tumor burden, liver function, performance status and comorbidities

Table 5 shows the multivariate analyses adjusted for baseline BCLC stage, ECOG-PS, diabetes and AHT in the same model, considering diabetes and AHT together and each one separately. The multivariate analysis adjusted for baseline BCLC stage, ECOG-PS, diabetes and AHT showed a statistically significant increased risk in the probability of eDAE in patients harboring *AGT2* (rs4762) AA genotype in the Northern Italy cohort (HR = 8.51, 95%CI: 1.78-40.54; *P* value = 0.007; AA *vs* GG; and HR = 5.61, 95%CI: 1.01-31.12; *P* value = 0.048; AA *vs* AG).

The same analysis was performed for *AGT2* (rs4762) AA genotype and DAE development. A statistically significant increased risk in the probability of DAE was observed in the Northern Italy cohort (HR = 5.97, 95%CI: 1.32-27.01; *P* value = 0.02; AA *vs* GG) and when considering all but the Northern Italy cohort together as a unique cohort (HR = 3.12, 95%CI: 1.2-8.14; *P* value = 0.02; AA *vs* AG, and HR = 2.73, 95%CI: 1.18-6.32: *P* value = 0.02; AA *vs* GG).

AGT1 (rs699) and AGT2 (rs4762) influence on survival

No statistically significant effect on survival was found for *AGT1* (rs699) or *AGT2* (rs4762) using univariate or multivariate models in any cohort or combination thereof (Supplementary Table 6 and

Table 4 Cox regression models for eDAE and DAE by *AGT1* (rs699)

Event	Centre	*AGT1* (rs699)	HR (95%CI)	*P* value	HR (95%CI) adjusted by BCLC + ECOG-PS	*P* value	HR (95%CI) adjusted by BCLC + ECOG-PS + AHT + DM	*P* value	HR (95%CI) adjusted for AHT + DM	*P* value	HR (95%CI) adjusted for DM	*P* value	HR (95%CI) adjusted for AHT	*P* value
eDAE	BCLC1 cohort	AA vs AG	2.31 (1.03-5.14)	0.04	2.3 (1.02-5.16)	0.04	2.34 (1.02-5.37)	0.04	2.33 (1.03-5.24)	0.04	2.45 (1.1-5.5)	0.03	2.24 (1-5.03)	0.049
		AA *vs* GG	1.68 (0.71-3.97)	0.2	1.69 (0.71-4)	0.2	1.64 (0.69-3.93)	0.3	1.65 (0.69-3.92)	0.3	1.75 (0.74-4.13)	0.2	1.62 (0.68-3.87)	0.3
		AG *vs* GG	0.73 (0.29-1.85)	0.5	0.73 (0.29-1.89)	0.5	0.7 (0.27-1.82)	0.5	0.71 (0.28-1.79)	0.5	0.71 (0.28-1.8)	0.5	0.72 (0.29-1.84)	0.5
	BCLC2 cohort	AA *vs* AG	0.66 (0.25-1.76)	0.4	0.63 (0.24-1.7)	0.4	0.71 (0.26-1.93)	0.5	0.72 (0.27-1.93)	0.5	0.72 (0.27-1.91)	0.5	0.68 (0.25-1.83)	0.5
		AA *vs* GG	1.13 (0.32-4.01)	0.9	1.08 (0.3-3.84)	0.9	1.35 (0.37-4.95)	0.7	1.36 (0.38-4.9)	0.7	1.32 (0.37-4.72)	0.7	1.13 (0.32-4)	0.9
		AG *vs* GG	1.71 (0.55-5.3)	0.4	1.7 (0.55-5.28)	0.4	1.89 (0.6-5.91)	0.3	1.89 (0.6-5.9)	0.3	1.85 (0.6-5.74)	0.3	1.66 (0.53-5.17)	0.4
	Northern Italy cohort	AA *vs* AG	0.8 (0.33-1.95)	0.6	0.75 (0.3-1.86)	0.5	1.02 (0.4-2.61)	0.9	0.96 (0.39-2.36)	0.9	0.83 (0.34-2.02)	0.7	0.91 (0.37-2.23)	0.8
		AA *vs* GG	0.9 (0.31-2.6)	0.8	0.71 (0.24-2.1)	0.5	0.96 (0.31-2.98)	0.9	1.22 (0.4-3.73)	0.7	0.96 (0.33-2.8)	0.9	1.12 (0.37-3.36)	0.8
		AG *vs* GG	1.12 (0.42-3.01)	0.8	0.95 (0.35-2.58)	0.9	0.94 (0.33-2.69)	0.9	1.27 (0.46-3.49)	0.7	1.15 (0.43-3.12)	0.8	1.23 (0.45-3.34)	0.7
	Naples cohort	AA *vs* AG	1.26 (0.54-2.95)	0.6	1.21 (0.51-2.86)	0.7	1.35 (0.56-3.27)	0.5	1.36 (0.57-3.25)	0.5	1.23 (0.52-2.93)	0.6	1.44 (0.61-3.39)	0.4
		AA *vs* GG	1.26 (0.34-4.66)	0.7	1.18 (0.31-4.43)	0.8	1.33 (0.35-5)	0.7	1.34 (0.36-4.96)	0.7	1.27 (0.34-4.68)	0.7	1.35 (0.37-5)	0.7
		AG *vs* GG	1 (0.28-3.51)	0.9	0.97 (0.28-3.43)	0.9	0.98 (0.28-3.49)	0.9	0.99 (0.28-3.49)	0.9	1.03 (0.29-3.65)	0.9	0.94 (0.27-3.3)	0.9
	BCLC2 cohort + Naples cohort + Northern Italy cohort	AA *vs* AG	0.87 (0.52-1.47)	0.6	0.85 (0.51-1.43)	0.5	0.84 (0.5-1.41)	0.5	0.85 (0.51-1.43)	0.6	0.87 (0.52-1.47)	0.6	0.85 (0.51-1.43)	0.6
		AA *vs* GG	1.05 (0.54-2.04)	0.9	0.95 (0.49-1.86)	0.9	0.92 (0.47-1.81)	0.8	1.01 (0.52-1.97)	0.9	1.05 (0.54-2.04)	0.9	1.01 (0.52-1.97)	0.9
		AG *vs* GG	1.2 (0.65-2.22)	0.6	1.12 (0.61-2.08)	0.7	1.1 (0.59-2.05)	0.8	1.18 (0.64-2.18)	0.6	1.2 (0.65-2.22)	0.6	1.18 (0.64-2.18)	0.6
	BCLC1 cohort + Naples cohort + Northern Italy	AA *vs* AG	1.35 (0.84-2.17)	0.2	1.35 (0.84-2.18)	0.2	1.33 (0.82-2.15)	0.2	1.31 (0.81-2.11)	0.3	1.35 (0.84-2.18)	0.2	1.3 (0.81-2.1)	0.3

	cohort	AA *vs* GG	1.19 (0.67-2.12)	0.6	1.13 (0.6-2.01)	0.7	1.08 (0.6-1.93)	0.8	1.1 (0.61-1.97)	0.8	1.19 (0.67-2.12)	0.6	1.09 (0.61-1.96)	0.8
		AG *vs* GG	0.88 (0.5-1.55)	0.7	0.83 (0.47-1.48)	0.5	0.81 (0.46-1.43)	0.5	0.84 (0.48-1.48)	0.6	0.88 (0.5-1.55)	0.7	0.84 (0.48-1.48)	0.6
	BCLC1cohort + BCLC2 cohort + Naples cohort	AA *vs* AG	1.32 (0.81-2.15)	0.3	1.29 (0.79-2.11)	0.3	1.3 (0.79-2.12)	0.3	1.31 (0.81-2.14)	0.3	1.33 (0.82-2.17)	0.3	1.31 (0.8-2.13)	0.3
		AA *vs* GG	1.4 (0.75-2.6)	0.3	1.38 (0.7-2.57)	0.3	1.44 (0.77-2.69)	0.3	1.45 (0.78-2.7)	0.2	1.46 (0.79-2.72)	0.2	1.38 (0.74-2.57)	0.3
		AG *vs* GG	1.06 (0.58-1.94)	0.9	1.06 (0.58-1.95)	0.9	1.11 (0.6-2.03)	0.8	1.1 (0.6-2.02)	0.8	1.1 (0.6-2.01)	0.8	1.06 (0.58-1.94)	0.9
DAE	BCLC1 cohort	AA *vs* AG	2.7 (1.27-5.75)	0.01	2.68 (1.25-5.77)	0.01	2.52 (1.16-5.47)	0.02	2.6 (1.21-5.57)	0.01	2.82 (1.32-6.06)	0.008	2.5 (1.17-5.35)	0.02
		AA *vs* GG	1.26 (0.62-2.58)	0.5	1.24 (0.61-2.55)	0.6	1.11 (0.53-2.31)	0.8	1.13 (0.55-2.35)	0.8	1.3 (0.63-2.66)	0.5	1.12 (0.54-2.32)	0.8
		AG *vs* GG	0.47 (0.21-1.05)	0.06	0.46 (0.2-1.06)	0.07	0.44 (0.19-1.01)	0.053	0.44 (0.19-0.98)	0.045	0.46 (0.2-1.03)	0.06	0.45 (0.2-1.01)	0.052
	BCLC2 cohort	AA *vs* AG	0.98 (0.43-2.2)	0.9	0.94 (0.42-2.13)	0.9	0.99 (0.43-2.26)	0.9	1.01 (0.45-2.3)	0.9	1.03 (0.45-2.32)	0.9	0.95 (0.42-2.16)	0.9
		AA *vs* GG	1.89 (0.59-6.04)	0.3	1.78 (0.55-5.76)	0.3	2.08 (0.63-6.85)	0.2	2.18 (0.67-7.03)	0.19	2.08 (0.65-6.66)	0.2	1.88 (0.59-6.01)	0.3
		AG *vs* GG	1.94 (0.64-5.9)	0.2	1.89 (0.62-5.77)	0.3	2.12 (0.69-6.49)	0.19	2.15 (0.7-6.57)	0.18	2.02 (0.66-6.15)	0.2	1.98 (0.65-6.05)	0.2
	Northern Italy cohort	AA *vs* AG	0.89 (0.39-2.06)	0.8	0.85 (0.37-1.98)	0.7	1.01 (0.42-2.41)	0.9	1 (0.42-2.33)	0.9	0.91 (0.39-2.11)	0.8	0.95 (0.41-2.22)	0.9
		AA *vs* GG	0.62 (0.25-1.57)	0.3	0.54 (0.21-1.37)	0.2	0.6 (0.23-1.6)	0.3	0.74 (0.28-1.92)	0.5	0.64 (0.25-1.62)	0.4	0.7 (0.27-1.79)	0.5
		AG *vs* GG	0.7 (0.3-1.62)	0.3	0.63 (0.27-1.48)	0.2	0.6 (0.25-1.43)	0.2	0.74 (0.32-1.72)	0.5	0.71 (0.3-1.63)	0.4	0.73 (0.31-1.69)	0.5
	Naples cohort	AA *vs* AG	1.29 (0.57-2.92)	0.5	1.23 (0.54-2.81)	0.6	1.35 (0.58-3.15)	0.5	1.38 (0.6-3.17)	0.5	1.23 (0.54-2.81)	0.6	1.49 (0.66-3.4)	0.3
		AA *vs* GG	1.38 (0.38-5.03)	0.6	1.28 (0.35-4.73)	0.7	1.45 (0.39-5.36)	0.6	1.49 (0.41-5.41)	0.6	1.39 (0.38-5.05)	0.6	1.51 (0.41-5.51)	0.5
		AG *vs* GG	1.07 (0.31-3.72)	0.9	1.04 (0.3-3.62)	0.9	1.08 (0.31-3.77)	0.9	1.08 (0.31-3.79)	0.9	1.13 (0.32-3.96)	0.9	1.01 (0.29-3.52)	0.9
	BCLC2 cohort + Naples cohort + Northern Italy cohort	AA *vs* AG	1 (0.62-1.61)	0.9	0.98 (0.61-1.57)	0.9	0.95 (0.59-1.54)	0.9	0.97 (0.6-1.56)	0.9	1.01 (0.63-1.62)	0.9	0.96 (0.6-1.55)	0.9
		AA *vs* GG	1.13 (0.61-	0.7	1.04 (0.56-1.92)	0.9	0.98 (0.53-1.81)	0.9	1.05 (0.57-1.95)	0.9	1.12 (0.61-2.07)	0.7	1.05 (0.57-1.95)	0.9

		2.08)											
	AG *vs* GG	1.13 (0.63-2)	0.7	1.06 (0.59-1.89)	0.8	1.02 (0.57-1.83)	0.9	1.09 (0.61-1.94)	0.8	1.12 (0.63-1.99)	0.7	1.09 (0.61-1.95)	0.8
BCLC1 cohort + Naples cohort +Northern Italy cohort	AA *vs* AG	1.43 (0.91-2.24)	0.12	1.43 (0.91-2.24)	0.12	1.39 (0.88-2.19)	0.15	1.36 (0.87-2.14)	0.18	1.44 (0.92-2.26)	0.11	1.35 (0.86-2.12)	0.19
	AA *vs* GG	0.94 (0.57-1.56)	0.8	0.9 (0.54-1.51)	0.7	0.82 (0.49-1.38)	0.5	0.83 (0.49-1.39)	0.5	0.94 (0.57-1.57)	0.8	0.82 (0.49-1.38)	0.5
	AG *vs* GG	0.66 (0.4-1.09)	0.1	0.63 (0.38-1.05	0.08	0.59 (0.36-0.99)	0.04	0.61 (0.37-1.01)	0.052	0.66 (0.4-1.08)	0.1	0.61 (0.37-1.01)	0.053
BCLC1 cohort + BCLC2 cohort + Naples cohort	AA *vs* AG	1.54 (0.98-2.41)	0.06	1.49 (0.95-2.34)	0.08	1.48 (0.94-2.32)	0.09	1.52 (0.97-2.37)	0.07	1.55 (0.99-2.43)	0.055	1.5 (0.96-2.35)	0.07
	AA *vs* GG	1.35 (0.78-2.32)	0.3	1.3 (0.75-2.25)	0.3	1.32 (0.76-2.28)	0.3	1.35 (0.78-2.33)	0.3	1.39 (0.81-2.4)	0.2	1.3 (0.76-2.24)	0.3
	AG *vs* GG	0.88 (0.51-1.51)	0.6	0.87 (0.51-1.5)	0.6	0.89 (0.52-1.54)	0.7	0.89 (0.52-1.54)	0.7	0.9 (0.52-1.54)	0.7	0.87 (0.5-1.49)	0.6

eDAE: early Dermatological adverse events; DAE: Dermatological adverse events; HR: Hazard ratio; 95%CI: 95% confidence interval; BCLC: Barcelona Clinic Liver Cancer; ECOG-PS: Eastern Cooperative Oncology Group Performance Status; AHT: Arterial hypertension; DM: Diabetes mellitus.

Supplementary Table 7).

DISCUSSION

The aim of Precision Oncology is to decide the treatment to be recommended to a specific patient according to the individualized evaluation of the clinical, biochemical and hopefully, molecular profile. It is common to focus all the attention on the genomic abnormalities of cancer to define the best intervention, but it is well known that, patients' genetic background, irrespective of the tumor, is involved in the efficacy and safety of any therapeutic intervention. The best example is the clearance related to the glucuronidation activity resulting in fast and slow elimination of drugs and their metabolites[18]. Response to inflammation or tolerance to antiangiogenic agents is also influenced by genetic background and most cancer treatments have targets affecting several of these separate domains. In some instances, these non-cancer effects may become a surrogate of drug activity and even be correlated with improved outcomes as already described in the introduction.

This multicenter international study explored whether specific genetic variants, as identified by SNP analysis, may be linked to the development of AEs that have been associated with improved outcome. This is not only the case for DAEs in patients with HCC treated with sorafenib[12,19], as has been extensively proven, but also when using other TKIs such as regorafenib[20]. Furthermore, the association of DAEs with improved outcome is also being reported when using chemotherapy or

Table 5 Cox regression models for eDAE and DAE by *AGT2* (rs4762)

Event	Center	*AGT2* (rs4762)	HR (95%CI)	*P* value	HR (95%CI) adjusted for BCLC + ECOG-PS	*P* value	HR (95%CI) adjusted for BCLC + ECOG-PS + AHT + DM	*P* value	HR (95%CI) adjusted for AHT + DM	*P* value	HR (95%CI) adjusted for DM	*P* value	HR (95%CI) adjusted for AHT	*P* value
eDAE	BCLC1 cohort	AA *vs* AG	1.14 (0.22-5.89)	0.9	0.98 (0.19-5.12)	0.9	0.97 (0.18-5.04)	0.9	1.09 (0.21-5.64)	0.9	1.15 (0.22-5.95)	0.9	1.11 (0.21-5.72)	0.9
		AA *vs* GG	0.84 (0.2-3.53)	0.8	0.73 (0.17-3.15)	0.7	0.71 (0.16-3.1)	0.7	0.81 (0.19-3.4)	0.8	0.8 (0.19-3.39)	0.8	0.84 (0.2-3.54)	0.8
		AG *vs* GG	0.73 (0.28-1.91)	0.5	0.74 (0.28-1.94)	0.5	0.74 (0.28-1.97)	0.6	0.74 (0.28-1.94)	0.6	0.7 (0.27-1.82)	0.5	0.76 (0.29-1.98)	0.6
	BCLC2 cohort	AA *vs* AG	3.71 (0.62-22.39)	0.2	3.52 (0.58-21.5)	0.2	4.8 (0.74-31.28)	0.1	4.81 (0.74-31.24)	0.1	4.78 (0.76-29.88)	0.09	4.46 (0.7-28.35)	0.11
		AA *vs* GG	4.43 (1.01-19.39)	0.048	4.24 (0.95-19.06)	0.06	6.14 (1.28-29.55)	0.02	6.28 (1.32-29.95)	0.02	6.25 (1.35-28.89)	0.02	5.34 (1.15-24.86)	0.03
		AG *vs* GG	1.19 (0.35-4.08)	0.8	1.21 (0.35-4.15)	0.8	1.28 (0.37-4.45)	0.7	1.31 (0.38-4.47)	0.7	1.31 (0.38-4.47)	0.7	1.2 (0.35-4.08)	0.8
	Northern Italy cohort	AA *vs* AG	2.72 (0.57-13.1)	0.2	3.21 (0.64-15.99)	0.15	5.61 (1.01-31.12)	0.048	3.43 (0.69-16.96)	0.13	2.69 (0.56-12.97)	0.2	3.2 (0.66-15.6)	0.15
		AA *vs* GG	4.54 (1.05-19.64)	0.04	5.15 (1.17-22.63)	0.03	8.51 (1.78-40.54)	0.007	5.51 (1.25-24.33)	0.02	4.72 (1.09-20.48)	0.04	4.93 (1.13-21.41)	0.03
		AG *vs* GG	1.67 (0.69-4.02)	0.3	1.6 (0.66-3.9)	0.3	1.52 (0.6-3.82)	0.4	1.61 (0.66-3.9)	0.3	1.75 (0.73-4.24)	0.2	1.54 (0.63-3.73)	0.3
	Naples cohort	AG *vs* GG	1.2 (0.48-3.01)	0.7	1.2 (0.48-3.02)	0.7	1.25 (0.5-3.15)	0.6	1.26 (0.5-3.16)	0.6	1.2 (0.48-3)	0.7	1.29 (0.51-3.23)	0.6
	BCLC2 cohort + Naples cohort + Northern Italy cohort	AA *vs* AG	2.76 (0.92-8.27)	0.07	2.95 (0.97-9.84)	0.06	2.78 (0.9-8.56)	0.07	2.61 (0.87-7.86)	0.09	2.75 (0.92-8.25)	0.07	2.61 (0.87-7.86)	0.09
		AA *vs* GG	3.5 (1.27-9.67)	0.02	3.8 (1.36-10.58)	0.01	3.67 (1.31-10.3)	0.01	3.39 (1.22-9.37)	0.02	3.5 (1.27-9.66)	0.02	3.38 (1.22-9.37)	0.02
		AG *vs* GG	1.27 (0.73-9.67)	0.4	1.29 (0.74-2.25)	0.4	1.32 (0.75-2.32)	0.3	1.3 (0.74-2.27)	0.4	1.27 (0.73-2.22)	0.4	1.3 (0.74-2.27)	0.4
	BCLC1 cohort + Naples cohort +Northern Italy cohort	AA *vs* AG	1.66 (0.65-4.9)	0.4	1.63 (0.55-4.85)	0.4	1.53 (0.51-4.57)	0.5	1.54 (0.52-4.57)	0.4	1.66 (0.56-4.9)	0.4	1.54 (0.52-4.57)	0.4
		AA *vs* GG	1.83 (0.67-5.03)	0.2	1.73 (0.63-4.77)	0.3	1.7 (0.62-4.69)	0.3	1.8 (0.65-4.94)	0.3	1.85 (0.67-5.08)	0.2	1.79 (0.65-4.93)	0.3
		AG *vs* GG	1.1 (0.65-1.86)	0.7	1.06 (0.63-1.81)	0.8	1.11 (0.65-1.9)	0.7	1.17 (0.69-1.97)	0.6	1.11 (0.66-1.88)	0.7	1.16 (0.69-1.97)	0.6

	BCLC1 cohort + BCLC2 cohort + Naples cohort	AA *vs* AG	1.67 (0.55-5.09)	0.4	1.6 (0.53-4.87)	0.4	1.61 (0.53-4.92)	0.4	1.66 (0.55-5.06)	0.4	1.71 (0.56-5.19)	0.4	1.63 (0.54-4.95)	0.4
		AA *vs* GG	1.7 (0.62-4.67)	0.3	1.67 (0.61-4.59)	0.3	1.68 (0.61-4.63)	0.3	1.7 (0.62-4.67)	0.3	1.7 (0.62-4.67)	0.3	1.68 (0.61-4.62)	0.3
		AG *vs* GG	1.01 (0.57-1.81)	0.9	1.04 (0.58-1.86)	0.9	1.04 (0.58-1.86)	0.9	1.02 (0.57-1.82)	0.9	0.99 (0.56-1.78)	0.9	1.03 (0.58-1.84)	0.9
DAE	BCLC1 cohort	AA *vs* AG	2.8 (0.78-10.01)	0.1	2.45 (0.68-8.81)	0.2	2.73 (0.74-9.99)	0.13	3.09 (0.85-11.2)	0.09	2.85 (0.79-10.22)	0.11	2.86 (0.8-10.28)	0.11
		AA *vs* GG	1.82 (0.64-5.16)	0.3	1.61 (0.56-4.64)	0.4	1.89 (0.64-5.57)	0.2	2.12 (0.74-6.1)	0.16	1.79 (0.63-5.08)	0.3	2.03 (0.71-5.78)	0.19
		AG *vs* GG	0.65 (0.27-1.56)	0.3	0.66 (0.27-1.59)	0.4	0.69 (0.28-1.72)	0.4	0.69 (0.28-1.68)	0.4	0.63 (0.26-1.52)	0.3	0.71 (0.29-1.71)	0.4
	BCLC2 cohort	AA *vs* AG	3.83 (0.64-23.05)	0.1	3.71 (0.61-22.68)	0.2	3.91 (0.62-24.73)	0.15	4.05 (0.65-25.33)	0.14	4.49 (0.73-27.55)	0.1	3.79 (0.61-23.44)	0.15
		AA *vs* GG	3.22 (0.75-13.76)	0.1	3.27 (0.74-14.38)	0.1	3.74 (0.82-17.15)	0.09	3.7 (0.82-16.76)	0.09	4.04 (0.91-18)	0.07	3.18 (0.72-14.13)	0.13
		AG *vs* GG	0.84 (0.25-2.8)	0.8	0.88 (0.26-2.96)	0.8	0.96 (0.28-3.24)	0.9	0.92 (0.27-3.06)	0.9	0.9 (0.27-3.01)	0.9	0.84 (0.25-2.8)	0.8
	Northern Italy cohort	AA *vs* AG	2.85 (0.59-13.73)	0.2	3.28 (0.66-16.21)	0.1	4.71 (0.89-24.91)	0.07	3.4 (0.69-16.77)	0.13	2.83 (0.59-13.64)	0.2	3.13 (0.65-15.21)	0.16
		AA *vs* GG	3.68 (0.86-15.63)	0.08	4.15 (0.96-17.87)	0.06	5.97 (1.32-27.01)	0.02	4.41 (1.02-19.03)	0.046	3.97 (0.93-16.94)	0.06	3.8 (0.89-16.16)	0.07
		AG *vs* GG	1.29 (0.55-3.01)	0.6	1.26 (0.54-2.96)	0.6	1.27 (0.53-3.05)	0.6	1.3 (0.55-3.05)	0.6	1.4 (0.6-3.29)	0.4	1.21 (0.52-2.84)	0.7
	Naples cohort	AG *vs* GG	1.12 (0.45-2.77)	0.8	1.11 (0.45-2.76)	0.8	1.12 (0.45-2.79)	0.8	1.13 (0.46-2.82)	0.8	1.12 (0.45-2.77)	0.9	1.16 (0.47-2.88)	0.8
	BCCL2 cohort + Naples cohort + Northern Italy cohort	AA *vs* AG	2.79 (0.93-8.35)	0.07	3.04 (1-9.21)	0.049	2.72 (0.88-8.34)	0.08	2.54 (0.84-7.63)	0.1	2.74 (0.92-8.21)	0.07	2.56 (0.85-7.7)	0.09
		AA *vs* GG	2.96 (1.08-8.13)	0.03	3.27 (1.18-9.05)	0.02	3.07 (1.1-8.56)	0.03	2.81 (1.02-7.73)	0.045	2.94 (1.07-8.07)	0.04	2.83 (1.03-7.78)	0.04
		AG *vs* GG	1.06 (0.62-1.83)	0.8	1.07 (0.62-1.86)	0.8	1.13 (0.65-1.96)	0.7	1.11 (0.64-1.92)	0.7	1.07 (0.62-1.85)	0.8	1.11 (0.64-1.91)	0.7
	BCLC1 cohort + Naples cohort + Northern Italy cohort	AA *vs* AG	2.82 (1.13-7.07)	0.03	2.9 (1.15-7.32)	0.02	2.7 (1.06-6.84)	0.04	2.66 (1.06-6.69)	0.04	2.81 (1.12-7.05)	0.03	2.68 (1.07-6.74)	0.04
		AA *vs* GG	2.86 (1.24-6.58)	0.01	2.84 (1.23-6.54)	0.01	2.85 (1.24-6.57)	0.01	2.94 (1.28-6.77)	0.01	2.91 (1.27-6.7)	0.01	2.94 (1.28-6.77)	0.01
		AG *vs* GG	1.01 (0.61-	0.9	0.98 (0.59-1.63)	0.9	1.06 (0.63-1.77)	0.8	1.1 (0.67-1.83)	0.7	1.04 (0.63-1.71)	0.9	1.1 (0.66-1.82)	0.7

		1.68)											
BCLC1 cohort + BCLC2 cohort + Naples cohort	AA *vs* AG	2.94 (1.14-7.6)	0.03	2.85 (1.1-7.39)	0.03	3.12 (1.2-8.14)	0.02	3.21 (1.23-8.34)	0.02	3.05 (1.18-7.9)	0.02	2.9 (1.12-7.5)	0.03
	AA *vs* GG	2.49 (1.08-5.73)	0.03	2.48 (1.08-5.72)	0.03	2.73 (1.18-6.32)	0.02	2.75 (1.19-6.34)	0.02	2.54 (1.1-5.85)	0.03	2.51 (1.09-5.77)	0.03
	AG *vs* GG	0.85 (0.49-1.48)	0.6	0.87 (0.5-1.52)	0.6	0.87 (0.5-1.53)	0.7	0.86 (0.49-1.5)	0.6	0.83 (0.48-1.45)	0.5	0.87 (0.5-1.51)	0.6

eDAE: early Dermatological adverse events; DAE: Dermatological adverse events; HR: Hazard ratio; 95%CI: 95% confidence interval; BCLC: Barcelona Clinic Liver Cancer; ECOG-PS: Eastern Cooperative Oncology Group Performance Status; AHT: Arterial hypertension; DM: Diabetes mellitus.

immunotherapy not only in liver cancer but also in other tumor types[3-5].

