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Editorial Board Member of *World Journal of Hepatology*, Lemonica Koumbi, MSc, PhD, Postdoctoral Fellow, Department of Nutritional Sciences and Dietetics, International Hellenic University (IHU), Thessaloniki 57400, Thessaloniki, Greece. lemonica.koumbi@gmail.com

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*WJH* mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

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*REVIEW*

# Impact of direct-acting antiviral regimens on hepatic and extrahepatic manifestations of hepatitis C virus infection

Iman Ibrahim Salama, Hala M Raslan, Ghada A Abdel-Latif, Somaia I Salama, Samia M Sami, Fatma A Shaaban, Aida M Abdelmohsen, Walaa A Fouad

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**Iman Ibrahim Salama, Ghada A Abdel-Latif, Somaia I Salama, Aida M Abdelmohsen, Walaa A Fouad,** Department of Community Medicine Research, National Research Center, Giza 12622, Dokki, Egypt

**Hala M Raslan,** Department of Internal Medicine, National Research Center, Giza 12622, Dokki, Egypt

**Samia M Sami, Fatma A Shaaban,** Department of Child Health, National Research Center, Giza 12622, Dokki, Egypt

**Corresponding author:** Iman Ibrahim Salama, MD, Professor, Department of Community Medicine Research, National Research Center, El Tahrir Street-Doki-Giza_Egypt, Giza 12622, Dokki, Egypt. salamaiman@yahoo.com

## Abstract

Hepatitis C virus (HCV) is a common cause of liver disease and is associated with various extrahepatic manifestations (EHMs). This mini-review outlines the currently available treatments for HCV infection and their prognostic effect on hepatic manifestations and EHMs. Direct-acting antiviral (DAA) regimens are considered pan-genotypic as they achieve a sustained virological response (SVR) > 85% after 12 wk through all the major HCV genotypes, with high percentages of SVR even in advanced fibrosis and cirrhosis. The risk factors for DAA failure include old males, cirrhosis, and the presence of resistance-associated substitutions (RAS) in the region targeted by the received DAAs. The effectiveness of DAA regimens is reduced in HCV genotype 3 with baseline RAS like A30K, Y93H, and P53del. Moreover, the European Association for the Study of the Liver recommended the identification of baseline RAS for HCV genotype 1a. The higher rate of hepatocellular carcinoma (HCC) after DAA therapy may be related to the fact that DAA regimens are offered to patients with advanced liver fibrosis and cirrhosis, where interferon was contraindicated to those patients. The change in the growth of pre-existing subclinical, undetectable HCC upon DAA treatment might be also a cause. Furthermore, after DAA therapy, the T cell-dependent immune response is much weaker upon HCV clearance, and the down-regulation of TNF-α or the elevated neutrophil to lymphocyte ratio might increase the risk of HCC. DAAs can result in reactivation of hepatitis B virus (HBV) in HCV co-infected patients. DAAs are effective in treating HCV-associated mixed cryoglobulinemia, with clinical and immunological responses, and have rapid and high

effectiveness in thrombocytopenia. DAAs improve insulin resistance in 90% of patients, increase glomerular filtration rate, and decrease proteinuria, hematuria and articular manifestations. HCV clearance by DAAs allows a significant improvement in atherosclerosis and metabolic and immunological conditions, with a reduction of major cardiovascular events. They also improve physical function, fatigue, cognitive impairment, and quality of life. Early therapeutic approach with DAAs is recommended as it cure many of the EHMs that are still in a reversible stage and can prevent others that can develop due to delayed treatment.

**Key Words:** Hepatitis C virus; Hepatic; Extrahepatic; Direct-acting antivirals; Impact

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**Core Tip:** Direct-acting antivirals (DAAs) are achieving an over 85% sustained virological response in treating hepatitis C virus (HCV) infection. The risk factors for DAAs failure include old males, cirrhosis, and the presence of resistance-associated substitutions mainly in genotypes 1a and 3. The higher rate of hepatocellular carcinoma after DAA therapy may be due to offering DAA regimens to patients with advanced liver fibrosis and cirrhosis, where using interferon was contraindicated. The change in the growth of pre-existing subclinical, undetectable hepatocellular carcinoma upon DAA treatment might be a cause. DAAs are effective in treating HCV-associated mixed cryoglobulinemia, thrombocytopenia, rheumatological, renal, and cardiovascular diseases.

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## INTRODUCTION

The worldwide prevalence of chronic hepatitis C virus (HCV) infection is estimated to be 58 million people, and 1.5 million individuals get new HCV infection annually. The World Health Organization stated that, about 290 thousand patients died from hepatitis C-related complications in 2019[1]. In 2016, the World Health Assembly adopted the Global Health Sector Strategy on viral hepatitis. This strategy is directed towards eliminating both viral hepatitis B and C infections. To achieve the target objective, this will require the diagnosis of 90% of the infected patients, followed by treatment of 80% of the diagnosed individuals[2]. HCV leads to acute and chronic hepatitis, progressing to lifelong liver cirrhosis and cancer, and is associated with several extrahepatic manifestations (EHMs)[1]. The aim of antiviral treatment is HCV eradication, thus preventing disease progression and reducing the EHMs. This mini-review outlines the currently available treatments for HCV infection and their prognostic effect on hepatic manifestations and EHMs.

## HEPATITIS C VIRUS

HCV possesses a single-stranded RNA genome that encodes a polyprotein, which is processed into ten proteins: E1, E2, core, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B[3]. The structural proteins E1, E2, and core are components of the virion and the nonstructural proteins NS3/4A, NS5A, and NS5B are involved in viral genome replication[4,5]. To enter the host cell, HCV requires a cascade of synchronized and sequentially ordered events where the virus binds to many receptors. HCV particles circulate, as lipoviroparticles (LVPs), in association with low-density lipoprotein (LDL) and very-LDL (VLDL) components, including apolipoproteins (such as Apo-B, Apo-AI, Apo-CI, and Apo-E)[6]. HCV core, E1, E2, and P7 are essential for cell-free and cell-to-cell viral transmission[7]. HCV recognition is initiated by Toll-like receptor 3 and retinoic acid-inducible gene I[8].

HCV infects hepatocytes through cell-free and cell-to-cell viral transmission (Table 1). LVPs circulate in the sinusoidal blood, and through sinusoidal endothelial fenestration, they become in contact with receptors on the basolateral membrane of hepatocytes[9]. The virus envelope glycoproteins and virus-associated lipoprotein components (particularly apoE) of LVPs attach to hepatocyte basolateral membranes through interaction with highly sulfated proteoglycans, particularly syndecans, LDL receptor (*LDLr*), and scavenger receptor class B type I (SR-BI) on the cell surface[10]. SR-B1, as both an

**Table 1 Current diagnostic and tools to assess liver disease stages and severity in hepatitis C virus infected patients**

| Diagnostic tool | Early fibrosis stages (METAVIR less than F2) | Fibrosis 2 | Fibrosis 3 | Compensated cirrhosis | Decompensated cirrhosis | Ref. |
|---|---|---|---|---|---|---|
| HCV antibody | Positive | Positive | Positive | Positive | Positive | AASLD and IDSA [30] |
| Quantitative HCV RNA (viral load) | Positive | Positive | Positive | Positive | Positive | |
| Platelet count < 150000/mm$^3$) | Normal | Normal | Normal | < 150000/mm$^3$ | < 150000/mm$^3$ | |
| Total and direct bilirubin, ALT & AST | Normal/elevated | Normal/elevated | Normal/elevated | Elevated | Elevated | |
| Child- Pugh | -------- | -------- | -------- | Class A (scores 5-6) | Class B (scores 7-9); Class C (scores 10-15) | |
| FIB-4 Score | < 1.45 | ≥ 1.45 but < 2.67 | ≥ 2.67 but < 3.25 | ≥ 3.25 | > 3.25 | Filozof *et al*[31] |
| Fibroscan by transient elastography | 5.3 kPa | 7.4 kPa | 9.1 kPa | 13.2 kPa | 13.2 kPa | Platon *et al*[32] |
| Fibro test | < 0.48 | 0.48 - 0.58 | > 0.58 but < 0.74 | > 0.74 | > 0.74 | Laboratory Corporation of America[33] |
| Enhanced liver fibrosis test | < 7.7 | 7.7 | 9.8 | 11.3 | 11.3 | Lichtinghagen *et al*[34] |
| Aspartate aminotransferase to platelet ratio index | < 0.77 | 0.77 | 0.77 | ≥ 0.83 | ≥ 0.83 | Lin *et al*[35] |
| Liver nodularity and/or splenomegaly | Negative | Negative | Negative | Positive | Positive | AASLD and IDSA [30] |
| Prior liver biopsy | F0: No fibrosis; F1: Portal fibrosis without septa | F2: Portal Fibrosis with few septa | F3: Numerous septa without cirrhosis | F4: Cirrhosis | F4: Cirrhosis | |

HCV: Hepatitis C virus.

entry factor and an attachment factor, has been shown to bind viral envelope proteins[11]. Knockdown of individual gene of *LDLr* or *SR-B1* had a moderate impact on HCV infection. While, knockdown of genes of both receptors resulted in a much more pronounced effect[12].