The results of our multicenter study confirm that the genetic background of patients plays a key role in the emergence of specific events that are linked to a distinct outcome under HCC treatment. Previously, different SNPs were reported to be potentially associated with survival outcomes[16,17] while others were identified as significantly associated with a higher likelihood of DAEs affecting the angiotensin gene and its *AGT2* (rs4762) variant.

Our results confirmed that the distribution of the *AGT* genetic variants studied, *AGT1* (rs699) and *AGT2* (rs4762), was comparable across patients from northern and southern Italy and those from Barcelona, and confirmed that the frequency of reference and alternative alleles follow the reported distribution for the European population[21,22].

Although rs699 and rs4762 could not be associated with AHT events in our patients, the most relevant finding is the identification of *AGT2* (rs4762) AA genotype as a predictor of DAE development [HR = 5.97; *P* value = 0.0201] in the Northern Italy cohort and its validation in the remaining 3 cohorts when they were considered as one unique cohort [HR = 3.12 (95%CI: 1.2-8.14); *P* value = 0.02 and HR = 2.73 (95%CI: 1.18-6.32); *P* value = 0.02].

AGT2 (rs4762) is a missense variant that codes for the replacement of threonine by methionine with no reported clear association with blood AGT protein levels. *AGT2* (rs4762) has been associated with renal dysplasia, a potentially benign disease[22]. However, published data suggest that rs4762 may be associated with an increased risk of mortality in patients with heart failure[23] and with the development of intracranial hemorrhage in stroke patients[24]. Available data at this moment do not allow to unequivocally associate an increase in blood AGT levels with rs4762 polymorphism, but it is speculated that it could induce Renin-Angiotensin System (RAS) activation. The RAS is a key regulator of systemic homeostasis by controlling salt-water balance and consequently, blood pressure. Interestingly, several studies have also unveiled the activation of this system in several peripheral tissues (tRAS)[25] and organs including skin and liver[26]. Since activation of tRAS is associated with tissue regeneration, inflammation and fibrosis[27] and considering that all of these are key components of tumor development, tRAS activation is likely to play a role in carcinogenesis. A review by Ager EI and collaborators[28] describes the potential contribution of tRAS activation in cancer development and progression putting the emphasis not only on tumor angiogenesis, but also on inflammation and fibrosis. Considering that the components of the tRAS pathway are also participating in physiological

and pathological wound healing and fibrosis processes that are particularly important in skin homeostasis[29,30], DAE development in our patients with rs4762 AA genotype may be considered a consequence of tRAS activation at the skin level.

The role of genetic variants in the components of the RAS pathway has been extensively reported in the past years and some of these roles involve response to anti-neoplastic treatments, disease prognosis and patient survival. In that sense, it is already known that *ACE* I/D rs4646994, a variant of the Angiotensin-Converting Enzyme (ACE), has been associated with prediction of response to bevacizumab in metastatic breast and colorectal cancer patients[31]. The *AGT*rs5050 GG genotype[32] is reported to be linked to poor prognosis in patients with astrocytoma. A very interesting *in silico* study by Goswami and colleagues analyzed 354 SNPs in the *AGT* gene[33] in order to predict those variants that are pathogenic and how amino acid substitutions would impact protein function. In this study, *AGT2* rs4762 was categorized mainly as a damaging *AGT* SNP with controversial results on its pathogenicity or disease identity. Thus, the importance of genetic variants is determined by the levels and/or functionality of the protein they code for. Along these lines, Feng *et al*[34] proposed that cancer tissue levels of *ACE2* correlates with immune infiltrates and these would affect the prognosis of cancer patients. In another study, Urupet *et al*[35] suggested that low expression of the *AGT* gene and high expression of an HLA-class II gene (*HLADQA1*) were independent predictors associated with response in glioblastoma patients treated with bevacizumab.

AGT2 (rs4762) has been associated with an increased risk of AHT in several studies[36,37] but this association remains controversial as the results could not be confirmed in other series of individuals analyzed[38]. We were not able to identify an association between *AGT2* rs4762 and AHT in our patients not even when analyzing the impact of concomitant medication that the BCLC1 and BCLC2 cohort patients received for AHT that included IEACA (renin angiotensin aldosterone axis inhibitor) (Supplementary Table 8). This could be related to the low frequency of *AGT2* rs4762 in patients who developed this AE [0 (0%) in the BCLC1 and Northern Italy cohorts, 1 (1.27%) in the BCLC2 cohort and 2 (2.9%) in the Naples cohort].

However, in our cohort, the impact of *AGT2* (rs4762) was maintained when the multivariate was adjusted for history of AHT.

To the best of our knowledge, the relationship between *AGT2* rs4762AA genotype and DAE development in HCC patients under sorafenib treatment has not been previously reported. This is a 'proof-of-concept' study to identify a novel genetic marker to screen for patients with good outcome. It would be interesting for our results to be validated in other cancer types besides HCC or even in different therapeutic approaches. If this were to be the case, *AGT2* (rs4762) should be considered a good prognosis marker instead of being only a predictor of DAE development. The retrospective profile of the study did not allow us to assess analysis related to radiological response as the radiological follow-up between the cohorts was different, and this could be seen as a limitation of the study. However, we prefer to be conservative and avoid overestimating the role of DAEs on the radiological outcome.

In conclusion, our findings open the window to explore individual genetic susceptibility as prognostic factors or predictors of treatment outcome, and to unveil novel mechanisms triggered by oncological treatment and their potential link to tumor response and patient survival.

CONCLUSION

DAE development in HCC patients receiving TKIs could be explained by the *AGT2* (rs4762) gene variant. If validated in other anti-oncogenic treatments, it might be considered a good prognosis marker.

ARTICLE HIGHLIGHTS

Research background

In hepatocellular carcinoma (HCC), patients regardless of the chosen treatment, the development of dermatologic adverse events (DAEs) is associated with better outcome. The underlying mechanism of these effects is unknown.

Research motivation

Distinct genetic variants could have an effect to the likelihood of developing DAEs in patients treated with TKIs for advanced HCC.

Research objectives

The objective of this study was to evaluate the association of two specific *AGT* gene single-nucleotide polymorphisms, rs699 and rs4762, in DAE development.

Research methods

Four cohorts were used to assess the effect, as training and external validation, of the effect of *AGT1* (rs699) and *AGT2* (rs4762) on the development of DAEs in patients with advanced HCC.

Research results

AGT2 (rs4762) AA genotype was related to an increased risk of DAEs development in the Northern Italy cohort in a multivariate model adjusted for clinically relevant factors such as BCLC stage, ECOG-PS, diabetes and arterial hypertension (AHT). This effect was externally validated in the validation cohort (combining BCLC1, BCLC2 and Naples cohorts).

Research conclusions

The development of DAEs in patients treated with TKIs for advanced HCC could be explained by the *AGT2* (rs4762) SNP.

Research perspectives

The *AGT2* (rs4762) SNP could be proposed as a valuable predictive marker if a similar effect is found in other anti-oncogenic treatments.

FOOTNOTES

Author contributions: Reig M, Boix L, Iavarone M, Bruix J and Torres F designed the conceptualization; Reig M, Torres F and Sapena V performed the methodology; Sapena V and Torres F performed the formal analysis; Reig M, Boix L, Iavarone M, Bruix J and Sapena V performed the research; Sapena V and Sanduzzi Zamparelli M performed data curation; Sapena V, Boix L and Reig M wrote the original draft; Sapena V, Iavarone M, Boix L, Facchetti F, Guarino M, Sanduzzi Zamparelli M, Granito A, Samper E, Scartozzi M, Corominas J, Marisi G, Diaz A, Casadei-Gardini A, Gramantieri L, Lampertico P, Morisco F, Torres F, Bruix J, Reig M contributed analytic tools and reviewed and edited the manuscript; Reig M, Bruix J, Iavarone M and Morisco F performed expert supervision; Reig M and Iavarone M performed project administration; Reig M, Bruix J and Boix L searched funding acquisition.

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

Conflict-of-interest statement: Víctor Sapena: Travel grants from Bayer; Massimo Iavarone: Bayer, Gilead Sciences, BMS, Janssen, Ipsen, MSD, BTG-Boston Scientific, AbbVie, Guerbet, EISAI, Shionogi; Loreto Boix: Speaker fees from Bayer; Marco Sanduzzi Zamparelli: Speaker fees and travel funding from Bayer; Travel grant from BTG, Eisai and MSD; Mario Scartozzi: Speakers Bureau and Advisory board Bayer, EISAI, MSD, AMGEN, Merck, Sanofi; Alba Díaz: Speaker fees from Bayer; Andrea Casadei-Gardini: Speakers Bureau and Advisory board Bayer, EISAI, MSD, Ipsen, AstraZeneca, GSK; Pietro Lampertico: Speaking bureau/advisor for Abbvie, Eiger, Bristol-Myers Squibb, Gilead, GlaxoSmithKline, Merck/ Merck Sharp & Dohme, MYR Pharma, Roche; Ferran Torres: DSMB fees from Basilea Pharmaceutica International and ROVI; educational fees from Janssen and Ferrer; Jordi Bruix: Consultancy fees from Arqule, Bayer, Novartis, BMS, BTG-Biocompatibles, Eisai, Kowa, Terumo, Gilead, Bio-Alliance/Onxeo, Roche, AbbVie, Merck, Sirtex, Ipsen, Astra-Medimmune, Incyte, Quirem, Adaptimmune, Lilly, Basilea, Nerviano; Research grants from Bayer and BTG; Educational grants from Bayer and BTG; Lecture fees from Bayer, BTG-Biocompatibles, Eisai, Terumo, Sirtex, Ipsen; María Reig: Consultancy fees from Bayer, BMS, Roche, Ipsen, AstraZeneca, UniversalDX and Lilly; Lecture fees from Bayer, BMS, Gilead, Lilly and Roche; Research grants (to the institution) from Bayer, Roche and Ipsen; and the rest of the authors have no conflicts of interest to report.

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ORIGINAL ARTICLE

Observational Study

Hepatobiliary phases in magnetic resonance imaging using liver-specific contrast for focal lesions in clinical practice

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Abstract

BACKGROUND

Challenging lesions, difficult to diagnose through non-invasive methods, constitute an important emotional burden for each patient regarding a still uncertain diagnosis (malignant x benign). In addition, from a therapeutic and prognostic point of view, delay in a definitive diagnosis can lead to worse outcomes. One of the main innovative trends currently is the use of molecular and functional methods to diagnosis. Numerous liver-specific contrast agents have

been developed and studied in recent years to improve the performance of liver magnetic resonance imaging (MRI). More recently, one of the contrast agents introduced in clinical practice is gadoxetic acid (gadoxetate disodium).

AIM

To demonstrate the value of the hepatobiliary phases using gadoxetic acid in MRI for the characterization of focal liver lesions (FLL) in clinical practice.

METHODS

Overall, 302 Lesions were studied in 136 patients who underwent MRI exams using gadoxetic acid for the assessment of FLL. Two radiologists independently reviewed the MRI exams using four stages, and categorized them on a 6-point scale, from 0 (lesion not detected) to 5 (definitely malignant). The stages were: stage 1- images without contrast, stage 2- addition of dynamic phases after contrast (analogous to usual extracellular contrasts), stage 3- addition of hepatobiliary phase after 10 min (HBP 10'), stage 4- hepatobiliary phase after 20 min (HBP 20') in addition to stage 2.

RESULTS

The interobserver agreement was high (weighted Kappa coefficient: 0.81- 1) at all stages in the characterization of benign and malignant FLL. The diagnostic weighted accuracy (Az) was 0.80 in stage 1 and was increased to 0.90 in stage 2. Addition of the hepatobiliary phase increased Az to 0.98 in stage 3, which was also 0.98 in stage 4.

CONCLUSION

The hepatobiliary sequences improve diagnostic accuracy. With growing potential in the era of precision medicine, the improvement and dissemination of the method among medical specialties can bring benefits in the management of patients with FLL that are difficult to diagnose.

Key Words: Liver; Liver neoplasms; Liver transplantation; Medical oncology; Diagnostic imaging; Magnetic resonance imaging

Core Tip: The translational objective was to determine the value of hepatobiliary phases using gadoxetic acid as a liver-specific agent in magnetic resonance imaging (MRI) in the characterization of benign and malignant focal liver lesions (FLL) in clinical practice. Morphofunctional MRI with gadoxetic acid in addition to the usual dynamic phases after contrast medium (arterial, portal and transitional/ equilibrium) increased the proportion of hits for differentiation between benign and malignant FLL in relation to the definitive diagnosis. The results suggest a relevant impact on the definition of strategies for the approach of focal hepatic lesions, as well as in the assessment of the treatment employed.

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INTRODUCTION

The accurate characterization of focal liver lesions (FLL) has great clinical relevance. Although ultrasonography (US) and computed tomography (CT) are the most important diagnostic tools for screening FLL, magnetic resonance imaging (MRI) is a well-established diagnostic imaging method in clinical practice and produces images without ionizing radiation, with good spatial resolution and excellent tissue resolution, thus allowing a very reliable assessment. Challenging lesions, difficult to diagnose through non-invasive methods, constitute an important emotional burden for each patient regarding a still uncertain diagnosis (malignant x benign). In addition, from a therapeutic and prognostic point of view, delay in a definitive diagnosis can lead to worse outcomes. One of the main innovative trends currently is the use of molecular and functional methods. Combined with diffusion and dynamic studies of the liver after administration of a contrast medium, MRI stands out as the most accurate non-invasive imaging method for the detection and characterization of FLL[1].

Numerous liver-specific contrast agents have been developed and studied in recent years to improve the performance of liver MRI, specifically those that are captured by liver cells by hepatocytes (gadolinium-based compounds), such as gadobenate dimeglumine (Gd-BOPTA), mangafodipir trisodium (Mn-DPDP), or by Kupffer cells which are particles of super magnetic iron oxide. Recently, one of the contrast agents introduced in clinical practice is gadoxetic acid (gadoxetate), formed by gadolinium and the ligand ethoxybenzyl-diethylenetriaminepentaacetic acid (Gd-EOB-DTPA)[2]. The gadoxetic acid has hepatocellular uptake and biliary excretion (about 50% in healthy patients), which allows to carry out routine three-phase dynamic studies at first (arterial, portal and transitional/equilibrium), with the characteristics of the liver parenchyma and FLL similar to the extracellular gadolinium, such as gadopentetate dimeglumine (Gd-DTPA), followed by hepatobiliary assessment in the same exam[3-6]. Given the particular importance in each patient's outcome of the correct diagnosis of a challenging focal liver lesion, the recent introduction of this contrast medium in MRI and its potential uses, the objective was to determine the value of hepatobiliary phases (HBP) using gadoxetic acid as a liver-specific agent in MRI in addition to the non-contrast and dynamic phases after contrast in the characterization of benign and malignant FLL in clinical practice, including hepatocellular carcinoma (HCC) and metastases.

MATERIALS AND METHODS

Study design

Controlled diagnostic clinical trial. Identification of the study under the Universal Trial Number (UTN): U1111-1247-9655.

Inclusion criteria

Abdominal MRI exams with the use of a liver-specific contrast agent for the assessment of FLL characterized as challenging- assessments that had already been identified in previous exams (US and CT with contrast and/or MRI with conventional gadolinium), but that remained undetermined, requiring diagnostic complementation for clarification.

Exclusion criteria

(1) Absence of definitive diagnostic criteria for FLL; (2) Previous radiofrequency ablation and/or chemoembolization of the lesion to be analyzed; (3) Artifacts in the exam preventing adequate characterization of the lesion to be analyzed; and (4) Absence of detection of FLL in the MRI exam.

Criteria used for the definitive diagnosis

The definitive diagnostic criterion for malignant lesions [liver metastases and HCC) and adenomas was based on anatomopathological confirmation. The histopathological slides were blindly reviewed by an experienced pathologist at the liver transplant unit of the hospital. The criteria used for the definitive diagnosis of other benign lesions [focal nodular hyperplasia (FNH), cysts, and hemangiomas] was the histopathological assessment or the absence of changes in the imaging follow-up (CT or MRI) of two years without treatment.

Technical parameters

The exams were performed in a 1.5 T (Tesla) MRI scanner, with a 4-channel body sense coil. The patients were required to fast for 6 h, prior to scanning. Non-contrast T1-weighted sequences, in-phase and out-of-phase, and T2-weighted coronal sequences were performed. A dynamic study was conducted following injection of the contrast medium with T1-weighted sequences with fat saturation before and after intravenous injection of the contrast medium, with a dose of 0.1 mL/kg of weight (equivalent to 0.025 mmol/kg) in bolus, using an automatic injector, at a rate of 1.5 mL/s, followed by a flush of 20 mL of saline solution at the same rate of infusion. After the injection of gadoxetic acid, axial images and T1-weighted gradient echo sequences with fat saturation were obtained in these dynamic phases: arterial within 15 to 20 s after the start of the intravenous injection, portal after 60 s, transition after 120 s, and in the hepatobiliary phase within 10 and 20 min after the start of the intravenous injection. Between the transition phase and the hepatobiliary phase, T2-weighted images with and without fat saturation and diffusion-weighted sequences (DWI, *b*-value 1000) were acquired. The technical parameters used in each sequence are shown in Table 1.

Image analysis

Two radiologists (radiologist A with 5 years of experience in abdominal radiology, while radiologist B has more than 10 years) independently assessed the four stages of images in the following order: Stage 1: Non-contrast images (T1-pre-contrast; T2-weighted images with and without fat saturation; DWI, *b*-value 1000); Stage 2: Non-contrast images and dynamic phases following injection of gadoxetic acid (arterial, portal, and transition phase); Stage 3: Addition of hepatobiliary phase ten minutes (HBP10′)

Table 1 Technical parameters used in the sequences of magnetic resonance imaging exams

Parameter	T2	T2 with fat saturation	T1 "in-phase" and "out-of-phase"	Diffusion	T1-weighted images without contrast and after contrast
Sequence	Fast spin-echo	Fast spin-echo	Gradient- echo FFE	EPI	Gradient- echo 3D/ TFE
Free breathing	Yes	Yes	No	No	No
Matrix	268 × 184	300 × 261	236 × 161	152 × 150	168 × 228
Thickness (mm)	6.5	7	7	7	2.5
Spacing (Gap)	1.5	1	1	1	-
Turning angle	90	90	80	90	10
Field of view (AP, LL, CC)	297 × 335 × 222	363 × 400 × 223	353 × 400 × 223	380 × 380 × 239	295 × 400 × 225
Repeat time (ms)	5299	1299	104	2160	4.1
Echo time (ms)	160	80	4.6/2.3	80	2.0
Acquisition time	02:48	02:24	00:21	02:57	00:15
Number of excitations	2	2	1	4	1

FFE: Fast field echo; TFE: Turbo field echo; EPI: Echo planar imaging.

following the injection of gadoxetic acid in stage 2; Stage 4: Addition of hepatobiliary phase twenty minutes (HBP 20') following the injection of gadoxetic acid in stage 2. A 6-point scale was created by the author for the assessment of each focal liver lesion in each stage as follows: Score 0: Lesion not detected in this stage; Score 1: Definitely benign; Score 2: Probably benign; Score 3: Undetermined; Score 4: Probably malignant; Score 5: Definitely malignant. The total time of analysis for each observer was three months, respecting the time interval of fifteen days between stages to avoid the influence of previous findings, to thus obtain an independent assessment of each stage. The two radiologists blindly assessed clinical-laboratory data and definitive diagnoses, and each issued its own report according to the parameters proposed by the researcher. The objective was to carry out an independent double assessment and subsequent comparison. Each observer reported the number of lesions diagnosed for each stage, the location (Couinaud segmentation[7]), and the proposed scores for each stage. The findings of each observer were analyzed with an assessment of the interobserver agreement. The cases of disagreement were discussed, and a consensus was reached.

Statistical analysis

Only lesions that appeared in the same location at the different stages of MRI and in the criteria for definitive diagnosis were considered correctly detected and characterized by the observers. The method of generalized estimating equations (GEE)[8] was used to compare the stages. The estimates were calculated by maximum likelihood to weight the difference in the number of repetitions for each patient. The statistical review of the study was performed by a medical statistician. The receiver operating characteristic (ROC) curve for repeated measurements was used to assess the accuracy of each stage in relation to the definitive diagnosis[9]. The observations in each patient are not independent, and intra-patient correlation and variation were introduced in the analyses using a generalized linear mixed model. The accuracy of each stage was compared estimating a logistic regression model for repeated measurements using the method of GEE[10]. A level of significance was adopted to be 5%.

RESULTS

Characterization of lesions according to the criteria for the definitive diagnosis

After approval of the project by the Institutional Research Ethics Committee, it was found that 290 MRI exams had been performed consecutively during the study period in patients over 18 years of age who had used gadoxetic acid in the characterization of FLL that had already been identified in previous exams (US and CT and/or MRI with conventional gadolinium), that had undetermined characterization, requiring diagnostic complementation. The exclusion criteria are shown in Figure 1. Therefore, the final sample according to the criteria used for the definitive diagnosis was composed of 302 Lesions from 136 patients who performed MRI exams using gadoxetic acid for the assessment of FLL, with 160

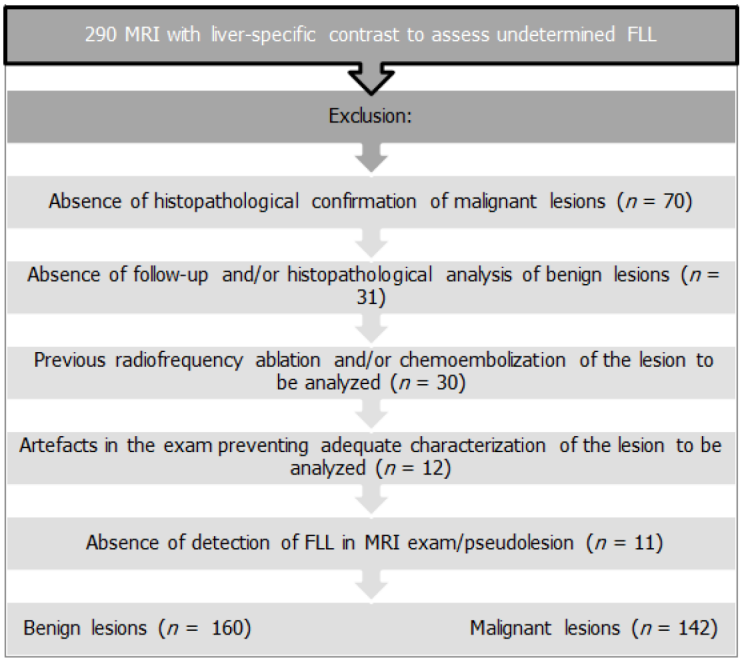

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Figure 1 Flowchart: Exclusion criteria. MRI: Magnetic resonance imaging; FLL: Focal liver lesions.

benign lesions (53.0%) and 142 malignant lesions (47.0%). Benign lesions included: FNH (n = 90; 56.2%); cysts (n = 36; 22.5%); hemangiomas (n = 22; 13.7%); adenomas (n = 12; 7.5%). Malignant lesions included: metastases (n = 87; 61.3%) and hepatocellular carcinomas- HCCs (n = 55; 38.7%). The number of lesions according to the criteria for the definitive diagnosis in each patient ranged from 1-5 Lesions (mean 2.4; SD 1.8). The diameter of the 160 benign lesions ranged from 0.4 cm to 8.8 cm (mean 2.7 cm; SD 1.9 cm). The diameter of the 142 malignant lesions ranged between 0.4 cm and 7.8 cm (mean 2.1 cm; SD 1.7 cm).

Characterization of patients

The final sample, based on the criteria used for the definitive diagnosis, was composed of 302 Lesions from 136 patients who performed MRI exams using gadoxetic acid for the assessment of FLL. Of these 136 patients, 80 (58.8%) were female, with a mean age of 43 years (SD 19). Personal history of cancer was present in 52.9% of patients (colorectal 95.5%; gastric 11.8%; breast 8.8%; prostate 8.1%; melanoma 7.3%; pheochromocytoma 4.4%).

Interobserver agreement

The weighted Kappa coefficient is used to describe the agreement between two or more observers when performing a nominal or ordinal assessment of the same sample and demonstrated high agreement (between 0.81 and 1) for all stages in the characterization of benign and malignant FLL. Of the total 302 Lesions, there was disagreement between observers in ten lesions in stage 1; eight lesions in stage 2; seven lesions in stages 3 and 4. For lesions where in there was no agreement between observers, the consensus of the radiologists was used for the final definition.

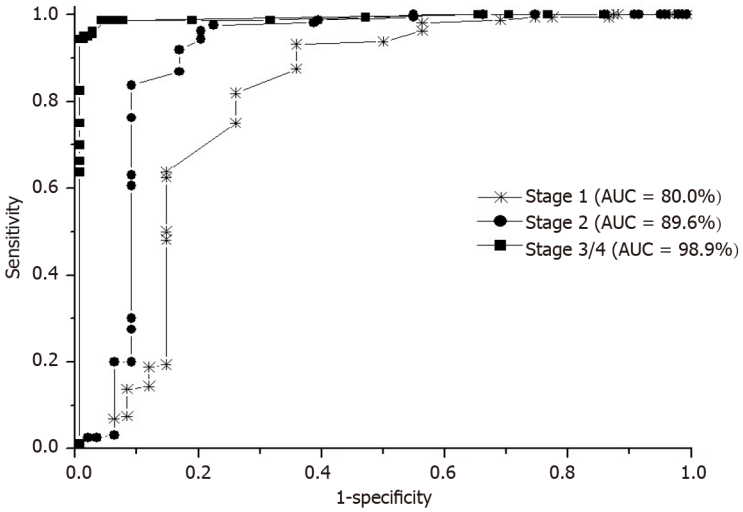
Diagnostic performance parameters

The accuracy weighted by the number of repetitions of lesions in each patient showed a good proportion of correct answers for differentiating between benign and malignant lesions (Figure 2). There were significant differences between the accuracy of the four stages (P = 0.0002, GEE, Figure 2).

The comparison of the weighted accuracy [area under the curve (AUC)] showed that the accuracy of stage 1 was lower than the accuracy of stages 2 and 3/4. The accuracy of stage 2 was lower than the accuracy of stages 3 and 4. There were no significant differences between stages 3 and 4 (Figure 2).

Results of the generalized estimation equations to study the size factor (numerical and categorization) in the stages

The characterizations in the stages of only the malignant lesions were associated with the numerical size (in cm) of the FLL. Each unit of increase in the size of the malignant lesion increases the chance of characterization with higher scores by 1.26 at each of all stages. Characterizations in the stages of only malignant lesions were associated with the size of the FLL categorized as < 1 cm and ≥ 1.0 cm.



Result of the comparison of the accuracy (AUC) between stages:
P = 0.0002 (GEE)
Differences between:
AUC1 x AUC2 *P* = 0.0131
AUC1 x AUC3 *P* < 0.0001
AUC2 x AUC3 *P* = 0.0059

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Figure 2 Receiver operating characteristic curves of each stage in relation to the definitive diagnosis and comparison of the accuracy between stages. AUC: Area under the curve; GEE: Generalized estimating equations.

Malignant lesions ≥ 1 cm are 2.4 times more likely to be characterized with higher scores at all stages than lesions < 1 cm (Table 2). Figure 3 shows subcentimetric metastasis in a cancer patient detected only in the hepatobiliary phases and a pseudo lesion.

DISCUSSION

The present study found a significant increase in the diagnostic reliability of malignant lesions (HCC and metastases) with the inclusion of stage 3 compared to stage 2. An ideal diagnostic tool by liver imaging should have a high diagnostic accuracy to provide an adequate therapeutic approach in malignant and benign cases. The MRI with gadoxetic acid has revealed excellent diagnostic performance for detecting metastases in recent meta-analyses[11-13]. The combined use of diffusion weighted sequences (DWI) and hepatobiliary phases in clinical practice is recommended in patients with potentially resectable liver metastases[14,15].

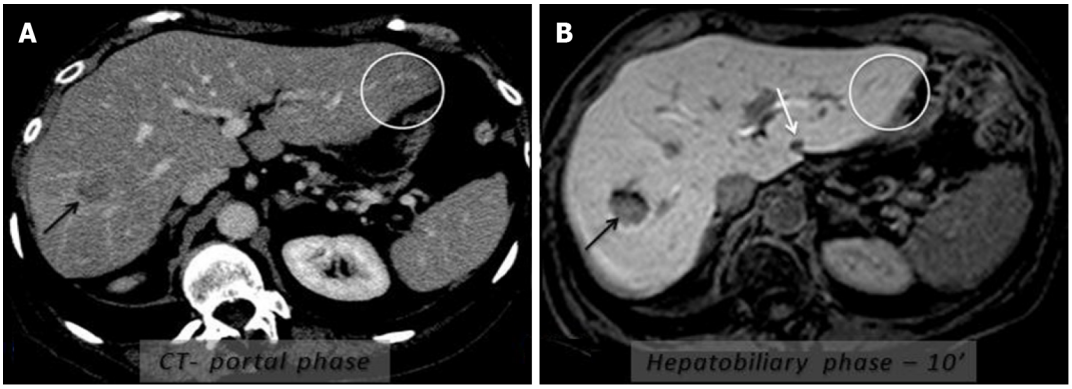
Still on the significant increase in the diagnostic reliability in the characterization of malignant lesions found in our study, the HCC is one of the few malignancies that can be diagnosed by imaging alone, without the need for confirmation by biopsy when the image is typical. Different guidelines established by medical groups and entities are used in patients at risk for HCC and reflect clinical and epidemiological differences, underlying etiologies of liver disease, socioeconomic background, and specificities of each region, such as surveillance and available therapeutic options[16-20]. The additional benefit of diffusion and a liver-specific contrast is recognized by the American College of Radiology (ACR) and is incorporated into the Liver Imaging Reporting and Data System (LIRADS)[21-25].

There was an increase in diagnostic reliability in the characterization of benign lesions with the addition of the hepatobiliary phases (stage 3) compared to stage 2. For benign lesions, a recent systematic review concludes that the low signal intensity in the hepatobiliary phases can help distinguish between adenomas and FNH[26].

Our research also showed the value of morphofunctional MRI with gadoxetic acid as a liver-specific contrast in the diagnosis of pseudo lesions, since 11 exams were excluded (3.8%) from the initial sample of 290 due to the absence of detectable lesions in the MRI exam. The lesions had been observed in other previous imaging methods, remaining undetermined. It is also noteworthy that 12 exams (4.1%) were excluded from the 290 of the initial sample due to artifacts preventing adequate characterization of the lesion to be analyzed, such as the phenomenon of "transient dyspnea". Studies relate this artifact to the use of gadoxetic acid, although the data is not consistent and the pathophysiology is not yet fully elucidated[27-29].

Table 2 Results of the generalized estimation equations to study the size factor (numerical and categorization) in the stages

Size effect	General *P* value	Benign *P* value	Malignant *P* value
Numerical (cm)	0.3785	0.1766	0.0025 OR = 1.2561 95%CI (1.0824; 1.4577)
(≥ 1 cm) x (< 1 cm)	0.2361	0.1476	0.0058 OR = 2.3691 95%CI (1.3001; 4.3171)



DOI: 10.4254/wjh.v14.i7.1459 **Copyright** ©The Author(s) 2022.

Figure 3 Use of hepatobiliary phases: Detection of small metastasis in a potential patient undergoing liver surgery. Computed tomography (CT) (A) and magnetic resonance imaging (B) (10-min hepatobiliary phase) showed metastatic lesion in the right lobe (arrow). However, the doubtful/suspected nodule for metastasis on CT was not confirmed in the hepatobiliary phase (isosignal-white circle, pseudolesion). However, another hyposignal nodule (white arrow) in the left lobe was well evidenced in the hepatobiliary phase (it had not been identified on CT), compatible with secondary involvement.

In this study, stages 3 and 4 showed identical results in the characterization of FLL. Although recommendations point to the acquisition of HBP20', some evidences suggest the possibility of earlier acquisition (HBP10') in the assessment of part of the cases of FLL[15,31-33]. Other cases individualized according to the diagnostic suspicion in clinical practice may require phases after 20 or possibly up to 30 minutes after contrast medium injection, for example the differentiation between biliary lesions and extra biliary cysts that do not communicate with bile ducts, such as duodenal duplication cysts, duodenal diverticula and pseudo cysts. The liver-specific contrast delineates the biliary tract demonstrating the communication of the biliary cystic lesions. Considering the complexity of the hepatic anatomy as well of the more refined surgical techniques, the previous Knowledge of the biliary anatomy and its variations becomes increasingly important in the preoperative planning. The anatomical and functional characterization of intra and extrahepatic biliary tract is provided through biliary excretion of the gadoxetic acid, and can reduces the occurrence of postoperative complications. In addition, hepatobiliary contrast-enhanced cholangiography allows for the accurate detection of postoperative complications (biliary fistulas, bilomas)[33-35].

Some considerations should be made about this study. The assessed MRI exams are from patients who are part of a cohort at the institutional FLL outpatient clinic; thus, the results of this research with an institutional-based sample may differ from results with population-based samples. Moreover, all images were acquired with the same parameter and the observers are familiar with the specific technical protocols, as in the usual clinical routine conditions.

Given the reality of the higher cost of liver-specific contrast in most countries, we highlight the value of morphofunctional MRI with the hepatobiliary phase, notably in specific situations after, for example, the diagnosis of a FLL has remained undetermined in previous exams (US and CT with contrast and/or MRI with extracellular contrast routinely used), as in the screening of patients in our study. The use and additional analysis in clinical practice of hepatobiliary stages (steps 3 and 4 in this study) as a criterion for information aggregation in relation to other sequences routinely performed in CT and MRI scans (stage 1: Non-contrast images and stage 2: Dynamic phases after contrast, analogous to the phases with extracellular contrast- arterial, portal and equilibrium/transition) may benefit a specific group of patients. Good cost-effective practices for the use of this methodology in morphofunctional MRI with liver-specific contrast may include, therefore, (1) The elucidation of possible pseudo-lesions (perfusion alterations x HCC, for example; most HCCs, except the well-differentiated ones, present hypo signal in the hepatobiliary phases) and and/or problem solving in patients with lesions with atypical characteristics by imaging; (2) The diagnosis of small metastatic lesions in potential patients for surgical treatment; (3) The search to complement information to increase diagnostic assertiveness in benign lesions still undetermined (hepatocellular x non-hepatocellular origin; or biliary lesions x extra biliary cysts); and (4) The definitive diagnosis in the non-invasive era of malignant lesions hitherto uncharacteristic in previous exams with routine extracellular contrast agents (either through the potential

increase in the LIRADS category in hepatocellular carcinomas or through a more assertive diagnosis of secondary liver involvement), as demonstrated herein. These applications mentioned above refer to the context more focused on FLL, without including the other important potential indications like those mentioned in the discussion of this study.

Other potential benefits in living laboratories integrating translational research and technological innovations have brought to light new uses of this methodology in morphofunctional MRI with liver-specific contrast, such as imaging biomarkers, outcome predictions and co-creation intelligences for the resolution and/or amelioration of specific diseases to patients, emerging as promising prospects. Further potential liver-specific contrast applications include assesment of liver fibrosis, the evaluation of the functional hepatic reserve before partial hepatectomy; evaluation of live donor's hepatic function as well as evaluation of early liver failure after transplantation. In another active area of investigation, morphofunctional MRI with liver-specific contrast may provide a system for stratifying patients according to risk of recurrence with a likely influence on the outcomes of locoregional HCC treatments [36]. The congruence of different knowledge is evident in medical practice and in the necessary advances.

CONCLUSION

The value of morphofunctional MRI with gadoxetic acid as a liver-specific contrast in addition to the usual dynamic phases after contrast medium (arterial, portal and transitional/equilibrium) was to increase the proportion of hits for differentiation between benign and malignant FLL in relation to the definitive diagnosis. The interobserver agreement was high (0.81-1). With growing potential in the era of precision medicine, the improvement and dissemination of the method among medical specialties can bring benefits in the management of patients with focal liver lesions that are difficult to diagnose.

ARTICLE HIGHLIGHTS

Research background

The accurate characterization of focal liver lesions (FLL) has great clinical relevance. Although ultrasonography (US) and computed tomography (CT) are the most important diagnostic tools for screening FLL, magnetic resonance imaging (MRI) is a well-established diagnostic imaging method in clinical practice and produces images without ionizing radiation, with good spatial resolution and excellent tissue resolution, thus allowing a very reliable assessment. One of the main innovative trends currently, is the use of molecular and functional methods.

Research motivation

Challenging lesions, difficult to diagnose through non-invasive methods, constitute an important emotional burden for each patient regarding a still uncertain diagnosis (malignant x benign). In addition, from a therapeutic and prognostic point of view, delay in a definitive diagnosis can lead to worse outcomes. Numerous liver-specific contrast agents have been developed and studied in recent years to improve the performance of liver MRI. More recently, one of the contrast agents introduced in clinical practice is gadoxetic acid (gadoxetate disodium).

Research objectives

To determine the value of hepatobiliary phases (HBP) using gadoxetic acid as a liver-specific agent in MRI in addition to the non-contrast and dynamic phases after contrast in the characterization of benign and malignant FLL in clinical practice, including hepatocellular carcinoma and metastases.

Research methods

Controlled diagnostic clinical trial. Two radiologists independently assessed the four stages of images in the following order: Stage 1: Non-contrast images (T1-pre-contrast; T2-weighted images with and without fat saturation; DWI, *b*-value 1000); Stage 2: Non-contrast images and dynamic phases following injection of gadoxetic acid (arterial, portal, and transitional phase); Stage 3: Addition of hepatobiliary phase ten minutes (HBP10′) following the injection of gadoxetic acid in stage 2; Stage 4: Addition of hepatobiliary phase twenty minutes (HBP 20′) following the injection of gadoxetic acid in stage 2. A 6-point scale was created by the author for the assessment of each focal liver lesion in each stage. The method of Generalized Estimating Equations (GEE) was used to compare the stages. The estimates were calculated by maximum likelihood to weight the difference in the number of repetitions for each patient. The receiver operating characteristic (ROC) curve for repeated measurements was used to assess the accuracy of each stage in relation to the definitive diagnosis.

Research results

The interobserver agreement was high (weighted Kappa coefficient: 0.81-1) at all stages in the characterization of benign and malignant FLL. The diagnostic weighted accuracy (Az) was 0.80 in stage 1 and was increased to 0.90 in stage 2. Addition of the hepatobiliary phase increased Az to 0.98 in stage 3, which was also 0.98 in stage 4.

Research conclusions

The value of morphofunctional MRI with gadoxetic acid as a liver-specific contrast in addition to the usual dynamic phases after contrast medium (arterial, portal and transitional/equilibrium) was to increase the proportion of hits for differentiation between benign and malignant FLL in relation to the definitive diagnosis.

Research perspectives

With growing potential in the era of precision medicine, the improvement and dissemination of the method among medical field can bring benefits in the management of patients with focal liver lesions that are difficult to diagnose. With the accumulation of experience, the use demonstrated herein and other potentials of morphofunctional MRI with liver-specific contrast as a new potential imaging tumor biomarker may be established, benefiting patients with challenging focal liver lesions. Other potential benefits in living laboratories have brought to light new uses of this methodology, such as outcome predictions and co-creation intelligences for the resolution and/or amelioration of specific diseases to patients, emerging as promising prospects. Further potential liver-specific contrast applications include assesment of liver fibrosis, the evaluation of the functional hepatic reserve before partial hepatectomy; evaluation of live donor's hepatic function as well as evaluation of early liver failure after transplantation. In another active area of investigation, morphofunctional MRI with liver-specific contrast may provide a system for stratifying patients according to risk of recurrence with a likely influence on the outcomes of locoregional HCC treatments. Also new translational studies similar to this one in other parts of the world added to the socioeconomic background and specificities of each region may bring benefits to this group of patients.

FOOTNOTES

Author contributions: Fernandes DA, Caserta NMG, and Boin IFFS designed the research study; Fernandes DA, Dal Lago EA, Oliver FA, and Loureiro BMC performed the research; Dal Lago EA, Oliver FA, Martins DL, Penachim TJ, Barros RHO, and Araújo-Filho JAB contributed analytic tools and analyzed the data; and All authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Efficacy and safety of COVID-19 vaccination in patients with cirrhosis

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Abstract

BACKGROUND

The clinical efficacy and safety of vaccination against novel coronavirus disease 2019 (COVID-19) in patients with cirrhosis have not been evaluated yet.

AIM

To evaluate the clinical efficacy and safety of vaccination against COVID-19 in patients with cirrhosis.

METHODS

This was a retrospective cohort study of patients with cirrhosis. The first cohort included patients vaccinated with Gam-COVID-Vac (Sputnik V); the second one consisted of unvaccinated controls.

RESULTS

The study included 89 vaccinated patients and 148 unvaccinated ones. There were 4 cases of COVID-19 in the vaccinated group and 24 cases in the unvaccinated group (P = 0.035). No severe cases of COVID-19 were revealed in the vaccinated group, while there were 12 ones in the unvaccinated group (P = 0.012) with 10 deaths detected (P = 0.012). The vaccine efficacy was 69.5% (95% confidence

interval [CI]: 18.5%-94.4%) against symptomatic cases of COVID-19, 100% (95%CI: 25.1%-100.0%) against severe cases, and 100% (95%CI: 1.6%-100.0%) against death associated with COVID-19. The efficacy of full vaccination with revaccination against symptomatic cases of COVID-19 was 88.3% (95%CI: 48.0%-99.6%). The overall mortality rate was higher in the unvaccinated group than in the vaccinated group (17.1% *vs* 3.0%; P = 0.001). Higher Child-Turcotte-Pugh class cirrhosis (hazard ratio [HR] = 4.13, 95%CI: 1.82-9.35) and higher age (HR = 1.08, 95%CI: 1.04-1.15) were independent predictors of overall mortality, while vaccination had a protective effect (HR = 0.09, 95%CI: 0.01-0.76). There was no significant difference in liver-related mortality (P = 0.135) or the incidence of liver decompensation (P = 0.077), bleeding esophageal varices (P = 0.397), and vascular events (P = 0.651) between the two groups of patients.

CONCLUSION

Vaccination against COVID-19 in patients with cirrhosis is effective and safe.

Key Words: Coronavirus; Vaccination; Revaccination; Booster; SARS-CoV-2; Sputnik V

Core Tip: The aim of the study was to evaluate the clinical efficacy and safety of vaccination against novel coronavirus disease 2019 (COVID-19) in patients with cirrhosis. No severe cases of COVID-19 were revealed in the vaccinated group. The vaccine efficacy was 69.5% (95% confidence interval [CI]: 18.5%-94.4%) against symptomatic cases of COVID-19, 100% (95%CI: 25.1%-100.0%) against severe cases, and 100% (95%CI: 1.6%-100.0%) against death associated with COVID-19. There was no significant difference in liver-related mortality, or the incidence of liver decompensation, bleeding esophageal varices, and vascular events between the two groups of patients. Vaccination against COVID-19 in patients with cirrhosis is effective and safe.

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INTRODUCTION

The new coronavirus infection 2019 (COVID-19) has become a challenge to the health services. At the time of this writing, more than a quarter of a billion people have been infected with COVID-19, and more than 5 million of them have died. Despite all the efforts of doctors, mortality from this infection remains high and its prevention through vaccination is urgently needed.

Although the main vaccines used in the world were shown to be highly effective in preventing COVID-19[1-6], the change of the dominant strain to the new variants led to a significant decrease in vaccination efficiency[7]. In addition, the immune response to vaccination decreases over time[8]. Therefore, the need for revaccination came up for discussion[8].

The main vaccines against COVID-19 lead to a moderate incidence of side effects, which are short-term and not dangerous in the vast majority[1-6]. However, there were some concerns that vaccination of patients with cirrhosis may lead to the decompensation of liver function or provoke bleeding esophageal varices. Immune paralysis observed in cirrhosis may lead to decreased efficacy of vaccination against different infections[9].

Recent articles have shown that a subset of cirrhotic patients has a poor antibody response to COVID-19 vaccination[10] and that several cirrhotic patients develop COVID-19 after full vaccination[11].

Cirrhosis is associated with an increased risk of mortality due to COVID-19 compared to non-cirrhotic patients[12,13]. Therefore, experts from the European Association for the Study of the Liver recommended COVID-19 vaccination of patients with cirrhosis without waiting for the results of studies on the efficacy and safety of the procedure in this cohort of patients[14].

Gam-COVID-Vac (Sputnik V) is a Russian vector two-component vaccine against COVID-19 that has shown its high efficiency in phase 3 clinical trials[1], as well as in an independent national-level comparative study in Hungary[14]. However, these data were obtained before the arrival of the COVID-19 delta surges.

The aim of this study was to evaluate the clinical efficacy and safety of COVID-19 vaccination and revaccination with Sputnik V in patients with cirrhosis.

MATERIALS AND METHODS

This was a retrospective cohort study approved by the Ethics Committee of Sechenov University (Protocol 20-11) in accordance with the Helsinki Declaration.

Patients

The patients with cirrhosis, who were residents of Moscow, regularly monitored at the Clinic for Internal Diseases, Gastroenterology and Hepatology of Sechenov University or Consultative and diagnostic center № 2, did not undergo liver transplantation, and were alive as of June 1, 2021, were included in the study.

Patients, who caught COVID-19 before June 1, 2021 or who were vaccinated against COVID-19 with a vaccine other than Gam-COVID-Vac (Sputnik V), were excluded from the study. The diagnosis of cirrhosis was established based on biopsy data or a combination of clinical, laboratory, and instrumental data.

Exposure

Patients in the vaccination group were injected with Sputnik V intramuscularly at a standard dose (0.5 mL) twice with an interval of 21-37 d between the doses. Patients in the subgroup of revaccination received the third (booster) dose (first component) of Sputnik V 6-8 mo after taking the first component of the vaccine.

Patients in the control (unvaccinated) group did not receive COVID-19 vaccination by the end of the observation period (November 30, 2021) and were not diagnosed with COVID-19 before the beginning of the observation period (June 1, 2021).

There were no special criteria for the selection of patients in the vaccination group. Vaccination was carried out at the will of the patients themselves.

All patients received standard of care treatment for cirrhosis according to its etiology and complications. There was no significant difference between groups in drugs used for the treatment of cirrhosis.

Outcomes

The primary outcome was the development of symptomatic COVID-19 during the observation period (from June 1, 2021 to November 30, 2021). We chose this period because the delta variant almost completely replaced other variants of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and became dominant in Russia in June 2021. The third (from June to August 2021) and fourth (from September to November 2021) surges of COVID-19 associated with the delta variant occurred in Moscow during this period. A symptomatic COVID-19 case was considered if a patient had a positive polymerase chain reaction (PCR) test of oropharyngeal or nasopharyngeal swab for SARS-CoV-2 and symptoms and/or signs of COVID-19 (fever, weakness, cough, shortness of breath, anosmia, ageusia, *etc*.). Both inpatients and outpatients were assessed in the study.

Patients were considered fully vaccinated 2 wk after receiving the second dose of the vaccine.

COVID-19 severity classification was carried out in accordance with the current guidelines of the World Health Organization.

Secondary outcomes included death due to COVID-19, death associated with complications of cirrhosis (liver-related death), death from all causes, and the incidence of liver decompensation, bleeding esophageal varices, and vascular events (myocardial infarction, stroke, transient ischemic attack, pulmonary embolism, and abdominal thrombosis [thrombosis of the portal or hepatic veins]).

Death due to COVID-19 was considered if a patient had a positive PCR test for SARS-CoV-2 and had a significant lung damage (areas of ground glass and/or consolidation occupying more than 25% of lung volumes according to chest computed tomography) or a cytokine storm (serum C-reactive protein level more than 60 mg/L), regardless of whether liver decompensation or vascular events developed or not.

When evaluating the efficacy of revaccination, the vaccinated patients were considered unvaccinated 6 mo after the administration of the first dose of Sputnik V. We chose this period because it has been shown that the serum level of anti-SARS-CoV-2-spike-RBD IgG was significantly reduced 6 mo after vaccination against COVID-19 with Sputnik V compared with the results in the first 3 mo after this vaccination[8]. Moreover, these antibodies were not detected in almost 70% of persons 6 mo after this vaccination, although they were detected in 94% of persons 3 mo after this vaccination[8].

Information about vaccination, COVID-19 cases and their severity, patient death and its cause, and development of complications of cirrhosis and vascular events was taken from the Unified Medical Information and Analytical System, which accumulates almost all medical information about the residents of Moscow.

The liver function was assessed before the beginning of the observation period using the Child-Turcotte-Pugh (CTP) classification based on the data of the last check-up of the patient.

Statistical analysis

Statistical analyses was performed with STATISTICA 10 software (StatSoft Inc., United States). The data are represented as median [interquartile range]. The difference in continuous variables was assessed by Mann-Whitney test. Fisher's exact test was used to assess the difference in categorical variables. Survival was assessed using the Kaplan-Meier estimator and Cox test. A Cox regression model was used to assess the influence of factors on patient survival and hazard ratio (HR). A P-value ≤0.05 was considered significant.

Vaccine efficacy was estimated by 100 × (1−IRR), where IRR (incidence rate ratio) is the calculated ratio of cases of COVID-19 per 1 person-year of the observation in the vaccinated group to the corresponding illness rate in the unvaccinated group; 95% confidence interval (95%CI) for vaccine efficacy was obtained by the Baptista-Pike method (on-line calculator https://rdrr.io/cran/ORCI/man/BPexact.CI.html was used)[15].

RESULTS

After excluding patients vaccinated with other vaccines (6 patients vaccinated with the EpiVacCorona peptide vaccine and 1 vaccinated with the CoviVac inactivated vaccine) and those who had had COVID-19 before the observation period (n = 33), a total of 237 patients with cirrhosis were enrolled in the study. Eighty-nine (37.6%) patients were vaccinated with Sputnik V, of whom 39 received the vaccine before the beginning of the observation period, and the rest did it during this period (Figure 1). If the patient was vaccinated during the observation period, then the period before the first dose injection was counted as an unvaccinated period, as well as all events that developed during it.

There was no significant difference between vaccinated and unvaccinated patients in age, gender distribution, severity and etiology of cirrhosis, or the presence of significant comorbidity (Table 1).

COVID-19 was detected significantly more often in unvaccinated individuals than in vaccinated ones. COVID-19 occurred in 4 vaccinated patients: 17 d, 3.0, 6.1, and 7.2 mo after injection of the first dose of the vaccine. Thus, the first case should be considered as incompletely vaccinated, and the third and fourth ones as unrevaccinated. Severe COVID-19 was detected in 50.0% of unvaccinated patients infected with the coronavirus and in none of vaccinated patients. None of vaccinated patients died of COVID-19. Ten deaths due to COVID-19 were registered in the unvaccinated group, which accounted for 41.7% of patients with COVID-19 in this group. However, there was no significant difference in the incidence of mild and moderate cases of COVID-19 between vaccinated and unvaccinated patients (Table 2).

The efficacy of vaccination was 69.5% (95%CI: 18.5%-94.4%) against symptomatic cases of COVID-19, 100% (95%CI: 25.1%-100.0%) against COVID-19 severe cases, and 100% (95%CI: 1.6%-100.0%) against death due to COVID-19.

The overall mortality and mortality associated with COVID-19 were lower among the vaccinated patients than among the unvaccinated ones. There was no significant difference in liver-related mortality, as well as in the overall incidence of liver decompensation, bleeding esophageal varices, and vascular events (P = 0.651) between the two groups of patients (Table 2). Among patients with cirrhosis of CTP classes B and C, there were also no significant differences in the incidence of liver decompensation (44.0% *vs* 51.8% per person-year; P = 0.500) and bleeding esophageal varices (22.0% *vs* 16.1% per person-year; P = 0.504) between vaccinated and unvaccinated patients.

All cases of liver decompensation, bleeding esophageal varices, and transient ischemic attack in the vaccination group occurred later than 3 mo after vaccination and are extremely unlikely to be associated with it. The only patient in the vaccination group died more than 7 mo after vaccination from liver decompensation following bleeding esophageal varices.

Higher CTP class cirrhosis and higher age were significant predictors of overall mortality, while vaccination had a protective effect, according to the results of multiple Cox regression (Table 3).

During the observation period, 39 patients had to be revaccinated, as they had more than 6 mo after the injection of the first vaccine dose. Nineteen (43.8%) of them were revaccinated. There were no cases of COVID-19, liver decompensation, bleeding esophageal varices, or vascular event after revaccination. If we consider unrevaccinated patients 6 mo after the injection of the first vaccine dose as unvaccinated (adjustment for the need for revaccination), the efficacy of full vaccination among patients with cirrhosis against symptomatic cases of COVID-19 was 88.3% (95%CI: 48.0-99.6%).

The incidence of COVID-19 in unrevaccinated patients was not significantly different from that in unvaccinated patients (39.0% *vs* 29.2% per person-year; P = 0.661).

There were no cases of COVID-19 or deaths among vaccinated CTP class A cirrhosis patients (20.8 person-years). Among unvaccinated CTP class A cirrhosis patients (40.4 person-years), COVID-19 developed in 8 (19.8% per person-year) ones, and in 3 (7.4% per person-year) of them it was severe and resulted in death. The efficacy of vaccination against symptomatic cases of COVID-19 was 100.0%

Table 1 Main characteristics of enrolled patients by group

	Vaccinated (*n* = 89)	Unvaccinated (*n* = 148)	*P* value
Age, yr	59 [48-68]	57 [47-64]	0.161
Male/Female	50/39	66/82	0.055
Child-Turcotte-Pugh class A	52 (58.4%)	72 (48.6%)	0.092
Child-Turcotte-Pugh classes B and C	37 (41.6%)	76 (51.4%)	
Etiology of cirrhosis			
Hepatitis B virus	3 (3.4%)	9 (6.1%)	0.275
Hepatitis C virus	16 (18.0%)	29 (15.6%)	0.449
Alcohol	41 (46.0%)	56 (37.8%)	0.094
Metabolic associated liver disease	5 (5.6%)	7 (4.7%)	0.492
Autoimmune hepatitis	1 (1.1%)	9 (6.1%)	0.059
Primary biliary cholangitis	9 (10.1%)	8 (5.4%)	0.136
Primary sclerosing cholangitis	1 (1.1%)	2 (1.4%)	0.684
Wilson disease	1 (1.1%)	1 (0.7%)	0.611
Other	0	6 (4.1%)	0.057
Mixed	12 (13.5%)	21 (14.2%)	0.521
Comorbidity			
Diabetes mellitus	18 (20.2%)	21 (14.2%)	0.151
Ischemic heart disease	7 (7.9%)	6 (4.1%)	0.170
Cancer	9 (10.1%)	12 (8.1%)	0.381
Hepatocellular carcinoma	1 (1.1%)	4 (2.7%)	0.379
Asthma	3 (3.4%)	2 (1.4%)	0.274
Chronic obstructive pulmonary disease	5 (5.6%)	2 (1.4%)	0.071

(95%CI: 16.1-100.0%) among CTP class A cirrhosis patients.