Attachment to SR-BI helps bind of LVPs to cluster of differentiation 81 (CD81), claudin-1 (CLDN1), and occludin (OCLN)[13]. Interaction of HCV with CD81 causes activation of epidermal growth factor receptor signaling and facilitates CD81 diffusion and formation of the HCV-CD81-CLDN1 complex[14]. This complex then interacts with OCLN, which mediates the clathrin-dependent internalization through interacting with GTPase dynamin[15]. Other entry factors have been demonstrated, such as CD36 which interacts directly with HCV E1 protein[16]. In addition, TIM-1/human hepatitis A virus cellular receptor 1/CD365 has been identified as a contributing factor to LVP attachment through interaction with phosphatidylserine exposed on the HCV envelope[17]. This interaction may enhance viral attachment and subsequent interaction with the main entry factors[18]. HCV uses cortactin (an actin-binding protein at the cell periphery) for its assembly to promote viral proliferation and controls cortactin phosphorylation to facilitate cell invasion. Cortactin may be involved in hepatic cell migration, so it may be a potential target to interfere with the HCV cellular pathogenesis[19].

SR-B1 has also a prominent role in cell-to-cell transmission. This type of transmission assists immune evasion and persistence. Cell-to-cell transmission may be the main route of HCV dissemination in chronically infected patients[20]. LIM and SH3 protein 1 (LASP-1) is a specific adhesion protein that plays an important role in the regulation of cell migration, proliferation, and protein-protein interactions. LASP-1 is an HCV NS5A-interacting partner. Both LASP-1 and NS5A are localized in the cytoplasm of HCV infected cells. RNA and protein levels of LASP-1 were increased in these cells, indicating that LASP-1 may be involved in HCV-induced liver pathogenesis[21].

HCV can also infect and replicate in other cell types, such as peripheral blood mononuclear cells (PBMCs) and bone marrow cells through cell-to-cell transmission[22]. HCV infects PBMCs and other cells through the interaction with CD81 molecules on the cell surface[23], allowing replication of HCV in the extrahepatic tissues, which is facilitated by the expression of miR-122[24]. B lymphocytes, particularly CD27+ memory B cells, can resist apoptosis and may serve as an HCV reservoir[25]. Infection of PBMCs with HCV leads to dysregulation of the signaling pathway mediators such as STAT-1 and IRF-1

and alterations in cytokine and chemokine production, including IL-1, IL-6, IL-8, and IL-10. Persistent HCV RNA and its antigens, combined with chronic immune activation, lead to exhaustion of PBMCs that become defective and more prone to programmed cell death[26]. Liu *et al*[27] prepared cell culture-derived infectious HCV particles (HCVcc) using Huh7 cells transfected with HCV RNA. They found that HCV entry into macrophages depends mainly on its phagocytic activity and does not depend on its cell receptors. Knockdown of CD81 had a minimal effect on the entry of HCVcc into macrophages. Exosomes have been demonstrated to contain HCV-RNA. However, the mechanism responsible for the transmission of HCV genomic RNA through exosomes is still not clarified[28].

## EVALUATION OF SEVERITY OF LIVER DISORDERS BEFORE AND AFTER THERAPY

Before starting direct-acting antiviral (DAA) therapy, liver disease severity should be assessed to detect clinically unapparent advanced fibrosis (METAVIR score F3) or cirrhosis (METAVIR score F4). In patients with cirrhosis, portal hypertension and esophageal varices should also be assessed[29]. These are important steps, as the choice of DAA regimens, prognosis, and hepatocellular carcinoma (HCC) surveillance every 6 months depend on the stage of fibrosis. Table 1 summarizes some of the current available HCV diagnostic and staging tests according to AASLD and IDSA[30], Filozof *et al*[31], and other studies[32-35]. Liver stiffness measurement (LSM) using transient elastography can assess the degree of liver fibrosis and portal hypertension. Aspartate aminotransferase to platelet ratio index and fibrosis-4 (FIB-4) are simple, inexpensive, and reliable panels of fibrosis biomarkers that can be used. However, these panels may be less sensitive among African patients. Both LSM and biomarkers are expected to be efficient in distinguishing cirrhosis *vs* no fibrosis, with the lower ability for intermediate degrees of fibrosis. The combination of blood biomarkers or the combination of LSM and a blood test may improve accuracy[36].