Among the fully vaccinated patients with cirrhosis of CTP classes B and C adjusted for the need for revaccination (11.6 person-years), there was 1 (8.6% per person-year) case of COVID-19 that was moderate. Among these unvaccinated and unrevaccinated patients (42.1 person-years), there were 18 (42.8% per person-year) cases of COVID-19 (with 2 cases that developed later than 6 mo after the first dose of vaccine injection), including 9 (21.4% per person-year) severe ones, of which 7 (16.6% per person-year) resulted in death. The efficacy of full vaccination with revaccination against symptomatic cases of COVID-19 was 79.9% (95%CI: 11.4-99.5%) among CTP B and C cirrhosis patients.

Among patients with cirrhosis of CTP classes B and C, overall mortality was significantly lower in the vaccinated group than in the unvaccinated group (7.7% *vs* 26.4% per person-year; $P = 0.010$).

DISCUSSION

Patients with cirrhosis have a high risk of poor outcome of COVID-19. The high mortality rate (34.0%) among these patients was shown in the first study on this topic[15]. In our study, the mortality rate among unvaccinated patients with cirrhosis was 38.4%, which is significantly higher than the mortality rate among patients with COVID-19 in the general population of Moscow over the same period (about 4%). Thus, the prevention of the development of COVID-19 in this group of patients is an urgent task for health care system.

The presence of impaired immune function in patients with cirrhosis[9] has raised concerns that vaccination against COVID-19 may be of lower efficacy. In a recent study, it was shown that antibodies to SARS-CoV-2 were not found 4 wk after vaccination in 3.8% of patients with cirrhosis, and were too low in 19% of them[10]. Interestingly, the percentage of insufficient responders to the vaccine did not differ significantly between patients with cirrhosis and pre-cirrhotic stages of chronic liver disease[10]. There are publications describing COVID-19 in vaccinated cirrhotic patients. COVID-19 occurred in 6 patients with cirrhosis later than 2 wk after receiving the second dose of vaccine (criterion for full

Table 2 Outcomes by group (*n* [per patient-yr])

	Vaccinated (*n* = 33.8 patient-yr)	Unvaccinated (*n* = 82.1 patient-yr)	*P* value
COVID-19 cases			
Total	4 (11.8%)/3 (8.9%)[1]	24 (29.2%)	0.035/0.013[1]
Mild	1 (3.0%)	7 (8.5%)	0.260
Moderate	3 (8.9%)/2 (5.9%)[1]	5 (6.1%)	0.431/0.666[1]
Severe	0	12 (14.6%)	0.012
Death			
Overall	1 (3.0%)	14 (17.1%)	0.001
Associated with COVID-19	0	10 (12.2%)	0.012
Liver-related	1 (3.0%)	4 (4.9%)	0.135
Non-COVID-19 complications (cases)			
Liver decompensation	4 (11.9%)	21 (25.6%)	0.077
Bleeding esophageal varices	2 (5.9%)	8 (9.7%)	0.394
Myocardial infarction	0	1 (1.2%)	0.707
Pulmonary embolism	0	1 (1.2%)	0.707
Stroke	0	0	-
Transient ischemic attack	1 (3.0%)	0	0.293
Abdominal thrombosis	0	0	-

[1]All vaccinated/fully vaccinated. COVID-19: Coronavirus disease 2019.

Table 3 Analysis of predictors of overall mortality among included patients with cirrhosis

Predictor	*P* value	Hazard ratio
Age	0.001	1.08 (95%CI: 1.04-1.15)
Vaccination	0.027	0.09 (95%CI: 0.01-0.76)
Child-Turcotte-Pugh class	0.001	4.13 (95%CI: 1.82-9.35)
Diabetes mellitus	0.363	
Ischemic heart disease	0.595	
Cancer	0.751	
Asthma	0.342	
Chronic obstructive pulmonary disease	0.851	

vaccination). Half of them required hospitalization, but none of them needed admission to the intensive care unit and none of them died[11]. In our study, COVID-19 developed only in 3 fully vaccinated patients and was also non-severe.

Our study is the first that describes the clinical efficacy of COVID-19 vaccination in cirrhosis. It was 69.5% against symptomatic cases of COVID-19 and 100% against severe cases and death due to COVID-19. However, the immune response to vaccination fades over time and antibodies to SARS-CoV-2 are retained in the blood only in one third of healthy persons 6 mo after the administration of the first dose of the Gam-COVID-Vac vaccine[8]. Therefore, it is not surprising that 2 out of 3 fully vaccinated patients who caught COVID-19 did it later than 6 mo after the injection of the first vaccine dose in our study. The incidence of COVID-19 in unrevaccinated patients was not significantly different from that of unvaccinated patients. Thus, revaccination of patients with cirrhosis within the sixth month after the injection of the first vaccine dose is highly recommended. None of the revaccinated patients caught COVID-19. The efficacy of full vaccination with revaccination against symptomatic COVID-19 was 88.3%.

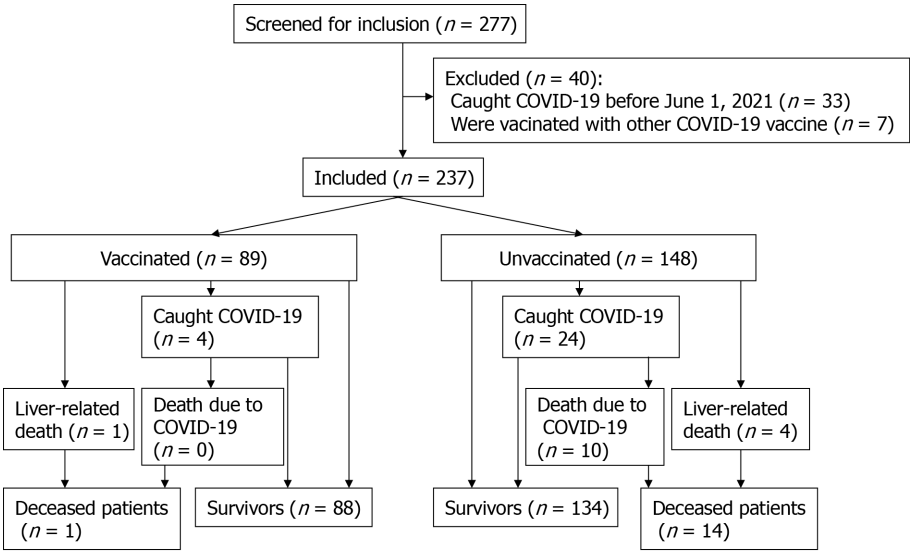

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Figure 1 CONSORT 2010 flow diagram. COVID-19: Coronavirus disease 2019.

High efficacy of vaccination was also observed in patients with cirrhosis of CTP classes B and C, which, taking into account the need for revaccination, was almost 80%.

Interestingly, the incidence of non-severe COVID-19 did not differ between the vaccinated and unvaccinated groups. Thus, vaccination protects against severe COVID-19 and death from this disease. The development of non-severe COVID-19 in vaccinated persons with cirrhosis is quite possible and should not be considered as an indicator of the ineffectiveness of vaccination.

Since COVID-19 is characterized by the development of thrombotic complications[16], there were concerns that vaccination against this infection could also contribute to their development, especially in persons with compromised hemostasis system which includes patients with cirrhosis[18]. Although a large study has shown that vaccination with certain types of vaccines is associated with an increased risk of developing thrombotic complications, this risk is negligible[19]. Therefore, one of the objectives of our study was to assess the risk of developing vascular thrombotic complications of vaccination in cirrhosis. In our study, the development of these complications was rare and their incidence did not differ significantly between vaccinated and unvaccinated patients.

The most discussed complications of COVID-19 vaccination are immune thrombotic thrombocytopenia[20] and myocarditis[21]. In our study, there were no cases of these complications, which, however, can be explained by the small number of included patients and the extremely rare reported incidence of these events[20-21].

We did not observe the onset of liver decompensation or bleeding esophageal varices associated with vaccination. The incidence of these events as well as mortality associated with complications of cirrhosis did not differ significantly between groups of vaccinated and unvaccinated patients.

Thus, we can state the excellent safety of Gam-COVID-Vac vaccination in patients with cirrhosis, including patients with CTP classes B and C cirrhosis.

Analyzing the overall mortality, we found that vaccination is an independent factor predicting the survival of patients with cirrhosis.

The need for revaccination should be emphasized. In our study, 2 out of 3 cases of COVID-19 in fully vaccinated patients were within 2 mo after 6 post-vaccination months, while there was only 1 this case within this six-month post-vaccination period.

The strength of our study is that it is the first to describe the efficacy and safety of vaccination against COVID-19 among patients with cirrhosis in the time of the delta variant dominance.

Although we tested only one vaccine in our study, we believe that the remaining major COVID-19 vaccines have a similar effect in patients with cirrhosis, as their efficacy was comparable in a recent national-level Hungarian study[14].

The limitation of our work is its retrospective nature. However, it is hardly possible to conduct randomized controlled trials on this topic in the pandemic. Another limitation is the fact that patients themselves decided whether they would be vaccinated or not, which can lead to selection bias. However, as shown in Table 1, the vaccinated and unvaccinated groups did not differ significantly in the main indicators.

CONCLUSION

Vaccination of patients with cirrhosis against COVID-19 with Gam-COVID-Vac is effective and safe. Revaccination should be carried out within the sixth month after the injection of the first dose of the vaccine.

ARTICLE HIGHLIGHTS

Research background

Patients with cirrhosis have a high risk of poor prognosis when developing novel coronavirus disease 2019 (COVID-19).

Research motivation

The clinical efficacy and safety of vaccination against the COVID-19 in patients with cirrhosis have not been evaluated yet.

Research objectives

To evaluate clinical efficacy and safety of vaccination against COVID-19 in patients with cirrhosis.

Research methods

This was a retrospective cohort study of patients with cirrhosis. The first cohort included patients vaccinated with Gam-COVID-Vac (Sputnik V); the second one consisted of unvaccinated controls.

Research results

There were 4 cases of COVID-19 in the vaccinated group and 24 cases in the unvaccinated group (P = 0.035). No severe cases of COVID-19 were revealed in the vaccinated group, while there were 12 ones in the unvaccinated group (P = 0.012) with 10 deaths detected (P = 0.012). The vaccine efficacy was 69.5% (95%CI: 18.5%-94.4%) against symptomatic cases of COVID-19, 100% (95%CI: 25.1%-100.0%) against severe cases, and 100% (95%CI: 1.6%-100.0%) against death associated with COVID-19. There was no significant difference in liver-related mortality, or the incidence of liver decompensation, bleeding esophageal varices, and vascular events between the two groups of patients.

Research conclusions

Vaccination against COVID-19 in patients with cirrhosis is effective and safe.

Research perspectives

The effectiveness of vaccinating patients with cirrhosis against COVID-19 with different vaccines should be compared.

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FOOTNOTES

Author contributions: Ivashkin V, Ismailova A, and Maslennikov R designed the research; all authors performed the research and analyzed the data; Ivashkin V, Ismailova A, Dmitrieva K, and Roman Maslennikov R wrote the paper.

Institutional review board statement: This study was approved by the Ethics Committee of Sechenov University (Protocol 20-11) in accordance with the Helsinki Declaration.

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ORIGINAL ARTICLE

Observational Study

Pre-sarcopenia and Mac-2 binding protein glycosylation isomer as predictors of recurrence and prognosis of early-stage hepatocellular carcinoma

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Abstract

BACKGROUND

The Mac-2 binding protein glycosylation isomer (M2BPGi), a fibrosis marker in various liver diseases, is reportedly a prognostic marker in patients with hepatocellular carcinoma (HCC) who underwent hepatectomy.

AIM

To evaluate whether the M2BPGi value, M2BP, and pre-sarcopenia before radiofrequency ablation (RFA) could be useful recurrence and prognostic markers in patients with early-stage HCC.

METHODS

In total, 160 patients with early-stage primary HCC treated with RFA were separately analyzed as hepatitis C virus (HCV)-positive and HCV-negative. Factors contributing to recurrence and liver-related death, including M2BP, M2BPGi, and skeletal muscle mass index, were statistically analyzed. Eighty-three patients were HCV-positive and 77 were HCV-negative.

RESULTS

In HCV-positive patients, only des-γ-carboxy-prothrombin ≥ 23 mAU/mL was a significant poor prognostic factor affecting survival after RFA. In HCV-negative patients, M2BPGi ≥ 1.86 cutoff index was significantly associated with tumor recurrence, while M2BP was not. M2BPGi ≥ 1.86 cutoff index (hazard ratio, 4.89; 95% confidence interval: 1.97-12.18; $P < 0.001$) and pre-sarcopenia (hazard ratio,

3.34, 95% confidence interval: 1.19-9.37; P = 0.022) were independent significant poor prognostic factors in HCV-negative patients.

CONCLUSION

In HCV-negative patients with primary HCC treated with RFA, lower M2BPGi contributed to a lower tumor recurrence rate and longer survival period. Pre-sarcopenia contributed to the poor prognosis independently in HCV-negative patients. These factors might be useful recurrence and prognostic markers for early-stage primary HCC.

Key Words: Mac-2 binding protein; Mac-2 binding protein glycosylation isomer; Pre-sarcopenia; Primary hepatocellular carcinoma; Radiofrequency ablation

Core Tip: Hepatocellular carcinoma (HCC) is prone to recurrence, even if cured at an early stage. Pre-sarcopenia is a poor prognostic factor in the elderly population. The usefulness of the Mac-2 binding protein glycosylation isomer (M2BPGi) to treat HCC has recently attracted attention. In this study, we investigated the recurrence and prognostic factors in patients who underwent radiofrequency ablation for early-stage HCC. Based on our data, pre-sarcopenia and higher M2BPGi, but not M2BP, were useful predictors of the recurrence and poor prognosis of early-stage primary HCC in hepatitis C virus-negative patients.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is an important health problem affecting approximately 900000 new cancer cases worldwide. In 2020, > 800000 people died from HCC, accounting for approximately 8.3% of cancer deaths[1]. HCC often results from cirrhosis or chronic liver injury caused by background diseases, such as hepatitis C virus (HCV), hepatitis B virus (HBV), primary biliary cholangitis (PBC), autoimmune hepatitis (AIH), alcoholic liver disease, and nonalcoholic steatohepatitis (NASH). In the last 25 years, treatment for viral hepatitis has made great strides. Notably, HCV can be eliminated in almost all cases using direct-acting antivirals (DAAs). Although HBV is still an important risk factor that accounts for approximately 50% of the causes of HCC, the proportion of non-viral liver diseases, especially steatohepatitis, as the causative disease of HCC is increasing[2].

HCC is prone to recurrence, even if cured at an early stage. In the Barcelona Clinic Liver Cancer (BCLC) staging system[3-5], which is widely used in the treatment of HCC, early-stage HCC is classified as stage 0 or A. BCLC stage 0 is defined as very early stage, for single nodule ≤ 2 cm, Child-Pugh A, Eastern Cooperative Oncology Group Performance status (PS) 0. BCLC stage A is defined as the early stage and is the case of maximum tumor diameter ≤ 3 cm, number of tumors ≤ 3, Child-Pugh A-B, and PS 0. Liver transplantation is considered in some unresectable cases of stage A disease, but resection and ablation are often recommended as curative treatments. In recent years, a median overall survival > 6 years has been expected for early-stage liver cancer patients undergoing BCLC-0 of A liver resection and ablation[6]. However, even in the case of liver resection for early-stage HCC, the prognosis is poor in cases of portal hypertension[7,8].

Radiofrequency ablation (RFA) is the most widely used local therapy for HCC treatment. It has been reported that the 4-year local recurrence rate after RFA in the early stage is approximately 5%-10% and the 5-year survival rate is approximately 70%[9-13]. However, it has been reported that cases with impaired liver function and/or bad tumor conditions (large tumor diameter and large number of tumors) have a poor prognosis[13].

In recent years, many studies have demonstrated that sarcopenia is a poor prognostic factor in patients with chronic liver disease and HCC, because it is related to frailty, loss of function, and low quality of life. Sarcopenia is diagnosed using both muscle power loss and muscle volume loss according to the Japan Society of Hepatology (JSH) diagnostic guidelines or European diagnostic guidelines[14-16]. Pre-sarcopenia is defined as muscle volume loss without muscle power loss, and has been reported

to be a poor prognostic factor in the elderly population[17].

In addition, the usefulness of the Mac-2 binding protein glycosylation isomer (M2BPGi), or Wisteria floribunda agglutinin (WFA)-positive M2BP, which was first reported as a fibrosis marker in HCV patients, to treat HCC has recently attracted attention[18]. M2BPGi is a serum marker predicting fibrosis in HCV and other liver diseases, such as HBV, AIH, PBC, and NASH[19-22]. It is also a useful predictor of HCC in various liver diseases[23-27].

In this study, we investigated the usefulness of pre-sarcopenia, M2BPGi, and M2BP as recurrence and prognostic factors in patients who underwent RFA for early-stage HCC.

MATERIALS AND METHODS

Patients and data collection

A total of 202 patients underwent RFA for primary HCC between 2001 and 2017 at Hokkaido University Hospital, 160 of whom were diagnosed with BCLC stage 0 or A and followed up > 6 mo after RFA. Patients with HCV-RNA positive were classified to "HCV-positive" group and HCV-RNA negative were classified to "HCV-negative" group. Blood chemistry data, tumor factors (tumor number, size, and form), and clinical symptoms including ascites, pleural effusion, and hepatic encephalopathy were obtained before RFA.

Percutaneous RFA procedure

Percutaneous RFA was performed using a cooled-tip electrode (Cool-Tip; Ablation Systems, Covidien, Boulder, Colombia, CO) after ultrasonography (US) planning. RFA was performed by experienced operators under real-time ultrasound guidance. In some cases, we used a contrast-enhanced US technique or a real-time visual support system to detect the tumor more clearly. Moreover, in some cases, artificial ascites or pleural fluid can prevent thermal injury to extrahepatic organs or avoid the lungs in the tracking line. The ablation time, including three occurrences of roll-off, was 3-12 min. The ablated lesion and ablative margin were assessed using dynamic computed tomography (CT) or magnetic resonance imaging (MRI) 1-4 d after RFA.

Follow up and definition of recurrence of HCC

Because of the early detection of local and distant recurrence, the first imaging test (dynamic CT or MRI) after RFA was performed 4-8 wk after RFA. After the initial evaluation, follow-up by imaging (dynamic CT or MRI) and serum tumor markers such as alpha-fetoprotein (AFP), lens culinaris agglutinin-A reactive AFP isoform (AFP-L3), and des-γ-carboxy-prothrombin (DCP) were performed every 3-4 mo. Chest CT was regularly performed to detect distant metastases.

The treatment for recurrence and the definition of deaths

For HCC recurrence, appropriate treatment was performed according to liver cancer treatment guidelines[28-31]. Deaths due to liver cancer, liver failure (including acute or chronic liver failure), hemorrhage due to gastroesophageal varices, and infections associated with spontaneous bacterial peritonitis were defined as liver-related deaths. Deaths other than liver diseases, such as other organ cancers, ischemic heart disease, and pneumonia, were analyzed as survival sensors.

The diagnosis of pre-sarcopenia

Pre-sarcopenia was assessed according to the sarcopenia assessment criteria of the JSH guidelines for sarcopenia in liver disease[14]. Skeletal muscle mass index (SMI) calculated using simple methods[14, 16]. In particular, the left-right sum of the long axis times the short axis of the iliopsoas muscles at the level of the third lumbar vertebra (L3) divided by height squared. This method has been reported to correlate well with SMI calculated using a muscle mass measurement software.

Measurement of M2BPGi and M2BP

M2BPGi levels were measured in the conserved serum before RFA and at 1 mo after RFA. M2BPGi detection was based on a lectin-antibody sandwich immunoassay (Sysmex Co., Kobe, Japan) and expressed as a cutoff index (COI), with a range of 0.1-20 COI as previously reported[18].

M2BP was measured in conserved serum using enzyme-linked immunosorbent assay methods (Human Mac-2 binding protein (Mac-2bp) Assay Kit, Immuno-Biological Laboratories Co., Ltd., Fujioka, Japan).

Statistical analysis

Statistical analyses were performed using EZR software[32]. The Mann-Whitney U test was used to analyze continuous variables. Fisher's exact test was used for univariate analysis of ordered variables. The Kaplan-Meier method was used to determine recurrence and survival rates, and the log-rank test was used to analyze differences. The median value was used as the cutoff. For the multivariate analysis

of factors related to recurrence and survival, Cox proportional hazards models with stepwise methods using *P* value were used.

Ethical considerations

The study protocol was approved by the Institutional Ethics Committee of Hokkaido University (IRB-No. 015-1412) and conformed to the ethical guidelines of the Declaration of Helsinki.

RESULTS

Patient characteristics

As shown in Figure 1, 202 patients underwent RFA for primary HCCs. Of these, 160 cases were classified as BCLC stage 0 or A, and the data were analyzed. Eighty-three patients were classified into the HCV-positive group, and 77 patients were classified into the HCV-negative group. The ratio of older age and Child-Pugh Grade B was higher in the HCV-positive group than in the HCV-negative group. Serum transaminase and fibrosis-4 (FIB-4) index were higher in the HCV-positive group than in the HCV-negative group. In addition, the serum AFP and AFP-L3 levels were higher in the HCV-positive group. The median tumor diameter and number were not significantly different; however, they tended to be larger in the HCV-positive group than in the HCV-negative group. In contrast, the SMI of the HCV-positive group was significantly lower than that of the HCV-negative group (Table 1).

M2BP and M2BPGi values in HCV-positive and -negative patients

In the HCV-positive group, the median M2BP was 5385 ng/mL and that of M2BPGi was 4.94 COI. On the other hand, in the HCV-negative group, the median M2BP was 2745 ng/mL and that of M2BPGi was 1.86 COI. M2BP and M2BPGi levels were significantly higher in the HCV-positive group than in the negative group (Figure 2). Therefore, we used the median as the cutoff value in the following analysis for each group.

M2BPGi, not M2BP, is the risk factor of recurrence in HCV-negative patients

Next, we examined whether M2BP and M2BPGi could be predictive factors for HCC recurrence in primary HCC patients with BCLC stage 0 or A. M2BP could not be a predictive factor for HCC recurrence in each group, but M2BPGi could be a clinical predictor for HCC recurrence only in the HCV-negative group (Figure 3). Therefore, it is suggested that M2BPGi, but not M2BP, is a predictive factor for HCC recurrence in patients without current HCV infection.

Higher M2BPGi levels and pre-sarcopenia are risk factors for liver-related death in HCV-negative patients

For further analysis, we examined whether M2BP and M2BPGi could be predictive factors of liver-related death in BCLC stage 0 or A. In the HCV-positive group, older age (≥ 70 years), albumin-bilirubin (ALBI) grade 2 or 3, DCP ≥ 23 mAU/L, and AFP-L3 ≥ 10% were factors contributing to a negative effect on survival on univariate analysis. Only DCP ≥ 23 mAU/L was a factor contributing to a negative effect on survival on multivariate analysis, and higher M2BP and M2BPGi were not significant factors for a negative effect on survival in the HCV-positive group (Table 2). In contrast, in the HCV-negative group, M2BPGi ≥ 1.86 COI and pre-sarcopenia were significant factors contributing to a negative effect on survival (Table 3). In the HCV-negative patient group, M2BPGi, but not M2BP, was a poor prognostic factor (Figure 4). Similarly, pre-sarcopenia was a poor prognostic factor only in the HCV-negative group (Figure 5). Therefore, higher M2BPGi and pre-sarcopenia were poor prognostic factors in patients without active HCV infection.

DISCUSSION

In this study, we retrospectively analyzed the prognostic factors of early-stage HCC (BCLC stage 0-A) after RFA treatment. Here, we investigated the usefulness of M2BGi and M2BP as predictors of HCC recurrence and prognosis. As a result, M2BPGi and pre-sarcopenia were useful in HCC recurrence and as prognostic factors in patients without current HCV infection.

Many randomized controlled trials[11,33-40] have compared the treatment outcomes of hepatectomy and RFA for early-stage HCC, but there are few reports with high quality evidence[11,39,40]. In recent years, Ng *et al*[11] reported no statistically significant difference in recurrence-free survival between hepatectomy and RFA in 109 cases. In the SURF trial, hepatectomy and RFA for HCC with a Child-Pugh score ≤ 7, tumor diameter ≤ 3 cm, and tumor number ≤ 3 had equivalent recurrence-free survival[39]. Based on the above, RFA has almost the same therapeutic results as hepatectomy for BCLC stage 0/A HCC. Considering that RFA is less invasive than hepatectomy, it is expected to become a standard

Table 1 Patient characteristics

	HCV-positive (*n* = 83)	HCV-negative (*n* = 77)	*P* value
Sex (male/female)	45/38	50/27	0.20
Age (years)[1]	70 (44-90)	64 (41-88)	< 0.01
Tumor factors			
Tumor number (solitary/multiple)	63/20	68/9	0.07
Tumor size (mm)[1]	17 (8-30)	15 (6-30)	0.05
Tumor form (only boundary/others)	67/16	68/9	0.20
Stage (LCSG) (I/II/III)	39/38/6	45/27/5	0.35
Liver function			
Child-Pugh Score (5-6/7-9)	66/17	66/11	< 0.01
ALBI grade (1/2-3)	27/56	43/34	0.41
Blood data			
Platelet ($\times 10^4/\mu L$)[1]	10.2 (2.7-43.7)	11.8 (3.7-36.8)	0.04
AST (U/L)[1]	56 (18-139)	39 (16-100)	< 0.01
ALT (U/L)[1]	49 (12-155)	30 (9-87)	< 0.01
FIB-4 index[1]	5.90 (0.96-37.86)	3.61 (0.88-14.16)	< 0.01
APRI[1]	2.00 (0.15-15.06)	1.08 (0.28-4.32)	< 0.01
PT (%)[1]	84.6 (48.6-125.0)	81.3 (51.8-117.1)	0.51
Total bilirubin (mg/dL)[1]	0.9 (0.2-2.8)	0.9 (0.4-2.7)	0.56
Albumin (g/dL)[1]	3.7 (2.2-4.7)	4.0 (2.4-5.0)	< 0.01
AFP (ng/mL)[1]	17.4 (3.0-621.6)	6.4 (1.3-1962.9)	< 0.01
DCP (mAU/mL)[1]	23 (4-1086)	22 (7-6308)	0.66
AFP-L3 (%)[1]	5.1 (< 0.5-69.1)	< 0.5 (< 0.5-85.6)	0.03
M2BPGi (COI)[1]	4.94 (0.78-17.81)	1.86 (0.36-10.23)	< 0.01
M2BP (ng/mL)[1]	5385 (1460-22770)	2745 (865-12150)	< 0.01
SMI (cm^2/m^2)[1]	5.28 (2.62-11.75)	6.51 (2.58-10.89)	< 0.01
Pre-sarcopenia, *n* (%)	21 (25.3)	14 (18.2)	0.34
Observation period (mo)[1]	46 (6-157)	56 (6-185)	0.19

[1]Median (range).

APRI (AST to platelet ratio index) = AST/platelet. FIB4 index = (age × AST)/(platelet × alanine aminotransferase × 0.5). HCV: hepatitis C virus; LCSG: liver cancer study group; ALBI: albumin-bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; PT: prothrombin time; AFP: alfa-fetoprotein; DCP: des-γ-carboxyprothrombin; M2BPGi: Mac-2 binding protein glycosylation isomer; M2BP: Mac-2 binding protein; SMI: skeletal muscle mass index.

treatment.

However, it has been reported that local recurrence is observed in approximately 10% of cases in which a sufficient ablation area is obtained by RFA[41,42]. The risk factors for recurrence have also been reported. Shiina *et al*[13] reported that, in a large number of cases, a higher DCP was associated with local recurrence. Ectopic recurrence is associated with HCV positivity, Child-Pugh grade B or C, platelet counts ≤ 100000, higher AFP, higher DCP, large tumor diameter, and a large number of tumors. Thus, regarding the recurrence of HCC after RFA, not only tumor factors but also factors related to liver function are largely involved. Contrarily, factors related to survival after RFA including younger age, lack of portosystemic shunt, Child-Pugh grade A, lower bilirubin, lower ALBI score, higher albumin, higher prothrombin time, lower AFP, HBV positivity, lower neutrophil to lymphocyte ratio, small tumor diameter, and low tumor number have been reported in a meta-analysis[43]. Therefore, liver function and pretreatment tumor factors are considered important factors not only for recurrence but also for survival.