LSM importance after sustained virological response (SVR) remains uncertain. Several studies have reported the significant regression of LSM after treatment of HCV infection with DAAs[37]. However, it is still debatable whether the decrease of LSM and post-DAA HCV eradication are due to the suppression of viral necro-inflammatory activity or regression of liver fibrosis[38]. It is recommended that assessing the fibrosis stage after therapy using non-invasive tools should not be endorsed as they are unreliable in this setting[29].

## IMPACT OF DIRECT-ACTING ANTIVIRAL REGIMENS ON HCV INFECTION

Until 2011, pegylated interferon alpha (PEG-IFNα) with ribavirin (RBV) was the standard therapy for HCV infection, with an about 50% SVR[39]. The European Association for the Study of Liver Diseases (EASL)[29] recommended that the endpoint of therapy is undetectable HCV RNA either in serum or plasma by an assay with a lower limit of detection ≤ 15 IU/mL, 12 wk (SVR12) or 24 wk (SVR24) after the end of treatment[29]. In low-resource areas, as an alternative to HCV RNA, HCV antigen (HCV Ag) testing might be useful for diagnosis of active HCV infection and at the end of treatment[40].

The identification of HCV encoded proteins and their function allowed the development of highly effective DAA regimens against the NS3 protease, NS5A, and the NS5B polymerase[41]. The maximum effectiveness of therapy is obtained when the patients are treated at early stage before advanced liver fibrosis or cirrhosis[42,43]. According to the mechanism of action, DAAs can be classified into four different groups: NS3/4A protease inhibitors [Glecaprevir (GLE), Voxilaprevir (VOX), Grazoprevir, Paritaprevir (PTV), and Simeprevir (SIM)], NS5A protein inhibitors [Daclatasvir (DCV), Velpatasvir (VEL), Ledipasvir (LDV), Ombitasvir (OBV), Pibrentasvir (PIB), and Elbasvir], NS5B polymerase inhibitor-nucleoside analogue [Sofosbuvir (SOF)], and NS5B polymerase inhibitor-non-nucleoside analogue [Dasabuvir (DSV)]. These drugs are considered pan-genotypic as they achieve a SVR > 85% through all the major HCV genotypes[44]. All DAAs are effective for genotype 1 and 4 and SOF for genotype 2. While for genotype 3, SOF, DCV, and LDV are effective. For genotypes 5 and 6, a combination of two regimens (VEL/SOF and asunaprevir (ASV)/DCV/beclabuvir) is indicated. A review for 28 randomized clinical trials, enrolling more than 7000 HCV naïve patients, revealed that DAA regimens for 12 wk significantly increased SVR12 and SVR24 compared to placebo and HCV cure was achieved in about 90.5% of patients. DAAs were well tolerated with no increase in serious adverse effects[39].

DAAs are recommended for both naïve patients as well as those who failed to achieve SVR after prior treatment. In addition, treatment is recommended for patients with advanced fibrosis or cirrhosis, including decompensated cirrhosis. Guidelines for the global standard treatment established SOF + VEL or GLE/PIB as the first recommended drug regimen for naïve patients, irrespective to HCV genotype or the presence of compensated liver cirrhosis[29,45]. Moreover, lifelong monitoring for HCC is recommended for patients with advanced fibrosis and cirrhosis, even with SVR, as DAAs decrease, but does not eliminate the risk of HCC[29,46]. In a multicenter cohort study involving 868 HCV patients with liver cirrhosis treated with DAA regimens, SVR was attained at 90% in Child-Pugh A patients and

81% in Child-Pugh B/C patients. Within a median period of 28 months follow-up, 14% of patients with Child-Pugh A and 64% of those with Child-Pugh B/C developed disease progression[47]. The use of protease inhibitors is contraindicated in patients with decompensated cirrhosis or with prior episodes of decompensation. These inhibitors carry a substantially higher drug exposure and risk of toxicity due to their hepatic metabolization[48]. Thus, the fixed-dose combination of SOF and VEL is the treatment of choice for patients with decompensated (Child-Pugh B or C) cirrhosis or with compensated (Child-Pugh A) cirrhosis with prior episodes of decompensation[29]. Tables 2 summarizes the current recommended DAA regimens for treating HCV infection according to AASL/ADSA 2021[30].