Table 2 Factors contributing to survival in hepatitis C virus-positive patients

Subject	Univariate	Multivariate		
	P value	HR	95%CI	*P* value
Age (< 70/≥ 70 years)	0.08	1.92	0.94-3.94	0.074
Sex (Female/Male)	0.13			
ALBI grade (1/2,3)	0.06	1.81	0.84-3.90	0.129
Child-Pugh Score (5-6/7-15)	0.15			
Stage (LCSG) (I/II+III)	0.47			
Tumor number (solitary/multiple)	0.97			
Tumor form (only boundary/others)	0.43			
Tumor size (< 20 mm/≥ 20 mm)	0.54			
AFP (< 17.2/≥ 17.2 ng/mL)	0.12			
DCP (< 23/≥ 23 mAU/mL)	< 0.01	2.54	1.23-5.23	0.012
AFP-L3 (< 10/≥ 10%)	0.02	1.72	0.80-3.71	0.167
M2BPGi (< 4.94/≥ 4.94 COI)	0.26			
M2BP (< 5385/≥ 5385 ng/mL)	0.24			
APRI (< 2.0/≥ 2.0)	0.58			
FIB-4 index (< 4.5/≥ 4.5)	0.31			
Pre-sarcopenia (No/Yes)	0.28			

APRI (AST to platelet ratio index) = AST/platelet. FIB4 index = (age × AST)/(platelet × alanine aminotransferase × 0.5). HR: hazard ratio; CI: confidence interval; ALBI: albumin-bilirubin; LCSG: liver cancer study group; AST: aspartate aminotransferase; ALT: alanine aminotransferase; PT: prothrombin time; AFP: alfa-fetoprotein; DCP: des-γ-carboxyprothrombin; M2BPGi: Mac-2 binding protein glycosylation isomer; COI: cutoff index; M2BP: Mac-2 binding protein.

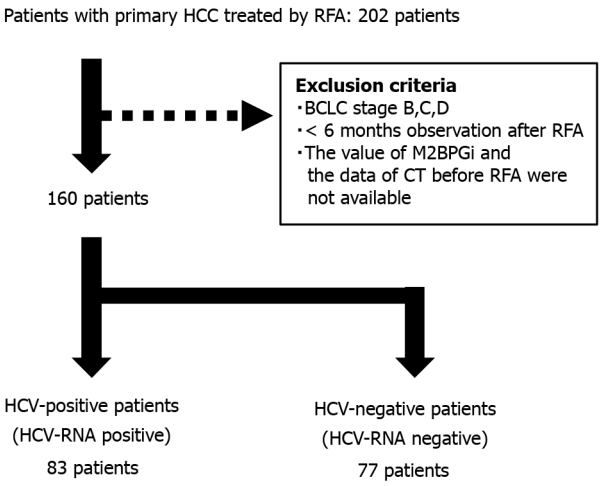

Figure 1 Patients' flow. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; BCLC: Barcelona Clinic Liver Cancer; CT: Computed tomography.

In this study, we focused on M2BPGi and muscle mass, which are not direct tumor factors and liver function. M2BP is a secreted glycoprotein of approximately 90 kDa that was originally reported as a ligand for galectin[44]. The serum concentration of M2BP has been reported to increase in various cancers, such as breast and lung cancers[45]. Furthermore, Kamada *et al*[46] reported its usefulness as a marker of liver fibrosis in patients with non-alcoholic fatty liver disease. M2BPGi has a sugar chain with an affinity for WFA and distinguishes the glycan structure of WFA-detectable M2BP. The usefulness of M2BPGi as a marker of liver fibrosis in patients with HCV infection was reported in 2013[18]. M2BPGi has also been reported to be useful as a fibrosis marker in various liver diseases[19-22]. However, the

Table 3 Factors contributing to survival in hepatitis C virus-negative patients

Subject	Univariate	Multivariate		
	P value	HR	95%CI	*P* value
Age (< 65/≥ 65)	0.03	-		
Sex (Female/Male)	0.88			
ALBI grade (1/2,3)	< 0.01	2.41	0.81-7.12	0.115
Child-Pugh Score (5-6/7-15)	< 0.01	-		
Stage (LCSG) (I/II+III)	0.91			
Tumor number (solitary/multiple)	0.54			
Tumor form (boundary/others)	0.11			
Tumor size (< 20 mm/≥ 20 mm)	0.74			
AFP (< 6.4/≥ 6.4 ng/mL)	0.64			
DCP (< 22/≥ 22 mAU/mL)	0.23			
AFP-L3 (< 10/≥ 10%)	0.29			
M2BPGi (< 1.86/≥ 1.86 COI)	< 0.01	4.89	1.97-12.18	< 0.001
M2BP (< 2745/≥ 2745 ng/mL)	0.92			
APRI (< 1.5/≥ 1.5)	0.04	-		
FIB-4 index (< 3.6/≥ 3.6)	< 0.01	1.86	0.63-5.44	0.257
Pre-sarcopenia (no/yes)	0.04	3.34	1.19-9.37	0.022

APRI (AST to platelet ratio index) = AST/platelet. FIB4 index = (age × AST)/(platelet × alanine aminotransferase × 0.5). HR: hazard ratio; CI: confidence interval; ALBI: albumin-bilirubin; LCSG: liver cancer study group; AST: aspartate aminotransferase; ALT: alanine aminotransferase; PT: prothrombin time; AFP: alfa-fetoprotein; DCP: des-γ-carboxyprothrombin; M2BPGi: Mac-2 binding protein glycosylation isomer; COI: cutoff index; M2BP: Mac-2 binding protein.

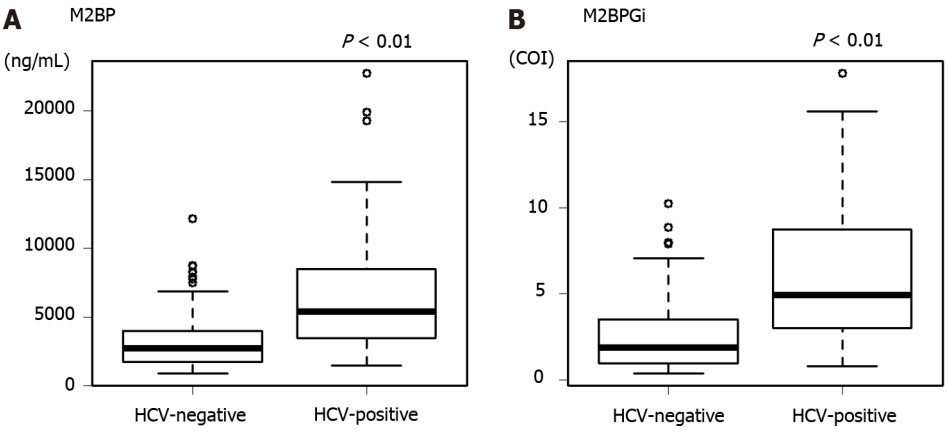

Figure 2 The value of Mac-2 binding protein and Mac-2 binding protein glycosylation isomer in hepatitis C virus-positive and -negative patients. A: The values of Mac-2 binding protein in hepatitis C virus (HCV)-negative and -positive groups; B: The values of Mac-2 binding protein glycosylation isomer in HCV-negative and -positive groups. The box charts for the Y-axis indicate the median as TextTitle lines in the boxes, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as lines for each edge. HCV: Hepatitis C virus; M2BPGi: Mac-2 binding protein; M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cutoff index.

M2BPGi value differs depending on the background liver disease, and it has been reported that the predicted cutoff value of METAVIR scoring system in the F4 stage is 5.2 COI for HCV, 3.1 COI for HBV, and 0.91 COI for NASH[47,48]. M2BPGi is an interferon (IFN)-simulated protein, and the amount of M2BPGi decreases after HCV eradication[49]. Therefore, it is suggested that M2BPGi is high in patients currently infected with HCV, even with the same degree of liver fibrosis. In this study, the median values differed significantly between the HCV-positive and HCV-negative patients. The M2BPGi levels were significantly higher in HCV-positive patients than in HCV-negative patients (Figure 2). Therefore,

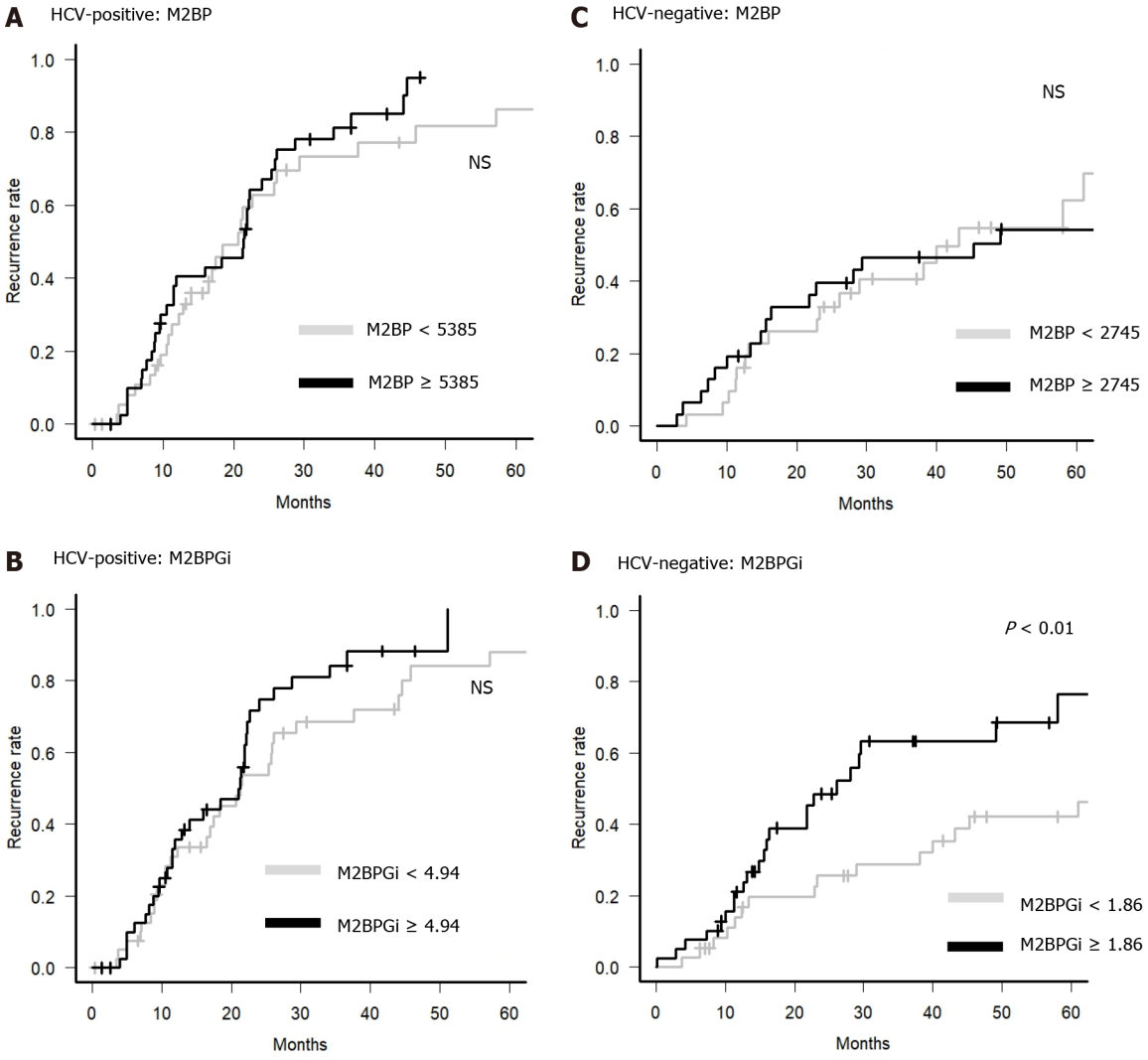

Figure 3 Recurrence rate according to the value of Mac-2 binding protein and Mac-2 binding protein glycosylation isomer. The hepatocellular carcinoma recurrence rate was divided into two groups according to the value of Mac-2 binding protein (M2BP) or M2BP glycosylation isomer (M2BPGi) before radiofrequency ablation. A: Hepatitis C virus (HCV)-positive group divided by M2BP value. The gray line indicates patients with M2BP < 5385 ng/mL, and the black line indicates patients with M2BP ≥ 5385 ng/mL; B: HCV-positive group divided by M2BPGi value. The gray line indicates patients with M2BPGi < 4.94 cutoff index (COI), and the black line indicates patients with M2BPGi ≥ 4.94 COI; C: HCV-negative group divided by M2BP value. The gray line indicates patients with M2B*P* < 2745 ng/mL, and the black line indicates patients with M2BP ≥ 2745 ng/mL; D: HCV-negative group divided by M2BPGi value. The gray line indicates patients with M2BP < 1.86 COI, and the black line indicates patients with M2BPGi ≥ 1.86 COI. HCV: Hepatitis C virus; M2BPGi: Mac-2 binding protein; M2BPGi: Mac-2 binding protein glycosylation isomer; NS: No significance.

we analyzed the M2BPGi values separately in HCV-positive and HCV-negative patients.

M2BPGi has also been reported as a useful marker for predicting the occurrence of HCC. Specifically, it has been reported as a marker for predicting HCC in HCV, HBV and post-HCV eradication cases[19,23,25,27,49-57]. In this study, M2BPGi significantly predicted recurrence in HCV-negative cases. In contrast, M2BP level was not be a predictor of recurrence. Progression of liver fibrosis is a risk factor for HCC. As M2BPGi reflects liver fibrosis, M2BPGi may be indirectly associated with the development of HCC. M2BPGi may show higher levels in HCV cases than in others, even at similar levels of liver fibrosis. This is because the inflammation caused by the current HCV infection might affect the M2BPGi value in the HCV-positive group. Therefore, predicting HCC recurrence may be difficult using the value of M2BPGi only in HCV-positive cases. Based on the results of this study, prediction of cases at a high risk for recurrence after RFA was possible in early-stage HCC by focusing on the value of M2BPGi in HCV-negative patients.

Furthermore, M2BPGi has been reported to be a useful marker for predicting the prognosis of patients after HCV eradication, hepatectomy, and transcatheter arterial chemoembolization[25,58,59]. In this study, we analyzed prognostic factors after RFA for early-stage HCC, focusing on M2BP, M2BPGi, and pre-sarcopenia. In HCV-positive cases, DCP that is one of the serum tumor markers of HCC was a significant poor prognosis factor. In contrast, in HCV-negative cases, M2BPGi and pre-sarcopenia were significant poor prognostic factors, but tumor factors (tumor number, size, form, and serum markers) were not. In addition, M2BP was not a prognostic predictor in either group. M2BPGi levels are affected

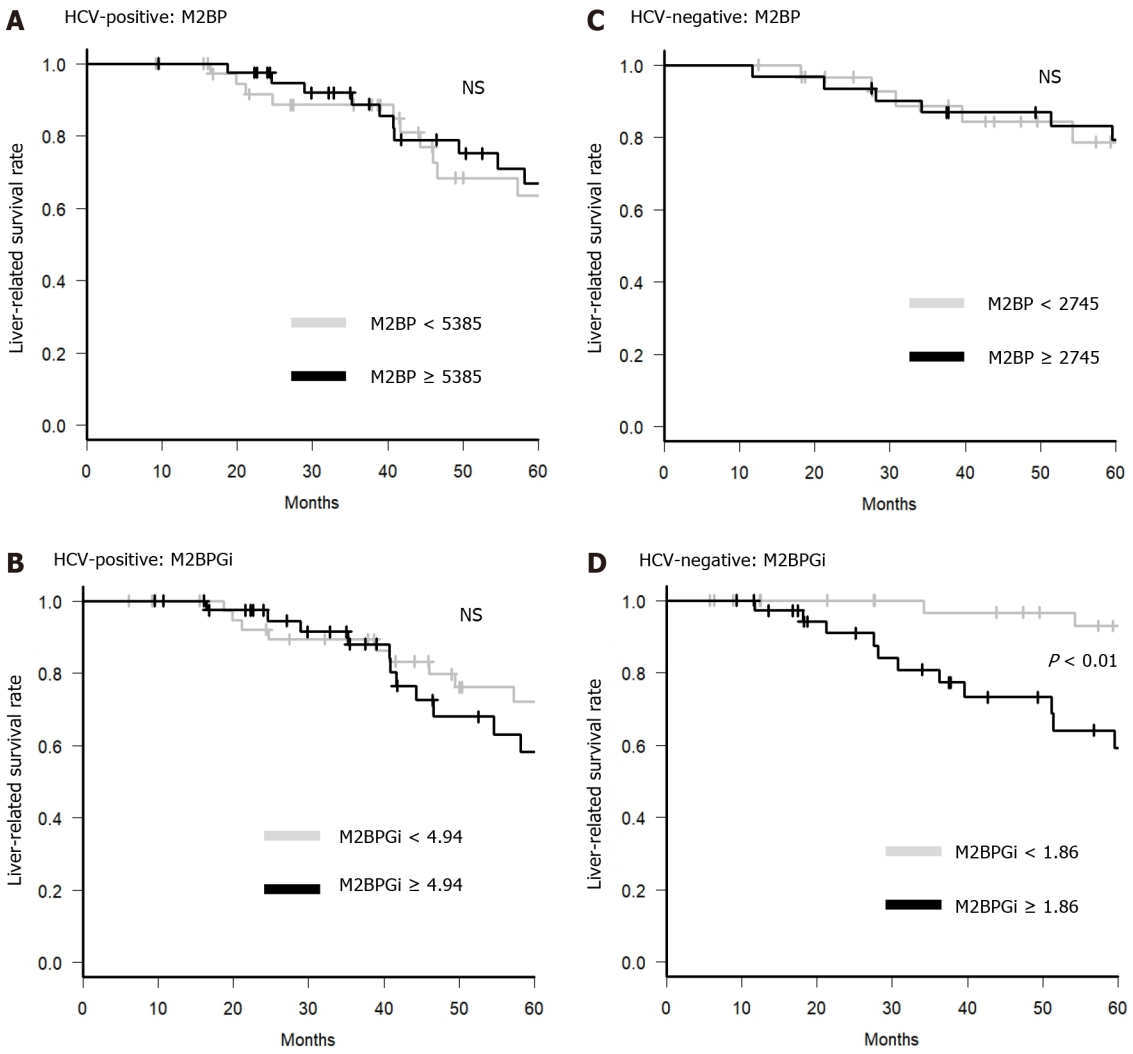

Figure 4 Survival rate according to the value of Mac-2 binding protein and Mac-2 binding protein glycosylation isomer. The liver disease-related death-free survival rate was divided into two groups according to the value of Mac-2 binding protein (M2BP) or M2BP glycosylation isomer (M2BPGi) before radiofrequency ablation. A: Hepatitis C virus (HCV)-positive patients divided by M2BP value. The gray line indicates patients with M2BP < 5385 ng/mL, and the black line indicates patients with M2BP ≥ 5385 ng/mL; B: HCV-positive patients divided by M2BPGi value. The gray line indicates patients with M2BPGi < 4.94 cutoff index (COI), and the black line indicates patients with M2BPGi ≥ 4.94 COI; C: HCV-negative patients divided by M2BP value. The gray line indicates patients with M2B*P* < 2745 ng/mL, and the black line indicates patients with M2BP ≥ 2745 ng/mL; D: HCV-negative patients divided by M2BPGi value. The gray line indicates patients with M2BPGi < 1.86 COI, and the black line indicates patients with M2BPGi ≥ 1.86 COI. HCV: Hepatitis C virus; M2BPGi: Mac-2 binding protein; M2BPGi: Mac-2 binding protein glycosylation isomer; NS: No significance.

by various factors, including acute liver failure, and are associated with liver inflammation, damage, and hepatocyte degeneration[60]. Furthermore, M2BPGi was reported to correlate with inflammatory cytokines and was reduced by steroid treatment in patients with autoimmune hepatitis[61]. In HCV-negative cases, high M2BPGi levels may indicate advanced fibrosis or coexistence of inflammation because these cases are not affected by HCV. Therefore, M2BPGi may be a predictor of liver-related death. Notably, M2BPGi was a more sensitive prognostic marker than other liver function or fibrosis markers such as ALBI and FIB-4 in HCV-negative patients. Thus, M2BPGi may be a marker that can predict poor prognosis, including the effects of other factors, such as inflammation and liver fibrosis.

Patients with chronic liver disease and sarcopenia have a significantly poorer prognosis[62]. Furthermore, it has been reported that in the elderly, pre-sarcopenia cases have a poorer prognosis than non-sarcopenia cases[17]. In this study, pre-sarcopenia was a significant poor prognostic factor in HCV-negative cases but was not a significant prognostic factor in HCV-positive cases. The reason for this might be related to the fact that HCV-positive patients had significantly less SMI than the HCV RNA-negative patient group (Table 1). Because muscle volume increases after IFN-free treatment in HCV-positive patients and HCV elimination suppresses pre-sarcopenia, the current HCV infection itself may contribute to pre-sarcopenia. In this study, the high proportion of cases of pre-sarcopenia and the elderly may have affected the observation that pre-sarcopenia was not a significant prognostic factor in HCV-positive cases[63,64].

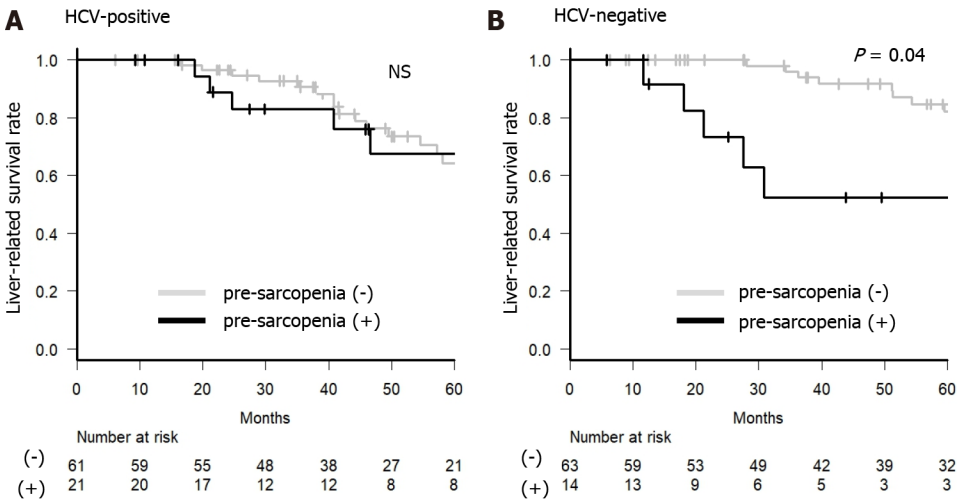

Figure 5 Survival rate with or without pre-sarcopenia. The liver disease-related survival rate was divided into two groups according to the presence of pre-sarcopenia before radiofrequency ablation. A: Hepatitis C virus (HCV)-positive group; B: HCV-negative group. The gray line indicates patients without pre-sarcopenia, and the black line indicates patients with pre-sarcopenia. The numbers under each group indicate the number at risk for each group. HCV: Hepatitis C virus; M2BPGi: Mac-2 binding protein; M2BPGi: Mac-2 binding protein glycosylation isomer; NS: No significance.

This study has several limitations. First, it was a retrospective observational study involving a single hospital and a small number of patients. Second, SMI was evaluated using only the simple CT method. Further studies with larger patient numbers and multicenter evaluations are needed.

CONCLUSION

In the near future, almost all HCVs will be eradicated by DAA treatment. Henceforth, almost no HCC cases were derived from the current HCV infection. In this study, we investigated the predictive factors of survival after RFA for HCC in BCLC stage 0 or A patients divided into two groups: HCV-RNA positive and negative. Pre-sarcopenia and M2BPGi, but not M2BP, might be useful tools for the prediction of survival in early-stage HCC in the era of HCV eradication.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is prone to recurrence, even if cured at an early stage. In recent years, many studies have demonstrated that sarcopenia is a poor prognostic factor in patients with chronic liver disease and HCC, because it is related to frailty, loss of function, and low quality of life. Pre-sarcopenia is defined as muscle volume loss without muscle power loss and is a poor prognostic factor in the elderly population. In addition, the usefulness of the Mac-2 binding protein glycosylation isomer (M2BPGi), or Wisteria floribunda agglutinin-positive M2BP, which was first reported as a fibrosis marker in hepatitis C virus (HCV) patients, to treat HCC has recently attracted attention.

Research motivation

The M2BPGi, a fibrosis marker in various liver diseases, is reportedly a prognostic marker in patients with HCC who underwent hepatectomy. In recent years, many studies have demonstrated that sarcopenia is a poor prognostic factor in patients with chronic liver disease and HCC, because it is related to frailty, loss of function, and low quality of life. Sarcopenia is diagnosed using both muscle power loss and muscle volume loss. Pre-sarcopenia is defined as muscle volume loss without muscle power loss and is a poor prognostic factor in the elderly population.

Research objectives

To investigate the usefulness of pre-sarcopenia, M2BPGi, and M2BP as recurrence and prognostic factors in patients who underwent RFA for early-stage HCC.

Research methods

In this study, 202 patients underwent radiofrequency ablation (RFA) for primary HCCs. Of these, 160

cases were classified as BCLC stage 0 or A, and the data were analyzed. Eighty-three patients were classified into the HCV-positive group, and 77 patients were classified into the HCV-negative group.

Research results

In HCV-positive patients, only des-γ-carboxy-prothrombin (DCP) ≥ 23 mAU/mL was a significant poor prognostic factor affecting survival after RFA. In HCV-negative patients, M2BPGi ≥ 1.86 cutoff index was significantly associated with tumor recurrence, but M2BP was not. M2BPGi ≥ 1.86 cutoff index (hazard ratio, 4.89; 95% confidence interval: 1.97-12.18; $P < 0.001$) and pre-sarcopenia (hazard ratio, 3.34, 95% confidence interval: 1.19-9.37; $P = 0.022$) were independent significant poor prognostic factors in HCV-negative patients.

Research conclusions

In HCV-negative patients with primary HCC treated with RFA, lower M2BPGi contributed to a lower tumor recurrence rate and longer survival period. Pre-sarcopenia contributed to the poor prognosis independently in HCV-negative patients.

Research perspectives

In the near future, almost all HCVs will be eradicated by DAA treatment. Almost no HCC cases were derived from the current HCV infection. Pre-sarcopenia and M2BPGi, but not M2BP, might be useful tools to predict survival in early-stage HCC in the era of HCV eradication.

FOOTNOTES

Author contributions: Nakai M and Morikawa K planned the contents of manuscript; Nakai M, Morikawa K, Hosoda S, Yoshida S, Kubo A, Tokuchi Y, Kitagataya T, Yamada R, Ohara M, Sho T, Suda G, Ogawa K, and Sakamoto N collected the data; Nakai M and Morikawa K analyzed the data and drafted the manuscript; all authors revised the manuscript.

Institutional review board statement: The study protocol was approved by the Institutional Ethics Committee of Hokkaido University (IRB no. 015-1412) and conformed to the ethical guidelines of the Declaration of Helsinki.

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

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

Data sharing statement: No additional data are available.

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

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Hepatitis C virus burden: Treating and educating people without prejudice

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Abstract

BACKGROUND

Hepatitis C virus (HCV) infection has a worldwide incidence of 1.1%. In Italy, 60% of people who inject drugs (PWIDs) and are receiving assistance for substance use disorder are infected with HCV. However, this subset of patients has extremely limited access to care due to multiple factors, including alcohol abuse, psychological comorbidities, and homeless status.

AIM

To describe the impact of our HCV-dedicated service for substance use disorder (SSUD) service on PWIDs receiving anti-HCV therapy.

METHODS

A dedicated, multidisciplinary team was set up at the SSUD of Trento in October 2020 to provide antiviral treatment to HCV RiboNucleic Acid-positive patients with an active or previous history of substance abuse. The treatment was followed by a health education program. Patients were treated with Direct-Acting Antivirals (DAAs). Data were retrospectively analyzed to assess the efficacy of our dedicated program in terms of therapy completion, HCV eradication, and

compliance (primary endpoint). The rate of HCV reinfection and DAA-related toxicity were also assessed (secondary endpoints).

RESULTS

A total of 40 patients were enrolled in the study: 28 (70.0%) were treated with Sofosbuvir/Velpatasvir, while 12 (30.0%) received Glecaprevir/Pibrentasvir. At the time of inclusion in the study, 36 patients were receiving opioid agonist maintenance therapy, whilst another 4 had just finished the treatment. 37.5% had a history of alcoholism and 42.5% received concomitant psychiatric treatment. All 40 patients (100.0%) completed the therapy cycle and 92.5% of patients adhered to the program. All patients tested negative for viral load at the end of the treatment. There were no significant drug interactions with common psychiatric treatments and no side effects were observed. The sustained virological response was achieved in 92.5% of cases with good tolerability, although two patients discontinued treatment temporarily. After HCV eradication, one patient died from an overdose, another from complications of cirrhosis, and one reinfection occurred.

CONCLUSION

Very high adherence to therapy and good tolerability was observed in our series of HCV patients treated at the SSUD, regardless of the substance abuse condition. Further validation in a larger population is required.

Key Words: Hepatitis C virus; Service for substance use disorder; Direct-acting antivirals; Sustained virologic response; Compliance; Tolerability

Core Tip: Hepatitis C virus (HCV) infection has an incidence of 1.1%, reaching 60% in Italy among people who inject drugs. This paper reports the impact of our HCV-dedicated program to provide antiviral treatment to HCV patients with a history of substance abuse. 40 patients were treated with direct-acting antivirals: 38 were receiving opioid agonist maintenance therapy, and 4 had just finished this treatment. 37.5% had a history of alcoholism, and 42.5% received concomitant psychiatric treatment. The therapy cycle was completed in all patients, and 92.5% adhered to post-therapy controls. All patients were HCV RNA-negative at the end of treatment, with a sustained virological response of 92.5%.

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INTRODUCTION

Hepatitis C virus (HCV) infection has an incidence of around 1.1% worldwide, making it one of the main issues in the area of public health[1]. Italy has the highest prevalence of HCV-positive patients in Europe, as well as the highest rate of complication-related deaths such as cirrhosis and hepatocellular carcinoma[2]. These conditions are associated with significant costs, particularly in terms of therapies, hospital admissions, hepatic transplantations, and associated extrahepatic manifestations and complications[3-6].

Direct-Acting Antivirals (DAAs) have demonstrated a high response rate as an anti-HCV treatment, resulting in effective curative therapy in 95%-96% of cases, and is an affordable and cost-effective treatment[7].

The World Health Organization (WHO) has set ambitious targets for the global elimination of HCV, including an 80% reduction in new chronic infections, a 90% reduction in the incidence of new infections, and an 80% increase in treatment by 2030[8].

People who inject drugs (PWIDs) currently bear the heaviest burden of HCV infection in Italy, with a prevalence of around 60%, increased morbidity and mortality, and limited access to care[9]. Microelimination in this subgroup of HCV patients is becoming a public healthcare priority in order to reduce circulation[10]. According to WHO data, PWIDs account for 23% of new infections, but this population is considered "hard to reach" for several reasons. Indeed, access to care is extremely limited for these

citizens due to social stigma, multifactorial frailty (*i.e.*, low compliance, alcoholic abuse, co-infection with HBV and HIV, homelessness, lack of a caregiver, and psychological and psychiatric issues), and poor use of standard medical channels[11,12].

Direct-Acting Antivirals are proven to be safe and effective in individuals with active substance abuse and those receiving opioid substitution therapy[13-15]. The SIMPLIFY study found that 92.5% of PWIDs with ongoing drug abuse achieved an overall sustained virologic response (SVR) following treatment with sofosbuvir and velpatasvir[16].

In Italy, approximately 50% of the population receiving assistance for substance use disorder is positive for HCV infection. These services in Italy could be considered a "hot spot" for HCV screening and treatment because they avoid the typical health care pattern, create a dedicated link to a care strategy for patient retention, curing the condition, and improving adherence to therapy and follow-up [11,17].

This approach would allow marginalized patients to undergo treatment in a standard care setting, accompanied throughout the whole process, thereby reducing loss to follow-up and low adherence. Since PWIDs are one of the main *reservoirs* of HCV infection, eradicating the virus in this "key subgroup" is crucial for halting its spread, improving public health, and reducing costs for the National Health System[18].

This study aims to prospectively describe the experience of our HCV-dedicated multidisciplinary program at the SSUD of Trento (Northeast Italy), which focuses on providing anti-HCV therapy in infected PWIDs with an active or past history of substance abuse.

MATERIALS AND METHODS

Study design and endpoints

This study, which had a prospective observational design and retrospective data analysis, included patients who met the following criteria: PWIDs attending our SSUD in Trento, having positive HCV-antibodies and HCV-RiboNucleic Acid (RNA) > 15 UI/mL, and were current or previous drug users.

The exclusion criteria were: age < 18 years old and the absence of informed consent.

The primary endpoint was the efficacy of our dedicated program in terms of therapy completion and PWIDs' adherence to post-treatment controls. Secondary endpoints included HCV eradication, the rate of HCV reinfection after treatment, and DAA-related toxicity.

In compliance with local legislation, the study protocol was approved by the local ethical committee (N. A785), and patients provided their informed consent for data acquisition.

The study is reported in accordance with the STROBE guidelines for observational studies and follows the criteria of the Declaration of Helsinki.

SSUD activities

In October 2020, a dedicated team was set up at the SSUD in Trento to provide opioid agonist therapy and DAA therapy to HCV RNA-positive patients with an active or past history of substance abuse.

The team consists of a hepatologist, a facility physician, and four dedicated nurses. The synergistic collaboration between the different specialists resulted in the development of a health education program, which included counseling on how to avoid reinfection.

Subjects are closely guided and monitored throughout treatment and follow-up. While some patients can take the treatment on their own and only attend the SSUD once a week, the vast majority receive daily treatment directly at the SSUD or a therapeutic rehabilitation facility. The team plans and organizes all the blood tests and visits, while also monitoring and enforcing adherence.

Opioid use disorder was diagnosed following the Diagnostic and Statistical Manual of Mental Disorders 5, and its severity was assessed before and after eradication therapy. Patients were classified as: (1) "Abstinent": The absence of other drugs other than opioid agonist maintenance therapy or occasional consumption; (2) "User": constant use without severe impairment of health and quality of life; and (3) "Abuser": constant consumption severely compromising health and quality of life and unable to maintain a normal social and work routine.

Antiviral therapy

Indications for DAA therapy followed the WHO criteria[19]. The HCV genotype was assessed before the start of treatment and the stage of liver disease was evaluated using transient elastography[20]. When this method was not feasible due to social and welfare-related reasons, liver stiffness was assessed using serum markers, such as the "Fibrosis 4 Score" (Fib-4), as recommended by the EASL Guidelines[20].

The treatment regimen was based on the standard 12-week oral schedule with Sofosbuvir/Velpatasvir or an 8-week oral Glecaprevir/Pibrentasvir regimen[20].

The patients received clinical monitoring each month throughout the treatment. Treatment efficacy was evaluated by detection of negative HCV RNA at the end of treatment and after 12 wk (SVR12), when possible, or by "delayed SVR" (SVR evaluated at any time after 12 wk).

Statistical analysis

Prospectively collected variables were extracted from electronic information flows and paper-based patient records and entered into an anonymous database. They included demographic information, concomitant substance abuse, comorbidities, adherence to the therapy schedule and follow-up controls, HCV RNA levels at the start and end of therapy, SVR12 or delayed SVR, rate of HCV reinfection, treatment pause, and side effects.

Statistical analysis was performed using a dedicated software program (Medcalc 15.6.1, www.medcalc.be).

The distribution of continuous variables was reported as the median and range.

SVR rates were evaluated through intention-to-treat analysis (considering all missing data as failures).

RESULTS

Patient characteristics

A total of 42 patients met this study's inclusion criteria. The final analysis included a total of forty patients, since two patients are still undergoing treatment. Patient characteristics are detailed in Table 1.

The majority of patients were male (77.5%). Elastography for the staging of liver disease could not be used in 5 patients, and the Fib-4 score was used instead for their evaluation. Four out of 35 patients (11.4%) were already suffering from liver cirrhosis at the start of treatment: three patients were diagnosed with compensated cirrhosis (Child-Pugh A) and one was described as decompensated (Child-Pugh B7).

Twenty-eight patients (70.0%) were treated with Sofosbuvir/ Velpatasvir, while 12 (30.0%) received Glecaprevir/Pibrentasvir. Table 2 reports treatment outcomes.

Recruited individuals had an active or past history of drug consumption and other psychoactive substances (*i.e.*, benzodiazepines).

Thirty-six of the 40 recruited patients were receiving opioid agonist maintenance therapy at study inclusion, whilst another 4 had just completed treatment.

A significant proportion of the study population (37.5%) had a history of alcohol consumption, whilst 42.5% received concomitant psychiatric treatment. Patients were generally prescribed benzodiazepines and neuroleptics, but six patients were prescribed selective serotonin reuptake inhibitors.

Treatment outcomes

Treatment outcomes are detailed in Table 2. The therapy cycle was completed by 40 patients (100%). All tested patients presented a negative viral load at the end of treatment and a sustained virologic response was observed in 92.5% (SVR12 + delayed SVR). Therapy was well-tolerated, except in two cases where the patients temporarily discontinued treatment and refused subsequent lab tests. Another patient elected to only be tested for HCV RNA at the end of treatment but refused all post-therapy controls. No significant drug interactions with commonly used psychiatric treatments or side effects were observed. One patient died of an overdose, another patient died of cirrhosis complications following HCV eradication and one reinfection was observed ten months after SVR12.

DISCUSSION

The present study demonstrates that a multidisciplinary, dedicated program to assist PWIDs during anti-HCV treatment can be a safe and effective method to improve their adherence to therapy and follow-up schedule. In detail, our service achieved 100% therapy completion, 92.5% adherence to post-treatment follow-up, and one loss at follow-up despite a negative HCV RNA 4 wk after treatment ended. Furthermore, 92.5% of cases responded to treatment with good tolerability of DAAs (100.0%). Therapy efficacy was also observed in people with concurrent drug and alcohol abuse, without any significant drug interactions with commonly used psychiatric treatments.

In comparison to previous studies, good adherence to treatment and the follow-up program was observed in our series, with no drop-outs due to toxicity[21-25]. In particular, Avramovic *et al*[24] reported a similar rate of virological response (92%) in PWIDs. This rate would have been even higher in our series (100.0% *vs* 96% for Avramovic *et al*[24]) if a "per-protocol" analysis had been performed by excluding any missing data or drop-outs from the calculation. Avramovic *et al*[24] also reported a high rate of loss to follow-up (17%) and reinfection (3.5%) in PWIDs with ongoing drug use treated for HCV. Adherence was higher in our series, with only one case of reinfection involving a patient who became homeless and had a history of ongoing alcohol abuse and psychiatric comorbidities. Our encouraging results are a direct outcome of the dedicated work of the SSUD of Trento's multidisciplinary team and its efforts to increase this fragile population's adherence to the anti-HCV program through a more vigilant and attentive approach. Adherence was difficult to maintain during the follow-up period,

Table 1 Patient characteristics at the start of treatment

Characteristic	*n* = 40
Gender [male; *n* (%)]	31 (77.5)
Age [yr; median (range)]	46.5 (24-63)
Severity of opioid use disorder	
Abstinent, *n* (%)	16 (40.0)
User, *n* (%)	21 (52.5)
Abuser, *n* (%)	3 (7.5)
Concomitant opioid agonist maintenance therapy	
Metadone, *n* (%)	35 (87.5)
Buprenorfine, *n* (%)	1 (2.5)
None (previous history), *n* (%)	4 (10.0)
History of alcohol abuse, *n* (%)	14 (35.0)
Concomitant psychoactive drugs, *n* (%)	17 (42.5)
HCV genotype	
1a, *n* (%)	21 (52.5)
3, *n* (%)	18 (45.0)
4, *n* (%)	1 (2.5)
Liver disease staging[1]	
F0, *n* (%)	8 (22.9)
F1, *n* (%)	12 (34.3)
F2, *n* (%)	6 (17.1)
F3, *n* (%)	5 (14.3)
F4, *n* (%)	4 (11.4)

[1]Available in 35 cases. Elastography could not be performed in the remaining 5 patients and disease staging was calculated using "Fibrosis 4 Score". HCV: Hepatitis C virus.

owing to the patients' general condition, poor access to the healthcare system, and a low peripheral venous heritage. However, our results demonstrate that a tailored treatment and follow-up plan, accompanied by close monitoring and constant contact to avoid alienation, can also be successful in treating HCV in PWIDs.

In the SIMPLIFY multicenter design study, Cunningham *et al*[21] demonstrated high adherence to anti-HCV therapy in PWIDs, measured using an electronic blister pack. Furthermore, a correlation was observed between non-adherence and recent stimulant injecting before and during DAA therapy, but with no impact on response to therapy. Our study corroborates these findings, as loss of compliance or delayed SVR was frequently associated with deterioration in the psychological/psychiatric situation and wealth status (*i.e.*, homelessness, self-isolation, loss of job, and the absence of a caregiver). However, the two subjects who discontinued DAA therapy for an extended period due to a boost in drug abuse associated with a worsening of their psychiatric conditions may also achieve negative HCV RNA at the end of treatment.

Two subjects in our population died following SVR12, including one who began the treatment with decompensated cirrhosis (Child-Pugh B7). Our patients were predominantly young (median age 46.5 years old), male (in line with the literature[26]), and mostly without clinically significant fibrosis. Eradicating HCV in a population with a high prevalence of infection and no or early-stage liver disease has a significant impact on public health by interrupting the vicious cycle of viral spread, progression to cirrhosis, and its complications, with significant cost-effectiveness.

Despite the strength of a real-world setting and prospective design, this study has a major limitation. The findings are the result of intensive and time-consuming work, with the program having been applied to a small population on a local scale thus far. The question then becomes whether this type of model could be scaled up to a larger and more complex field while maintaining reasonable costs and demand for human resources. Based on the encouraging results achieved thus far, our next step will be

Table 2 Management and outcomes of anti-hepatitis C virus treatment

Treatment outcomes	*n* = 40
Therapy management	
Self-administration at home, *n* (%)	16 (40.0)
Daily administration at the SUD, *n* (%)	21 (52.5)
In-patient in rehabilitation service, *n* (%)	3 (7.5)
Complete adherence to the programme, *n* (%)	37 (92.5)
Therapy completion, *n* (%)	40 (100)
Post-treatment controls, *n* (%)	37 (92.5)
HCV-RNA at end of treatment	
Negative, *n* (%)	39 (97.5)
Not assessed, *n* (%)	1 (2.5)
SVR12	21 (52.5)
Negative, *n* (%)*	28 (70.0)
Not assessed, *n* (%)	12 (30.0)
Delayed SVR	
Negative, *n* (%)[1]	9 (22.5)
Not assessed, *n* (%)	3 (7.5)
HCV reinfection, *n* (%)	1 (2.5)
Side effects, *n* (%)	0 (0)

[1]Intention-to-treat analysis. SVR: Sustained Virologic response; HCV: Hepatitis C virus.

to apply this multidisciplinary anti-HCV program to a larger PWID population and validate it in a larger-scale real-world setting.

CONCLUSION

In conclusion, targeted anti-HCV programs involving vulnerable infected patients, such as PWIDs, can be effective at improving patient compliance and eradicating infection with good tolerability. However, a larger prospective study is required to definitively confirm the efficacy of this initiative.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) infection has an incidence of around 1.1% worldwide, making it one of the main issues in the area of public health. Direct-Acting Antivirals (DAAs) have demonstrated a high response rate as an anti-HCV treatment, resulting in effective curative therapy in 95%-96% of cases, and is an affordable and cost-effective treatment. In Italy, approximately 50% of the population receiving assistance for substance use disorder (SSUDs) is positive for HCV infection. These services in Italy could be considered a “hot spot” for HCV screening and treatment because they avoid the typical health care pattern, create a dedicated link to a care strategy for patient retention, curing the condition, and improving adherence to therapy and follow-up.

Research motivation

To prospectively describe the experience of our HCV-dedicated multidisciplinary program at the SSUD of Trento (Northeast Italy), which focuses on providing anti-HCV therapy in infected "people who inject drugs" (PWIDs) with an active or past history of substance abuse.

Research objectives
To show the efficacy of our dedicated program in terms of therapy completion and PWIDs' adherence to post-treatment controls. Secondary endpoints included HCV eradication, the rate of HCV reinfection after treatment, and DAA-related toxicity.

Research methods
This study included: PWIDs attending our SSUD in Trento, with HCV-antibodies and HCV-RiboNucleic Acid (RNA) > 15 UI/mL, with history of substance abuse. In October 2020, a dedicated team was set up at the SSUD in Trento to provide opioid agonist therapy and DAA therapy to HCV RNA-positive to these patients. The team provided health education program, including counseling, planning of blood tests and visits. Indications for DAA therapy followed the World Health Organization criteria. The HCV genotype was assessed before treatment start and the stage of liver disease by transient elastography or Fibrosis 4 Score", as recommended by the EASL Guidelines. The treatment regimen was based on the standard 12 wk oral schedule with Sofosbuvir/Velpatasvir or an 8 wk oral Glecaprevir/Pibrentasvir regimen. Treatment efficacy was evaluated by negative HCV RNA at the end of treatment and after 12 wk (SVR12), or by "delayed SVR" (SVR evaluated at any time after 12 wk).

Research results
Forty patients were included in the study, with active or past history of drug consumption and other psychoactive substances (*i.e.*, benzodiazepines), and 37.5% with history of alcohol consumption. Twenty-eight patients (70.0%) were treated with Sofosbuvir/Velpatasvir, 12 (30.0%) received Glecaprevir/Pibrentasvir. The therapy cycle was completed by 40 patients (100%). All tested patients presented a negative viral load at the end of treatment and a sustained virologic response was observed in 92.5% (SVR12 + delayed SVR). Therapy was well-tolerated, except in two cases where the patients temporarily discontinued treatment and refused subsequent lab tests. Another patient elected to only be tested for HCV RNA at the end of treatment but refused all post-therapy controls. No significant drug interactions with commonly used psychiatric treatments or side effects were observed. One patient died of an overdose and another of cirrhosis complications following HCV eradication. One reinfection was observed ten months after SVR12.

Research conclusions
In conclusion, targeted anti-HCV programs involving vulnerable infected patients, such as PWIDs, can be effective at improving patient compliance and eradicating infection with good tolerability. However, a larger prospective study is required to definitively confirm the efficacy of this initiative.

Research perspectives
Despite the strength of a real-world setting and prospective design, this study has a major limitation. The findings are the result of intensive and time-consuming work, with the program having been applied to a small population on a local scale thus far. The question then becomes whether this type of model could be scaled up to a larger and more complex field while maintaining reasonable costs and demand for human resources. Based on the encouraging results achieved thus far, our next step will be to apply this multidisciplinary anti-HCV program to a larger PWID population and validate it in a larger-scale real-world setting.

FOOTNOTES

Author contributions: Elettra E and Menotti E shared the first authorship; Pravadelli C, Branz G, and Merola E designed the research and served as guarantors; Menotti E, Seligmann S, and Branz G participated in the data acquisition; Merola E, Pravadelli C, and Pertile R participated in the data analysis; all the authors participated in the interpretation of the data; Merola E, Menotti E, Pravadelli C, Branz G, and Michielan A drafted the initial manuscript; all the authors participated in a critical review of the article's important intellectual content and approved the final version.

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ORIGINAL ARTICLE

Prospective Study

Volumetric assessment of hepatic grafts using a light detection and ranging system for 3D scanning: Preliminary data

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Abstract

BACKGROUND

Liver transplantation has evolved into a safe life-saving operation and remains the golden standard in the treatment of end stage liver disease. The main limiting factor in the application of liver transplantation is graft shortage. Many strategies have been developed in order to alleviate graft shortage, such as living donor partial liver transplantation and split liver transplantation for adult and pediatric patients. In these strategies, liver volume assessment is of paramount importance, as size mismatch can have severe consequences in the success of liver transplantation.

AIM

To evaluate the safety, feasibility, and accuracy of light detection and ranging (LIDAR) 3D photography in the prediction of whole liver graft volume and mass.

METHODS

Seven liver grafts procured for orthotopic liver transplantation from brain deceased donors were prospectively measured with an LIDAR handheld camera and their mass was calculated and compared to their actual weight.

RESULTS

The mean error of all measurements was 17.03 g (range 3.56-59.33 g). Statistical analysis of the data yielded a Pearson correlation coefficient index of 0.9968, indicating a strong correlation between the values and a Student's *t*-test *P* value of 0.26. Mean accuracy of the measurements was calculated at 97.88%.

CONCLUSION
Our preliminary data indicate that LIDAR scanning of liver grafts is a safe, cost-effective, and feasible method of *ex vivo* determination of whole liver volume and mass. More data are needed to determine the precision and accuracy of this method.

Key Words: Light detection and ranging; Graft volume; 3dscan; *Ex vivo* volumetry; Liver grafts

Core Tip: Liver transplantation (LT) is the golden standard in the treatment of end stage liver disease. The main limiting factor in the application of LT is graft shortage and over the years, many strategies have been developed in order to increase graft availability, such as living donor liver transplantation and split liver transplantation. In these strategies, liver volume assessment is of paramount importance in the success of LT. In this preliminary proof-of-concept study, we evaluated the use of light detection and ranging (LIDAR) technology for *ex vivo* measurement of hepatic grafts. Preliminary data indicate that LIDAR scanning of liver grafts is a safe, cost-effective, and feasible method of *ex vivo* determination of whole liver volume and mass.

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INTRODUCTION

Liver transplantation (LT) has evolved into a safe life-saving operation and remains the golden standard in the treatment of end stage liver disease[1]. The main limiting factor in the application of LT in the vast range of diseases that progress to end stage liver failure, as well as in the developing transplant oncology, is graft shortage, affecting thousands of adult and pediatric patients[2].

Over the years, many strategies have been developed in order to alleviate graft shortage, such as living donor liver transplantation[3] and split liver transplantation[4]. In these strategies, liver volume assessment is of paramount importance, as size mismatch can have severe consequences in the success of LT[5].

Although several techniques have been developed in order to assess liver graft volumes, few data and methods can accurately calculate partial split graft volumes in split liver transplantation[6], especially in the scenario of donors that have not been subjected to abdominal imaging studies.

Reality capture, on the other hand, is the use of various technical means to capture a digital 3D model representation of a subject from the real world. Recent technological advancements have made reality capture hardware such as light detection and ranging (LIDAR) 3D technology available to the public at reasonable prices. This technology has a multitude of applications and its value has not been extensively explored in liver surgery and liver transplantation[7,8]. We conducted a preliminary proof-of-concept study in order to evaluate the feasibility, safety, and accuracy of 3D LIDAR scanning photography of whole liver grafts and the prediction of liver volume and mass.

MATERIALS AND METHODS

Seven liver grafts procured for orthotopic liver transplantation from brain deceased donors were prospectively measured in this single blind, ongoing study. During the standard back table procedure, grafts were weighed and their mass in grams was recorded using a DSW200D weight scale (DELMAC Group, Athens, Greece). Before graft storage in the traditional nylon bags, the graft was placed on a flat sterile surface and photographed using an Original Structure 3D Scanning Sensor from the Occipital company (Occipital inc., Boulder, United States) (Figure 1). This particular sensor can be adapted to any device with the iOS and iPadOS operating system (Figure 2A), using a special bracket suitable for each corresponding model of iPhone or iPad of the end user. For the purposes of this study, an iPad (6th generation; Apple Inc., California, United States) was used (Figure 2B). The structure sensor communicates with the iPad *via* a USB to a lightning cable, while the 3D scanning process is done using a suitable iPadOS compatible application provided by Occipital. This application provides the user with

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Figure 1 Liver graft measurement using an original structure 3D scanning sensor.

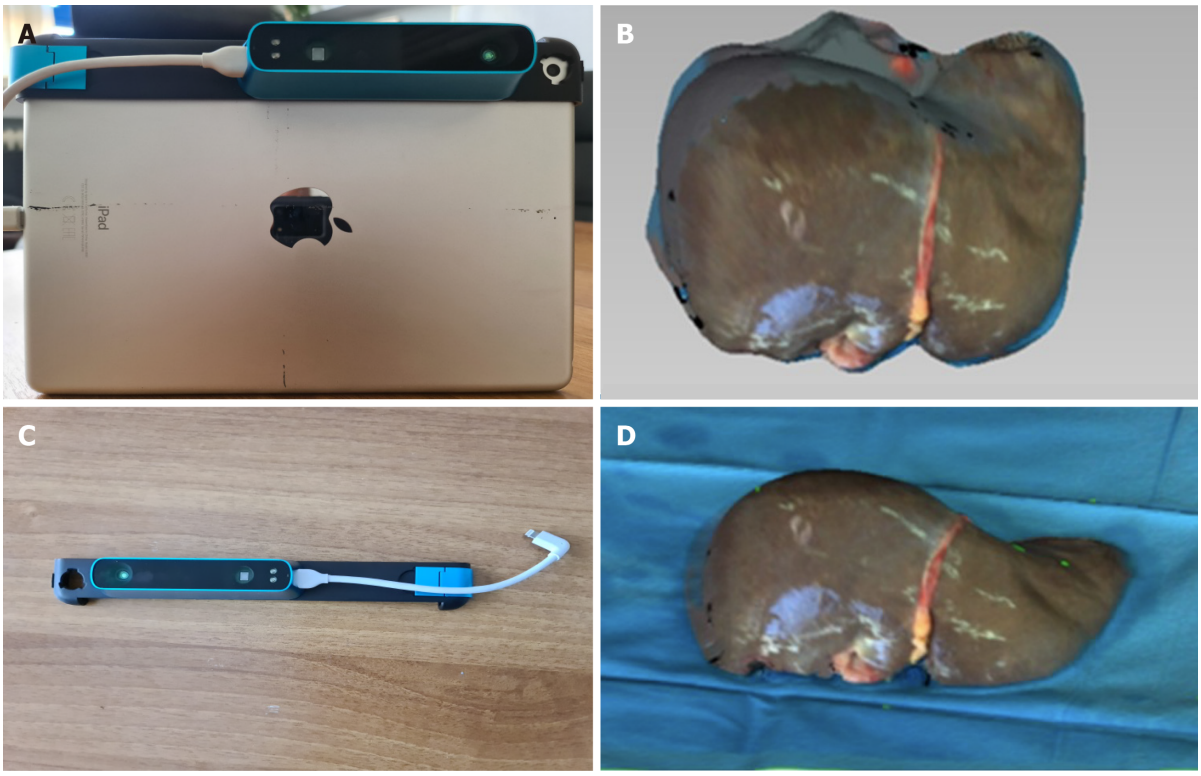

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Figure 2 The particular sensor can be adapted to any device with iOS and iPadOS operating system. A: The device used in the present study. The structure core sensor, the adjustment bracket, the USB communication cable, and the iPad (6th generation) are shown; B: The Occipital original structure sensor; C: An exported 3D model of a liver graft; D: The final 3D model of the liver graft.

the ability to convert the point cloud resulting from the scanning process into a Mesh 3D digital .obj format. The Occipital structure sensor is a mobile based structure light system (SLS). This SLS consists of a laser-emitting diode, an infrared radiation range projector, and an infrared sensor and the iPad's RGB sensor that provide measuring data to an included system on a chip (SOC) for processing. The output stream from the structure sensor alone consists of a point dataset, with a VGA resolution (640 × 480 pixels), where every pixel records the distance from sensor to the target. The infrared sensor records the reflectance intensity of the infrared (IR) light pattern projected by the IR projector onto the target while its SOC triangulates the 3D scene using specific algorithmic patterns. The main advantage of the above procedure is that the extraction of the 3D model does not require any kind of contact with the physical object (in our case the liver transplant). All measurements were conducted in fully sterile conditions with no contact with the grafts. All measurements lasted less than 3 min.