### *Direct-acting antiviral treatment failure and retreatment*

Risk factors for DAA failure include males with advanced liver fibrosis/cirrhosis, the presence of resistance-associated substitutions (RAS) in the region targeted by the received DAAs, and inadequacy of treatment. RAS linked to the *NS5A* gene are present at higher levels and persist for longer duration than those linked to the *NS3/4* gene[49]. The naturally occurring RAS do not affect treatment efficacy, as they are present in a minority of circulating HCV virions. RAS resulting from treatment are present in the majority of the circulating HCV quasispecies, which decrease the efficacy of re-treatment with the same DAA class[50]. For DAA regimens involving LDV/SOF and DCV/SOF, the identification of baseline RASs for HCV genotype, such as 1a, is recommended to decide the treatment duration or if RBV addition is needed[51]. Eventually, the adverse impact of baseline RAS could be decreased by increasing duration of treatment or optimizing DAA regimens. However, a considerable percent of treatment failures is triggered by RAS acquired during therapy[49]. This may be related to the relatively low barrier to resistance of the NS5A region and the high genetic barrier of SOF. Moreover, the third-generation NS3 inhibitors are expected to have intermediate genetic barrier in HCV genotypes 1a and 1b and very high in non-1 genotypes[52]. A meta-analysis on 6500 HCV infected patients, reported reduced effectiveness of GLE/PIB in HCV genotype 3 with baseline RAS like A30K, Y93H, and P53del. Testing RAS for genotype 3 HCV infection is mandatory to improve the prognosis of treatment outcome and selection of therapy[53].

Baseline RAS were only identified in the *NS5A* region in Iranian patients with HCV genotypes 1a and 3a with no RAS in the *NS5B* region[54]. Among 539 Italian HCV genotype 3 patients (417 DAA-naïve and 135 DAA-failed), Sanger sequencing of *NS3/NS5A/NS5B* at baseline samples showed a higher prevalence of *NS5A* RAS in DAA-failed (5/13, 38.5%) *vs* DAA-naïve (61/393, 15.5%, $P = 0.04$) patients. The presence of baseline Y93H and/or A30K was associated with SVR rate of 72.2% *vs* 95.7% among patients without *NS5A* RAS ($P = 0.002$). Chen *et al*[55] and Pisaturo *et al*[52] reported at least one RAS among over 85% out of the studied 220 HCV naïve patients with DAA-based treatment. However, according to the recommendation of international guidelines, massive testing for RAS detection before starting DAAs treatment is not needed, with some exceptions[29,45]. RAS testing before treatment is recommended for HCV genotype 3 infected patients with liver cirrhosis, as those without a baseline Y93H RAS in *NS5A* are eligible for SOF/VEL therapy. While, those with baseline Y93H RAS could be treated with SOF/VEL/VOX or SOF/VEL plus RBV[45]. However, according to EASL guidelines, the same therapeutic regimen should be used to all compensated cirrhotic patients regardless of viral genotype[29]. Currently, the US Food and Drug Administration (FDA) and European Medical Agency (EMA) approved two types of DAA regimens, SOF/VEL/VOX and GLE/PIB, to treat patients with previous experience of DAAs failure[36,46-48]. The effectiveness of the regimen SOF/VEL/VOX plus or minus RBV for 12 wk among patients with DAA failure revealed that SVR at 12 wk ranged from 91% to 100%. Most patients tolerated retreatment well[58].

### *Direct-acting antivirals and hepatocellular carcinoma*

The impact of DAAs on the development of HCC is controversial. Meanwhile, it should be noted that in all studies, the risk of HCC remained even after successful HCV treatment. A meta-analysis study revealed that, the incidence rate for a new HCC was 3.3% (95% confidence interval: 1.2-9%) per year after DAA treatment[57]. Some studies reported that, the risk of *de novo* HCC after DAA therapy was reduced, while other studies noted a much higher HCC risk mainly within the first year after DAA therapy than later[58,59]. A retrospective cohort study was carried out on 243 consecutive HCV patients who received PEG-IFN/RBV and were followed for a median of 9.3 years, and 263 HCV patients who received DAA treatment and were followed for a median of 4.1 years. It revealed that a considerably increased hazard was associated with DAA treatment[60]. A French study conducted on 1270 HCV patients revealed that, the differences of the occurrence of HCC after IFN and DAA regimens could be explained by the higher prevalence of Child-Pugh class B, portal hypertension, and diabetes among DAA-treated patients *vs* IFN-induced SVR patients. A time-dependent Cox model weighted by inverse probability of treatment was used to overcome selection bias. This model shows that DAAs were not significantly associated with an increase in the risk of HCC occurrence ($P = 0.73$), nor with a more aggressive pattern of presentation[61].