After completing the 3D reconstruction of the liver graft, the final .obj model is imported into the 3D Mesh and Point Cloud management and editing software, the 3D Slicer, a free, open source and multi-platform software package used for medical, biomedical, and related imaging research. A detailed view of an exported model participating in this study is shown in Figure 2C.

To extract the final volume of the liver model, the part of the surface on which the implant is placed (blue background) is removed from the model. The side of the graft that is in contact with the table is considered as a completely flat surface (Figure 2D). The complete flowchart of the procedure is presented on Figure 3.

In this study, mass and volume calculations were conducted by two separate teams that were blinded as to the other team's results and measurements.

LIDAR calculated volume was converted into mass using a fixed value of liver density defined by convention at 1.07 gr/mL[9,10] .

Calculated liver mass was compared to the actual weighted liver mass of each graft.

Statistical analysis

R studio for windows (R studio, Boston MA, United States) version 4.1.1 was used to perform all the statistical analyses employing packages "rstatix" and "tidyverse".

RESULTS

From June 2021 until January 2022, seven liver grafts from deceased donors were included in the study. The average donor age was 52.4 years, and the men-to-women ratio was 3:4. Apart from gender and age, we recorded weight, height, body mass index, and body surface area (BSA). Liver core biopsy was performed for all liver grafts as a standard practice in our department. Donor demographics are presented in Table 1. Graft weight was measured in grams (g). LIDAR imaging analysis provided the calculated graft volumes expressed in millilitres (mL). Considering the mean human liver density at 1.07 g/mL, calculated LIDAR volumes were converted to mass in grams by multiplying the volumes by 1.07. The theoretical volume of the grafts was also recorded using the Vauthey-Abdalla formula[11] [total liver volume = -794.41 + 1267.28 × body surface area). Table 2 depicts the results.

The mean duration of the measurement was 123 (74-171) s. No incidence was recorded during the procedure, which was conducted during the usual graft preparation by the surgical team. One graft was discarded due to severe steatosis. In the other six grafts, no cases of graft dysfunction or non-function were recorded in the subsequent transplantation.

LIDAR assisted graft volume and mass calculation results were compared with the actual weighed mass of the grafts. The mean error of all measurements was 17.03 g (range 3.56-59.33 g). Initially, data fluctuation analysis was performed for one factor (ANOVA). Average values, fluctuation, and degrees of freedom were calculated, and the null hypothesis (F < Fcit) was confirmed (Table 3). Statistical analysis of the data yielded a Pearson correlation coefficient index of 0.9968, indicating a strong correlation between the values and a Student's *t*-test *P* value of 0.26. Mean accuracy of the measurements was calculated at 97.88%. Results are depicted in Figure 4.

DISCUSSION

Liver graft mass and volume and their relations to recipient somatometric characteristics are essential factors for the outcome of LT. Although in standard whole liver adult to adult orthotopic LT, size is usually not an issue and the already existing methods of graft volume evaluation might be sufficient, accurate prediction of partial liver volumes in living donor[12] and split liver transplantation presents a more complex challenge[13]. Up to date, the main methods for partial liver volume calculation rely on preoperative imaging studies[14,15], which present their own set of challenges[16]. In the present work, we conducted a preliminary proof-of-concept study for the evaluation of the available handheld LIDAR technology for the evaluation of hepatic graft volume, as the first step in the development of a method that could eventually accurately estimate partial split liver volumes of grafts evaluated for split liver transplantation. The use of whole grafts aimed at calibrating the method and detecting eventual technical issues, as well as overcoming the technical issues associated with the split liver surgical technique and the fact that split liver transplantation is not currently performed in Greece. Our preliminary data tend to validate the concept of the study; however, it does not have a valuable clinical application *per se*, as whole liver mass and volume can be easily calculated by simply weighing the graft or by the water displacement method. However, due to the asymmetric structure of the liver, the calculation of partial liver volumes is more complex, and the existing mathematic formulas cannot accurately predict the segmental hepatic volumes that can vary considerably between patients[17], leaving the preoperative imaging studies of the graft in the form of either a computed tomography (CT) or magnetic resonance imaging (MRI) scan as the most used and valuable option. LIDAR assisted liver volumetry could add a useful tool for *ex vivo* partial liver volume calculation mainly in cases of split liver transplantation for donors that for various reasons did not have a pre-procurement CT or MRI study. Compared to traditional methods for liver volumetry such as CT and MRI, LIDAR volumetric assessment is more cost-effective, less time-consuming, and less operator-dependent. Triple phase liver

Table 1 Donor demographics.

N	Gender	Age (yr)	Cause of death	Graft steatosis (%)	Weight (kg)	Height (m)	BMI (kg/m^2)	BSA (m^2)
1	Female	59	IBI	> 10	60	1.6	23.43	1.76
2	Male	32	IBI	> 10	75	1.7	25.95	1.88
3	Male	64	SH	> 10	85	1.75	27.75	2.03
4	Female	63	ICH	60	70	1.6	27.34	2.06
5	Female	46	ICH	5	90	1.7	31.14	2.41
6	Female	54	ICH	20	120	1.75	39.18	1.63
7	Male	49	IBI	> 10	75	1.83	22.39	1.95

N: Donor number; BMI: Body mass index; BSA: Body surface area; IBI: Ischemic brain injury; SB: Subarachnoid haemorrhage; ICH: Intracerebral haemorrhage.

Table 2 Results

N	Graft weight (g)	LIDAR volume (mL)	Vauthey volume (mL)	LIDAR estimated graft mass (g)	LIDAR error (g)	LIDAR error (%)
1	1202	1179	1275.04	1261.53	59.53	4.95
2	1623	1490	1590.52	1594.30	-28.70	-1.77
3	2201	2090	1781.61	2236.30	35.30	1.60
4	1332	1248	1440.86	1335.36	3.36	0.25
5	1227	1141	1818.15	1220.87	-6.13	-0.50
6	1074	1040	2266.36	1112.80	38.80	3.61
7	1623	1482	1680.03	1585.74	-37.26	-2.30

Estimated graft mass = Light detection and ranging (LIDAR) volume mL × 1.07 gr/mL. The LIDAR error is calculated by subtracting the LIDAR estimated liver mass from the actual mass (weight) of the grafts. N: Donor number; g: Grams; LIDAR: Light detection and ranging.

Table 3 ANOVA: Single factor

Source of Variation	SD	df	MS	F	F crit
Between groups	225616.3945	2	112808.1972	0.861884735	3.554557
Within groups	2355938.641	18	130885.4801		
Total	2581555.035	20			

SD: Standard deviation; df: Degrees of freedom; MS: Mean squares; F crit: F critical.

CT scans or MRI scans can be difficult to obtain even in tertiary hospitals, let alone in the setting of a small rural donor hospital. Moreover, the multi-organ donor is not burdened with intravenous contrast media administration, which may affect kidney function. Liver 3D model capture using the LIDAR camera is performed *ex vivo*, just after backtable liver preparation, in less than 3 min and under sterile conditions. Actual volume measurement is done utilizing an open, free software package without the need of an expert radiologist. One obvious drawback in comparison to preoperative donor imaging is that the internal anatomy of the liver cannot be assessed and surgical plane planning is not possible. Another issue is that liver volume is measured during a state of non-perfusion, so liver mass and volume may differ if compared to a perfused organ *in vivo*[10]. LIDAR assisted volumetry showed a better accuracy than the theoretical volume calculation using the VAUTHEY formula. This is probably mainly due to the lack of precise donor data (mainly donor weight), as many rural hospitals do not have the ability to weigh bedridden patients and the donor weight data derive from crude estimation or medical records. Finally, the main flaw of the present study is the inability to scan the inferior surface of the liver and segment I, which lie against a flat surface, and by convention this surface is considered

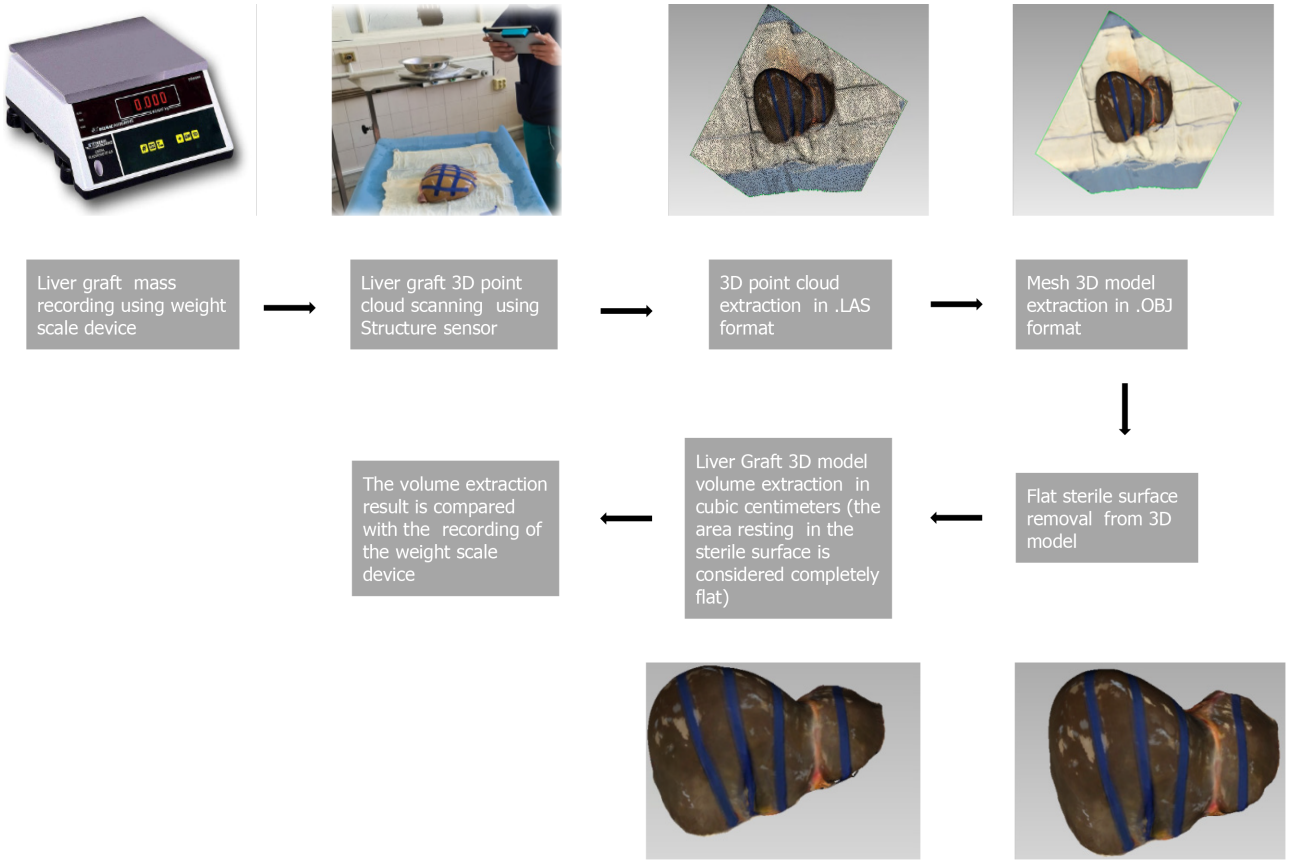

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Figure 3 Flowchart of the light detection and ranging assisted volumetric assessment of liver grafts.

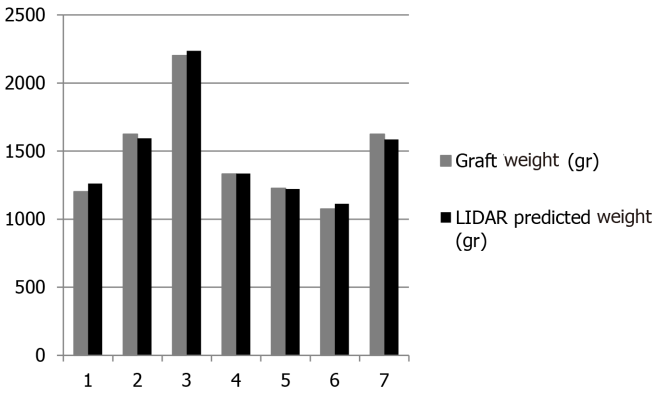

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Figure 4 Results of light detection and ranging assisted prediction of whole liver mass in grams in the seven liver grafts.

completely flat in our calculations. The subsequent steps in this ongoing study will be the refinement of the measuring technique, and the evaluation of the method in cadaveric livers with simulation of the *ex situ* splitting procedure and measurement of partial liver volumes (mainly left lateral section volumes), before moving in the actual setting of real world split liver transplantation.

CONCLUSION

Our preliminary data indicate that LIDAR scanning of liver grafts is a safe, cost-effective, and feasible method of *ex vivo* determination of whole liver volume and mass. More data are needed to determine the precision and accuracy of this method.

ARTICLE HIGHLIGHTS

Research background

Split liver transplantation is a viable option of increasing the number of available grafts, as one liver graft can yield two partial grafts for two donors. In this procedure, partial liver volume estimation, particularly left lateral segment volume estimation, is critical to the outcome of the procedure.

Research motivation

To assess the application of light detection and ranging technology in the *ex vivo* estimation of whole liver grafts.

Research objectives

To evaluate the feasibility, safety, and accuracy of 3D light detection and ranging (LIDAR) scanning photography of whole liver grafts and the prediction of liver volume and mass.

Research methods

Seven liver grafts procured for orthotopic liver transplantation from brain deceased donors were prospectively measured in this single blind, ongoing study. All measurements were conducted in fully sterile conditions with no contact with the grafts. LIDAR calculated volume was converted into mass using a fixed value of liver density defined by convention at 1.07 gr/mL. Calculated liver mass was compared to the actual weighted liver mass of each graft.

Research results

From June 2021 until January 2022, seven liver grafts from deceased donors were included in the study. Graft weight was measured in grams (g). LIDAR imaging analysis provided the calculated graft volumes expressed in millilitres (mL). Considering the mean human liver density at 1.07 g/mL, calculated LIDAR volumes were converted to mass in grams by multiplying the volumes by 1.07. Statistical analysis of the data yielded a Pearson correlation coefficient index of 0.9968, indicating a strong correlation between the values, and a Student's *t*-test *P* value of 0.26. Mean accuracy of the measurements was calculated at 97.88%.

Research conclusions

Our preliminary data indicate that LIDAR scanning of liver grafts is a safe, cost-effective, and feasible method of *ex vivo* determination of whole liver volume and mass. More data are needed to determine the precision and accuracy of this method.

Research perspectives

LIDAR assisted liver volumetry could add a useful tool for *ex vivo* partial liver volume calculation mainly in cases of split liver transplantation for donors that for various reasons did not have a pre-procurement computed tomography (CT) or magnetic resonance imaging (MRI) study. Compared to traditional methods for liver volumetry such as CT and MRI, LIDAR volumetric assessment is more cost-effective, less time-consuming, and less operator-dependent.

FOOTNOTES

Author contributions: Katsanos G and Karakasi KE contributed equally to this work; Katsanos G, Karakasi KE, and Tsoulfas G designed the research study; Katsanos G, Karakasi KE, Karolos IA, and Kofinas A performed the research; Antoniadis N and Karakasi KE conducted the data analysis and statistical analysis; Katsanos G, Tsoulfas G, and Tsioukas V analyzed the data and wrote the manuscript; Katsanos G, Kofinas A, and Tsoulfas G revised the manuscript; all authors have read and approved the final manuscript.

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CASE REPORT

Hepatitis B virus markers in hepatitis B surface antigen negative patients with pancreatic cancer: Two case reports

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Abstract

BACKGROUND

Hepatitis B virus (HBV) is a known carcinogen that may be involved in pancreatic cancer development. Detection of HBV biomarkers [especially expression of HBV regulatory X protein (HBx)] within the tumor tissue may provide direct support for this. However, there is still a lack of such reports, particularly in non-endemic regions for HBV infection. Here we present two cases of patients with pancreatic ductal adenocarcinoma, without a history of viral hepatitis, in whom the markers of HBV infection were detected in blood and in the resected pancreatic tissue.

CASE SUMMARY

The results of examination of two patients with pancreatic cancer, who gave informed consent for participation and publication, were the source for this study. Besides standards of care, special examination to reveal occult HBV infection was performed. This included blood tests for HBsAg, anti-HBc, anti-HBs, HBV DNA, and pancreatic tissue examinations with polymerase chain reaction for HBV DNA, pregenomic HBV RNA (pgRNA HBV), and covalently closed circular DNA HBV (cccDNA) and immunohistochemistry staining for HBxAg and Ki-67. Both subjects were operated on due to pancreatic ductal adenocarcinoma and serum HBsAg was not detected. However, in both of them anti-HBc antibodies were detected in blood, although HBV DNA was not found. Examination of the resected pancreatic tissue gave positive results for HBV DNA, expression of HBx,

and active cellular proliferation by Ki-67 index in both cases. However, HBV pgRNA and cccDNA were detected only in case 1.

CONCLUSION

These cases may reflect potential involvement of HBV infection in the development of pancreatic cancer.

Key Words: Pancreatic cancer; Pancreatic ductal adenocarcinoma; Hepatitis B virus; Previous hepatitis B; Anti-HBc; Hepatitis B virus X antigen

Core Tip: Hepatitis B virus (HBV) is a known carcinogen that may be involved in pancreatic cancer development. Detection of HBV biomarkers (especially expression of HBV regulatory X protein) within the tumor tissue may provide direct support for this. However, there is still a lack of such reports, particularly in non-endemic regions for HBV infection. We present two cases of HBsAg-negative patients with pancreatic ductal adenocarcinoma, in whom the markers of HBV were detected in blood and in the tumor tissue. This reflects potential role of the virus in the etiology and pathogenesis of pancreatic ductal adenocarcinoma.

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INTRODUCTION

Pancreatic cancer (PC) is one of the most prevalent cancers worldwide and its incidence rate is growing [1]. Among different types of pancreatic cancer, pancreatic ductal adenocarcinoma represents 90% of cases[2]. Despite difference in epidemiology observed across regions (incidence rates of 0.5-9.7 per 100000 people), it causes about 4% of all deaths per year globally[2]. PC is known for its aggressive nature with a low 5-year survival rate that does not exceed 9%[3].

Early detection of PC remains a challenge. Therefore, stratification of risk factors and identification of subjects at risk are actual. The known risk factors for PC are male sex, non-O (I) blood group, cigarette smoking, low physical activity, genetics and positive family history, presence of diabetes mellitus, obesity, dietary factors (high levels of red and processed meat, low fruits and vegetables consumption, and alcohol intake), and history of pancreatitis[4]. Association of PC with some infections, including hepatitis B virus (HBV) infection, has been described[5,6]. However, the results of these reports are controversial, and the mechanisms of HBV involvement in pathogenesis of PC are not fully clear.

HBV is a known carcinogen that causes up to 80% of cases of hepatocellular carcinoma in endemic regions[7]. Also, the virus may be involved in non-liver oncogenesis due to its ability to integrate into the genome of infected cells, to cause genomic aberrations and enhance expression of oncogenes or inhibit tumor suppressors[8]. Several reports have shown that replication of the virus may occur not only in the liver, but also in other organs, including the pancreas[9-11]. Moreover, pancreatic beta cells and hepatocytes develop from the ventral foregut endoderm during ontogenesis and thus may share characteristics favorable for HBV-induced tumor development[12]. Markers of previous or current HBV infection are commonly found in patients with PC, while HBV DNA and viral antigens have been detected in the pancreatic tumor tissues, suggesting a potential role of the infection in the etiology of this cancer[13-15]. However, most of these reports came from Asian countries, where HBV infection is prevalent, and most of subjects were HBsAg-positive. In contrast, uncertain results of the cohort studies performed in Europe (1 from Denmark and 2 from Sweden) make an association of the PC and HBV infection questionable[5,16-18]. Although the data of epidemiological studies are important, direct support of the involvement of HBV infection in PC development may be provided with the detection of HBV biomarkers [especially expression of HBV regulatory X protein (HBx)] within the tumor tissue. However, there is still a lack of such reports, especially in non-endemic regions for HBV infection.

Here we report two cases of patients with no history of HBV infection, admitted to the Moscow Clinical Research Center named after A.S. Loginov for pancreatic cancer treatment, who gave their consent for special examination and the use of the obtained data for scientific purposes, including publication of images.

CASE PRESENTATION

Chief complaints

Case 1: The patient was a 61-year-old white/Caucasian man, with blood type O (I). His complaints were non-remarkable.

Case 2: The patient was a 60-year-old white/Caucasian man, with blood type A (II) with no remarkable complaints.

History of present illness

Case 1: The patient was admitted for planned surgery in June 2019. Previous repeated screening blood tests on HBsAg were negative.

Case 2: The patient was admitted in February 2020 for planned surgical treatment due to previously diagnosed pancreatic cancer involving the superior mesenteric vein. Before surgery, he received seven courses of neoadjuvant chemotherapy according to the FOLFIRINOX scheme with no progression of the tumor.

History of past illness

Case 1: The patient's history of past illness was non-remarkable.

Case 2: The patient had a known history of chronic pancreatitis, type 2 diabetes mellitus, and obesity (body mass index 34.5 kg/m^2).

Personal and family history

Case 1: The patient had a history of alcohol abuse.

Case 2: The patient had a personal history of alcohol abuse and smoking experience for more than 20 years.

Physical examination

Cases 1 and 2: No notable deviations.

Laboratory examinations

Case 1: At admission, blood tests revealed signs of previous hepatitis B, but no markers of current HBV infection (Table 1). Methods used for standard and special examinations are described in Supplementary material[19-20].

Histological assessment of the resected tissue revealed ductal adenocarcinoma of the pancreas (pT1 G2 R0 N0 V0 Pn0)[21,22].

Case 2: At admission, no markers of current HBV infection were detected by blood tests. However, serum anti-HBc test was positive, suggesting that the patient had a previous hepatitis B (Table 1).

Morphological examination of the resected tissue identified pancreatic ductal adenocarcinoma with involvement of the duodenal wall (pT2 R0 N0 V0 Pn1 TRS 3)[21,22].

Imaging examinations

Case 1: Special examination of the resected pancreatic tissue in this case revealed markers of HBV replication and active cellular proliferation, as well as expression of HBx (shown in Table 1 and Figure 1).

Case 2: Examination of the resected pancreatic tissue gave positive result for HBV DNA, with no other markers of active viral replication (Table 1). However, immunohistochemistry revealed expression of HBx and high level of cellular proliferation by Ki-67 index (Table 1 and Figure 1).

FINAL DIAGNOSIS

In both cases, based on result of a complex examination, cancer of the head of the pancreas was diagnosed.

TREATMENT

Case 1: The patient underwent laparoscopic distal subtotal pancreatic resection with resection of the splenic vessels using the Warshaw technique.

Table 1 Results of special examination of blood and pancreatic tissue samples

	Subject 1	Subject 2
HBsAg (blood)	Negative	Negative
Anti-HBc (blood)	Positive	Positive
Anti-HBs (blood)	Positive	Negative
HBV DNA, IU/mL (blood)	Not detected	Not detected
HBV DNA, IU/mL (pancreatic tissue)	364	1183
pgRNA HBV, IU/mL (pancreatic tissue)	520	Not detected
cccDNA, copies/cell x 10^{-6} (pancreatic tissue)	314	Not detected
HBxAg (pancreatic tissue)	Positive	Positive
HBx - positive cells[1], % (pancreatic tissue)	3.4	3.7

[1]Median values by several fields of vision. HBsAg: Hepatitis B surface antigen; Anti-HBc: Antibody to hepatitis B core antigen; Anti-HBs: Antibody to hepatitis B surface antigen; pgRNA: Pregenomic RNA; cccDNA: Covalently closed circular DNA; HBxAg: Hepatitis B X antigen; HBV: Hepatitis B virus.

Case 2: The patient underwent gastropancreatoduodenal resection.

OUTCOME AND FOLLOW-UP

Cases 1 and 2: After discharge, both patients continued treatment offered by a local oncologist. No special treatment for silent HBV infection was required. The patients were advised to undergo regular check-ups to exclude reactivation of HBV infection: Alanine aminotransferase, HBsAg, and HBV DNA (in blood) at least once in 3 mo.

DISCUSSION

These two cases demonstrate the presence of HBV markers in HBsAg-negative patients with pancreatic cancer in non-endemic regions for the infection. Both of our patients had several known risk factors for PC development. We suppose that previous HBV infection could be an additional risk factor for PC. It is known that HBV infection, even resolved, may present a molecular basis for carcinogenesis. Carcinogenic mechanisms in HBsAg-negative persons with previous HBV infection may be related to transcriptional activity of episomal HBV genomes (cccDNA), which remains in the cell nucleus as a matrix for the life-long synthesis of new virions. In case 1, detection of not only HBV DNA but also cccDNA and pgRNA HBV (transcribed exclusively from cccDNA) suggests that this patient had a silent low-level replication of the virus in the pancreatic tissue. In case 2, pgRNA HBV and cccDNA were not detected despite a significant amount of HBV DNA in the pancreatic tissue. While no HBV replication in this patient was found, integrated HBV DNA could evidently cause the expression of HBx, which is similar to that observed in hepatocellular cancer[23]. This protein, detected in pancreatic tissue of both of our subjects, is considered to be the most pro-oncogenic[24]. It is assumed that HBx plays a major role in pathogenesis of liver cancer through nuclear translocation, protein-protein interactions, influence on transcription regulation, induction of chromosomal instability, control of proliferation, and transformation, invasion, and metastasis of tumor cells even in cases when HBV replication is absent[23, 24]. These mechanisms may also play a role in extra-hepatic cancer development. To our knowledge, there are only two studies that described HBx expression in pancreatic cancer tissues, both performed in a cohort of Asian patients in HBV endemic regions[11,25]. Song *et al*[11] reported that HBx expression was detected in ten out of ten subjects with PC and only three were HBsAg-negative.

Although the presence of HBV biomarkers in pancreatic adenocarcinoma tissue detected by PCR and immunohistochemistry does not allow proving causal relationship between the two conditions, it reflects potential involvement of the virus in the etiology and pathogenesis of pancreatic cancer. It may be important that Ki-67 proliferative index was more than 50% in both subjects. According to the literature, such values are relatively rare among PC patients (approximately 12%), and associated with more aggressive grade and poorer prognosis[20].

Together with data of the cohort studies, our cases may be important for the clinical practice. It is not yet clear whether universal testing of all patients with PC for anti-HBc and HBV DNA is necessary. However, these tests are reasonable when chemotherapy is planned, and when blood transaminases

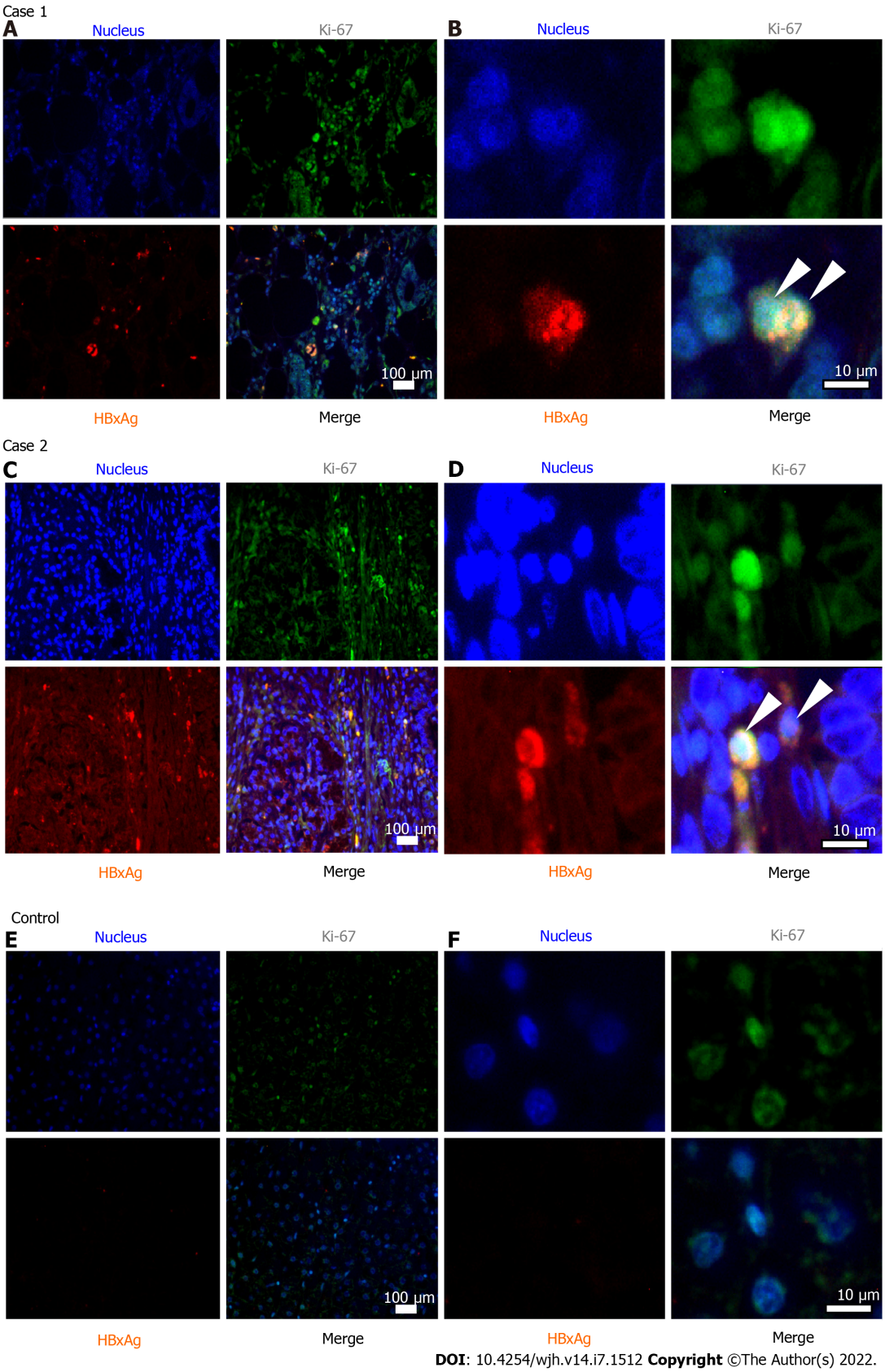

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Figure 1 Immunohistochemistry of resected pancreatic tissues. Case 1 and case 2 are subjects with pancreatic ductal adenocarcinoma and positive markers of hepatitis B virus (HBV) infection. Control refers to pancreatic tissue of a patient with pancreatic cancer, negative for markers of current and previous HBV infection (control case is not described). Samples were stained for Ki-67 protein (green fluorescence) and HBV regulatory X protein (HBx) (red fluorescence). Cell nuclei were counterstained with Hoechst33342 dye (blue). A, C and E: Images at magnification 10 ×; B, D and F: Images at magnification 100 ×. Arrows indicate HBx/Ki-67 co-stained cells. Median Ki-67 index (%): Subject 1 - 77, Subject 2 - 68, Control - 55.

flare on the mentioned treatment occurs[26,27].

Detection of HBV cccDNA in pancreatic tissue in HBsAg-negative subject in our report may support the need for revision of the statements of the Taormina Workshop (2018), which defines occult HBV infection as the presence of replication-competent HBV DNA in the liver and/or HBV DNA in the blood of people who test negative for HBsAg[28]. As extrahepatic replication of HBV DNA may occur in HBsAg-negative subjects (as shown in a number of studies and in our case 1), skipping a mention of specific organ for HBV DNA (cccDNA) detection seems reasonable.

CONCLUSION

The described cases may reflect potential involvement of HBV infection in the development of pancreatic ductal adenocarcinoma. Larger studies are necessary to assess the risk of pancreatic ductal adenocarcinoma in subjects with previous HBV infection and define HBV-associated mechanisms of cancerogenesis in them.

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FOOTNOTES

Author contributions: Batskikh S collected the data; Batskikh S and Morozov S analyzed the data; Kostyushev D performed PCR and immunohistochemistry, and prepared the figures; Morozov S and Batskikh S drafted the manuscript; all authors critically revised the manuscript and approved its final version.

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CASE REPORT

"Starry liver" - Von Meyenburg complex clinical case presentation and differential diagnosis discussion: A case report

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Abstract

BACKGROUND

Von Meyenburg complex (VMC) (*i.e.*, biliary hamartoma) is a rare congenital disorder characterized by multiple dilated cystic bile ducts, without clear trends in sex or age predominance. Due to the low number of published cases and the lack of recognized guidelines, the management of such patients remains a clinical challenge.

CASE SUMMARY

We present a case of symptomatic VMC that was diagnosed after imaging and histopathological examinations. Considering the patient's condition, a conservative treatment strategy was chosen. Instrumental, laboratory, and clinical follow-up demonstrated the stable condition of the patient receiving conservative treatment.

CONCLUSION

VMC is a potentially non-life threatening condition, but its recognition is crucial for the management of patients.

Key Words: Biliary hamartoma; Von Meyenburg complex; Liver polycystic disease; Ultrasonography imaging; Magnetic resonance imaging; Case report

Core Tip: Von Meyenburg complex (VMC) is a congenital bile duct malformation, which is asymptomatic and non-life threatening in most cases. As such, it remains underdiagnosed. Here, we report the features of VMC detected in a symptomatic patient by contrast-enhanced ultrasonography and magnetic resonance imaging. The prescribed treatment gradually improved the patient's condition. Throughout the surveillance period, the patient remained asymptomatic. In reporting this case study, we highlight the need for thorough differential diagnosis of VMC as well as a personalized treatment approach that considers disease complications, if present.

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INTRODUCTION

Biliary hamartoma or Von Meyenburg complex (VMC) is a congenital disorder that is characterized by multiple dilated cystic bile ducts. The discovery of such lesions in most cases is incidental, with an estimated incidence in the general population of 6%[1]. The formation of hamartomas has a genetic background and consists of remodeling of primitive ductal plate configurations[1]. The distinctive feature of these cystic hepatic lesions is that they do not communicate with biliary tracts, and are usually small (up to 1.5 cm), dimensionally similar with each other, and countless, producing a "starry sky" configuration[1]. Although in most cases hamartomas do not cause symptoms, VMC can have diverse clinical presentations, such as abdominal pain, fever, jaundice[2], and in rare cases, severe portal hypertension[3]. The condition can be diagnosed by ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI), contrast-enhanced magnetic resonance cholangiopancreatography (MRCP) in particular, where it appears as multiple irregularly-shaped lesions of about 10 mm diameter[2]. Multiple liver cystic lesions may cause diagnostic uncertainties, such as when mimicking liver metastasis on imaging[4]. In such cases, liver biopsy with subsequent histopathological evaluation is recommended[1]. Notably, blood analysis has not proven to be very useful due to the variability of findings and low specificity.

Complications of the condition include possible calcifications[5], portal hypertension[3], recurrent cholangitis with infectious complications[2] and malignization[1]. Currently no specific treatment is available, except treatment for symptomatic patients developing the abovementioned complications. Surveillance by US, MRI and/or MRCP is necessary, but clinical recommendations lack data on the frequency of check-ups[1].

Considering the variability of symptoms, imaging results, and possible inconclusiveness of histological evaluation, the knowledge of differential diagnostic features of the condition is essential to establish the correct diagnosis.

Here, we present a clinical case report, which is discussed from the point of differential diagnosis in order to combine the latest knowledge of clinicians, radiologists, and pathologists on biliary hamartoma.

CASE PRESENTATION

Chief complaints

A 57-year-old Caucasian woman complained of pain in the right hypochondriac region.

History of present illness

Fifteen days after a trip to Egypt, the patient was referred to a local hospital where her temperature was recorded at 38.5 °C. Laboratory examination showed increased cholestasis indices (alkaline phosphatase and gamma-glutamyl transferase > 5-6 times higher than the normal range) and acute inflammation of gall bladder as discovered during US. The patient was transferred to our unit for a second opinion.

History of past illness

The patient's medical history was unremarkable.

Personal and family history

The patient's family history was unremarkable.

Physical examination

Physical examination did not reveal any abnormality apart from painful sensations during palpation of the right hypochondriac area.

Laboratory examinations

Increased cholestasis indices were confirmed, and erythrocyte sedimentation rate and C-reactive protein were notably increased. Antinuclear, antimitochondrial, anti-smooth muscle actin, perinuclear anti-neutrophil cytoplasmic, and anti-*Saccharomyces cerevisiae* antibodies, and viral hepatitis markers were negative. Alpha fetoprotein, carcinoembryonic antigen, and carbohydrate antigen 19-9 were within normal limits.

Imaging examinations

Abdominal US scanning showed a coarse echostructure with irregularity of the liver surface, similar to that of a cirrhotic liver. Considering the marked inhomogeneity of the liver structure revealed by US scan, further contrast-enhanced US (CEUS) was performed (Figure 1), which showed some small areas of contrast washout during the portal phase. Due to the high suspicion of cirrhosis associated with neoplastic areas, an US-guided liver biopsy was performed (Figure 2). Histological examination hypothesized the diagnosis of liver hamartomas (small clusters of dilated biliary ducts surrounded by fibrous stroma with epithelial lining of biliary ducts formed by a single layer of cuboidal or flattened biliary epithelium) (Figure 3). Consequent MR cholangiography sequences enhanced with gadolinium-based contrast showed multiple hypointense nodular lesions on T1-weighted images, hyperintense lesions on T2-weighted images, and no enhancement in arterial phase after Gadolinium infusion. The lesions did not communicate with the biliary tract and a typical "starry sky" image was recorded (Figure 4). Based on the above-mentioned findings, the diagnosis of biliary hamartoma, first described by Von Meyenburg in 1918, was established.

FINAL DIAGNOSIS

VMC (*i.e.*, biliary hamartoma).

TREATMENT

The proposed treatment approach was conservative and included biliary salts (ursodeoxycholic acid 15 mg/kg of body weight), daily, with approved prolongation at each subsequent follow-up visit.

OUTCOME AND FOLLOW-UP

Further clinical, laboratory, and instrumental check-ups performed every 6 mo to date, showed a clinically stable condition with no progression noted on US imaging, as well as normal serum liver tests.

DISCUSSION

Biliary hamartoma or VMC belongs to a heterogeneous group of congenital diseases defined as "fibrocystic liver diseases." Such diseases are caused by anomalous development of ductal plate during embryogenesis. In addition to VMC, other fibrocystic diseases include congenital liver fibrosis, Caroli's disease (CD), polycystic liver disease (PCLD), and choledochal cysts[6].

From the embryogenetic point of view, in VMC as well as PCLD, malformation of the ductal plate is involved in little intrahepatic biliary tracts causing loss of continuity with the remaining biliary tree[7]. The consequence of such malformation is the absence of communication between typical cysts of the PCLD or VMC and biliary tree, which is different from some other "fibropolycystic liver diseases," such as CD. In CD, communication between cystic formations and the biliary tree is preserved since malformation of the ductal plate takes place at another time during embryogenetic development, hence involving other biliary ducts than those in PCLD or VMC.

The diagnosis of PCLD can be made by the identification of more than 20 Liver cysts on imaging modalities such as US, CT, and/or MRI, which do not communicate with the biliary tree. In cases where doubts exist regarding whether there is communication between cystic formation and the biliary tree, and consequently, the differential diagnosis among PCLD, VMC and CD is not possible, liver-specific contrast-enhanced MR cholangiography (functional MRI) will be of use. On such images, in the liver phase, biliary tracts are opacified with the contrast, and consequently, in the absence of communication

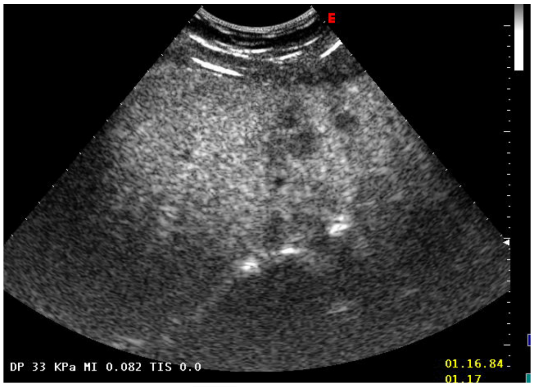

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Figure 1 Abdominal contrast-enhanced ultrasound imaging of the patient. Marked echostructural inhomogeneity of the liver.

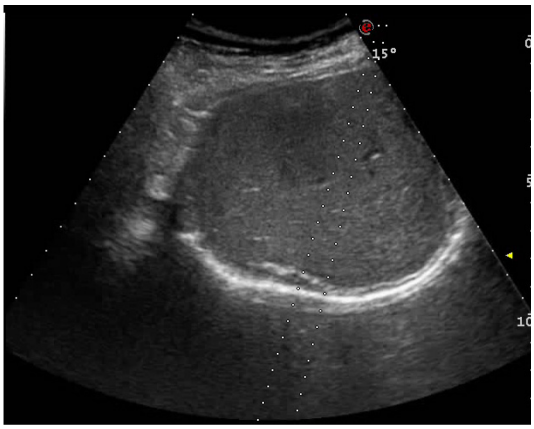

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Figure 2 Ultrasound-controlled multiple needle liver biopsy. The procedure was performed to obtain hepatic tissue for histopathological examination.

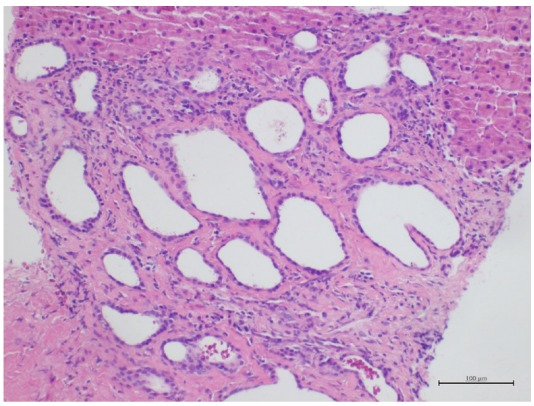

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Figure 3 Histopathological examination of the punctured liver biopsy specimen. Microphotograph of histological appearance of liver biopsy specimen showing in the peri-portal region a group of ductal structures embedded in a hyalinized stroma. The ductal structures appear variably dilated and focal microcystic. These ducts are lined with a cubic or flattened biliary epithelium. (hematoxylin-eosin staining, magnification × 20).

between biliary tracts and cysts, the latter will not be contrasted, unlike biliary tracts. While in the case of communication between cystic lesions and the intrahepatic biliary tree, the cystic formation is opacified, allowing the diagnosis of CD to be established[5]. In our presented case, MR cholangiography sequences allowed us to exclude CD, as the communication between cystic formation and biliary ducts was not preserved. Contrast-enhanced CT or MRI allowed us to study the vessels as well, allowing identification of the last distinctive feature of VMC and PCLD from CD (*i.e.*, "central dot sign" - tiny dots with strong contrast enhancement of the portal vein in the venous phase within the dilated hepatic

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Figure 4 Gadolinium contrast-enhanced magnetic resonance cholangiography. Multiple lesions were hyperintense on coronal thick slab T2 MIP. No communication between hamartomas and biliary ducts; noticeable typical formation of a "starry sky."

bile duct).

In biliary hamartoma as well as PCLD, cystic hepatic lesions that characterize the disease do not communicate with biliary tracts; however in VMC, such findings are usually smaller (up to 1.5 cm), countless, and dimensionally similar with each other compared with typical cystic formations in PCLD [4]. Therefore, in MR cholangiography, in addition to the lack of communication between hamartomas and biliary tracts, the typical formation of VMC is defined as a "starry sky," as can be seen in Figure 4.

Moreover, in the case of a patient who has liver lesions suspected of VMC or PCLD, it is necessary to obtain a thorough family history, as in approximately 90% of cases, PCLD is associated with autosomal dominant polycystic kidney disease (ADPKD) or autosomal dominant polycystic liver disease (ADPLD), which should be excluded to confirm isolated PCLD due to an inheritance pattern of ADPKD/ADPLD being autosomal-dominant[8,9]. The diagnosis of ADPLD in the setting of liver cysts is based on a family history of polycystic liver with a requisite number of liver cysts for a given age. Notably, liver cysts in ADPLD are often greater in quantity and size than those in ADPKD; hence, studies emphasize the necessity of the differential diagnosis between ADPKD and ADPLD as well[9]. An Italian study group reported a clinical case of a 54-year-old kidney transplant recipient with ADPKD in whom VMC was not previously recognized but visualized on routine ultrasound scan and confirmed with MRI 4 years after renal transplantation[10]. The authors emphasized that the similar pathological pathways of the two conditions as well as immunosuppressive therapy in the patient could lead to increased risk of malignization of the lesions; thus, thorough surveillance of such patients, preferentially by CEUS or MRI over routine US, is recommended[10]. It is worth mentioning that the use of contraceptive steroids or female hormone replacement therapy by postmenopausal women is another independent risk factor for developing PCLD, which should be considered during medical history collection while no similar considerations have been published for VMC[8].

In Table 1, we present the main differential diagnostic criteria of PCLD, CD, and VMC using a PubMed search with terms such as 'biliary hamartoma,' 'Von Meyenburg complex,' 'Caroli's disease,' and 'polycystic liver disease.'

By contrast, peribiliary cysts are cystic formations that are small in size (up to 20 mm) and localized along intrahepatic biliary ducts of the large caliber, in peribiliary spaces, with possible involvement of extrahepatic biliary tracts. Peribiliary cysts in the majority of cases are associated with liver cirrhosis, portal hypertension, portal thrombosis, and polycystic disease predominantly of the kidneys[3]. These little cysts do not show communication with corresponding biliary ducts; therefore, functional MR cholangiography is an accurate method to exclude biliary-cyst communication[3,11].

Liver-specific contrast-enhanced functional MR cholangiography is the most sensitive method for the diagnosis of intra- and extrahepatic biliary pathways and liver cystic lesions, allowing evaluation of their connection with the biliary tree[1,3,11].

While most patients with VMC remain asymptomatic, the elevation of inflammatory factors and liver function parameters (*i.e.*, gamma-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase) in serum could represent the only available biomarkers suggestive of the pathology. The approach to management of patients with VMC varies from regular follow-up, as in cases with asymptomatic course, to active treatment, as in cases of symptomatic or complicated disease course and which might include administration of ursodeoxycholic acid and/or antibiotics[12], as in our presented case.

Table 1 Differential diagnosis criteria of polycystic liver disease, Caroli's disease, and biliary hamartoma

	Polycystic liver disease	Caroli's disease	Biliary hamartoma (VMC)
Epidemiology	From 1 to 10 cases per 1000000; M:F = 1:6[13]	From 0.01 to 1 in 1000000[7,14]; M:F = 1:1 [14]	In up to 5.6% of autopsies, an estimated 6% of the general population[1]; M:F = 1:1
Symptoms associated with a disease	20% of patients have dyspnea, early satiety, abdominal distension, malnutrition, gastroesophageal reflux, hepatomegaly, portal hypertension, ascites, and variceal hemorrhage[15]	Rarely portal hypertension or hepatomegaly, fever, hepatolithiasis, and gallbladder stones[16]	Normally absent; In rare cases, fever, jaundice, abdominal pain, and variceal hemorrhage[11]
Blood examination findings	Elevated GGT, ALP, AST, and total bilirubin are reported in some serious cases[15]; Possible elevation of CA19-9 [17]	Transaminase levels may be slightly elevated; Thrombocytopenia and leukopenia if portal hypertension and/or hypersplenism are present; Leukocytosis and erythrocyte sedimentation rate may indicate cholangitis[16]	Possible elevation of liver enzymes (ALT, AST, ALP) and bilirubin; Rarely increased CA19-9[18]
Ultrasound/contrast-enhanced ultrasound	Hyperechoic areas in the subcapsular portion of the liver[11]; More than 20 hepatic cysts[19]; Larger in size compared to VMC[11]; Rarely uniform cysts, varying from < 1 mm to ≥ 12 cm in diameter; Diffuse dilatation of intra- and extrahepatic bile ducts[11]	Intrahepatic cystic anechoic areas in which fibrovascular bundles (composed of portal vein and hepatic arteries, which can be demonstrated by Doppler ultrasonography), stones and linear bridging or septum may be present[16]; Saccular or fusiform cystic dilatations of the intrahepatic bile ducts up to 5 cm in diameter often containing calculi[11]	Hypo- or hyperechoic lesions with comet-tail echoes, dot-sign; Small well-circumscribed lesions scattered throughout the liver with hypoechoic, hyperechoic, or mixed echogenicity depending on solid, cystic, or mixed components, respectively[12,19]; Hamartomas are relatively uniform in size[11]
Contrast-enhanced MRI, magnetic resonance cholangiopancreatography	Biliary ducts are not opacified with contrast; Cysts are round and smooth and are deformed but do not communicate with bile ducts[16]; Possible calcification of the walls of hepatic cysts[11]	Multiple small cystic formation is opacified with contrast; Present communication between the sacculi and bile ducts and diverticulum-like sacculi of the intrahepatic biliary tree; Cystic spaces are irregular in shape and communicate with biliary tree; Intrahepatic bile duct ectasia; Central dot sign[11,16]	Hypointense on T1-weighted images and hyperintense on T2-weighted sequences; Signal intensity is similar to the spleen but less intense than that of liver cysts[20]; MRI with gadolinium shows no communication between hamartomas and biliary ducts; Typical formation "starry sky"; Central dot sign[11]
Histological/ cytological evaluation	Multiple diffuse cystic lesions resembling solitary cysts, lined by cuboidal to flat biliary epithelium surrounded by fibrous stroma, with straw-colored fluid; 40% have identifiable VMCs[21]	Dilated bile ducts lined by cuboidal or columnar epithelium with fibrotic duct wall[22]	Bile ducts lined by cuboidal or flattened epithelium; Small to medium size[12]: Class 1: Mostly solid lesions with narrowing of biliary ducts; Class 2: Mixed solid/cystic lesions; Class 3: Mostly cystic lesions with ectasia of biliary ductsCytology findings of VMCs and bile duct adenomas are similar [12]
Treatment approach	No approved treatment; Optional pharmacological treatments of somatostatin receptor antagonists, mTOR inhibitor, vasopressin-2-receptor antagonists, estrogen receptor antagonists;surgical therapy: percutaneous cyst aspiration and sclerotherapy, laparoscopic cyst fenestration, segmental hepatic resection, and liver transplantation[15]	No specific treatment; Cholangitis must be handled with antibiotics, while cholestasis can be treated with ursodeoxycholic acid [23]; Antibiotics may stabilize the acute cholangitis; Drainage procedures with ERCP or PTC are important and sphincterotomy can aid biliary drainage and stone removal or subsequent passage and may decrease bouts of cholangitis; Lobectomy and liver transplantation[16,23]	VMC is considered a benign lesion that does not need any specific treatment unless complicated; In that case, a symptomatic treatment is prescribed (ursodeoxycholic acid or antibiotics)[12]
Complications, progression to cancer risk	Hemorrhage, infection, rupture, portal hypertension, jaundice, and end-stage liver disease[11,24]; Malignization is extremely rare[11]	Portal hypertension, cholangitis, sepsis, choledocholithiasis, hepatic abscess, cholangiocarcinoma and portal hypertension; Cholangiocarcinoma due to chronic inflammation of the biliary tree has been reported in 7%-14% of patients[11,16,23]	Rarely persistent upper right quadrant pain; Rare calcifications, portal hypertension infectious cholangitis[2,3,5]; Lesions do not tend to grow[4]; Less than 3% probability of developing cholangiocarcinoma[1]

ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate aminotransferase; CA19-9: Carbohydrate antigen 19-9; ERCP: Endoscopic retrograde cholangiopancreatography; F: Female; GGT: Gamma-glutamyl transferase; M: Male; MRI: Magnetic resonance imaging; mTOR: Mammalian target of rapamycin; PTC: Percutaneous transhepatic cholangiography; VMC: Von Meyenburg complex.

CONCLUSION

Biliary hamartoma is a predominantly asymptomatic liver formation that is often diagnosed incidentally. Some studies have proven that with time, the function of the affected liver can be altered,

although the formation bears a low risk of malignization. Thus, the knowledge of diagnostic features and differential diagnostic criteria are crucial for choosing the correct surveillance method, as currently no available international guidelines exist for standardizing the clinical diagnoses or guiding clinicians in the treatment approach and follow-up for VMC.

FOOTNOTES

Author contributions: De Sio I, Vitale LM, Niosi M, and De Sio C performed and interpreted the imaging findings and managed the patient; Priadko K reviewed the literature and drafted the manuscript; De Sio I contributed to manuscript drafting; Romano M was responsible for the revision and final approval of the manuscript; all authors issued final approval for the version to be submitted.

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RETRACTION NOTE

Retraction Note: Screening and identification of bioactive compounds from citrus against non-structural protein 3 protease of hepatitis C virus genotype 3a by fluorescence resonance energy transfer assay and mass spectrometry

Mahim Khan, Waqar Rauf, Fazal-E- Habib, Moazur Rahman, Mazhar Iqbal

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Abstract

Retraction note: Khan M, Rauf W, Habib F, Rahman M, Iqbal M. Screening and identification of bioactive compounds from citrus against non-structural protein 3 protease of hepatitis C virus genotype 3a by fluorescence resonance energy transfer assay and mass spectrometry. *World J Hepatol* 2020; 12(11): 976-992 PMID: 33312423 DOI: 10.4254/wjh.v12.i11.976. The online version of the original article can be found at https://www.wjgnet.com/1948-5182/full/v12/i11/976.htm.

Key Words: Non-structural protein 3; Hepatitis C virus; Genotype 3a; Fluorescence resonance energy transfer

Core Tip: We have decided to retract the above article for further consideration due to some misunderstandings in communication.

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TO THE EDITOR

In this manuscript, actually our study focus was to develop fluorescence resonance energy transfer (FRET) assay through expression of non-structural protein 3/4a (NS3/4A) protease of HCV genotype 3a, followed by the evaluation of extract and targeted pure natural products. However, we mistakenly used the expression vector that contains co-factor NS4A from genotype 1a. But whole story was built and described on the use of NS4A sequence/expression vector from the genotype 3a. The amino acid sequences of NS4A of the genotype 1a (KKGSVVIVGRIVLSGK) is significantly different from the genotype 3a (KKGCVVIVGHIELGK) that lead to the variation in the activity of NS3/4A protease[1].

We checked NS3/4A activity with co-factors from both genotypes (1a and 3a) and found a clear variation in the proteolytic activity of NS3 protease when fused to its respective co-factor NS4A. As mentioned earlier, in the published manuscript, by mistake we supplemented the full-length NS3 and NS4A-fused NS3 protease with a peptide derived from the NS4A of a genotype 1a virus that led to wrong interpretation and conclusion. Now we found that NS4A of a genotype 3a virus is really compatible with NS3 protease (3a) and exhibited much higher protease activity than the NS4A of a genotype 1a virus. Subsequently, this led to difference in the inhibitory concentration values of inhibitors (extracts and natural products) screened through the FRET assay. This significant variation in the activity assay has altered the downstream inhibitory activities of extracts and natural products. Regrettably, this situation has forced us to retract our paper[2] to conduct more experimentation and make the major correction in data, before we can consider its rewriting and publication.

FOOTNOTES

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