The higher rate of HCC after DAA therapy may be because they are the drugs of choice for treating old patients and those with liver cirrhosis and end-stage liver disease as IFN was not indicated to treat such patients[62]. The possible clarification of the elevated incidence of HCC after the start of DAA therapy, might be the change in the growth of pre-existing subclinical and undetectable HCC upon

**Table 2 Recommended direct-acting antiviral regimens for treatment of hepatitis C virus infection according to AASL/ADSA 2021**

| Treatment | No cirrhosis | | Compensated cirrhosis | | Decompensated cirrhosis |
|---|---|---|---|---|---|
| | Naïve HCV infected patient | Previously treated patients | Naïve HCV infected patients | Previously treated patients | |
| Sofosbuvir (400 mg)/Velpatasvir (100 mg) | 12 wk | Sofosbuvir (400 mg)/Velpatasvir (100 mg)/Voxilapevir (100 mg), 12 wk, for all genotypes. ALTERNATIVE: Glecaprevir (300 mg)/Pibrentasvir (120 mg), but not recommended for genotype 3 with Sofosbuvir/NS5A inhibitor | For genotypes 1, 2, 4, 5, and 6 & genotype 3 with NS5A-RAS Y93H negative, 12 wk, but not recommended for genotype 3 with NS5A-RAS Y93H positivity | Sofosbuvir (400 mg)/Velpatasvir (100 mg)/Voxilapevir (100 mg), 12 wk, for genotypes 1, 2, 4, 5, and 6; for genotype 3, 12 wk in addition to weight-based Ribavirin. ALTERNATIVE: Glecaprevir (300 mg)/Pibrentasvir (120 mg), but not recommended for genotype 3 with Sofosbuvir/NS5A inhibitor | Patients with HCV infection who have decompensated cirrhosis, *i.e.*, Child-Pugh class B or class C, should be referred to a medical practitioner with expertise in that condition, ideally in a liver transplant center |
| Glecaprevir (300 mg)/Pibrentasvir (120 mg) | 8 wk | 16 wk in addition to Sofosbuvir (400 mg) + weight-based Ribavirin ALTERNATIVE: 12 wk of Sofosbuvir (400 mg)/Velpatasvir (100 mg)/Voxilapevir (100 mg) | 8 wk | 16 wk in addition to Sofosbuvir (400 mg) + weight-based Ribavirin. ALTERNATIVE: 12 wk of Sofosbuvir (400 mg)/Velpatasvir (100 mg)/Voxilapevir (100 mg) in addition to weight-based Ribavirin | |
| Elbasvir (50 mg)/Grazoprevir (100 mg) | 12 wk for genotype 1b | 12 wk Sofosbuvir (400 mg)/Velpatasvir (100 mg)/Voxilapevir (100 mg). However, Glecaprevir/Pibrentasvir for 16 wk is not recommended as an alternative for this group of patients | 12 wk for genotype 1B | NA | |

HCV: Hepatitis C virus; RAS: Resistance-associated substitutions; NA: Not applicable

DAA treatment[60]. A high HCC risk after DAA treatment was also reported, especially in individuals with uncharacterized liver nodules[63]. Owusu Sekyere *et al*[64] detected a reduced HCC specific tumor response upon DAA-induced HCV clearance. In HCV patients who subsequently developed HCC, the T cell-dependent immune response was much weaker, indicating their important role in inhibiting tumor growth. DAA therapy for HCV was associated with a weakening of the strength of HCC-specific CD8+ but not CD4+ T cell responses in cirrhotic patients *in vitro*. Moreover, a mechanism like cellular behavior after eradication of HCV by DAA therapy may increase the HCC growths as detected in early test models[59]. Recently, Lu *et al*[65] concluded that the down-regulation of tumor necrosis factor α (TNF-α) after successful DAA therapy increases the risk of HCC and the inhibition of TNF-α might attenuate the host immune surveillance against tumor cells. These findings might provide a clue for the pathogenesis of HCC and a strategy for HCC surveillance based on risk stratification. In Egypt, HCC was found to be significantly aggressive in HCV patients treated with DAAs, especially among those with an elevated neutrophil to lymphocyte ratio ($P$= 0.012)[66]. It is recommended to screen for HCV every 6 months for cases with cirrhosis and every 12 months for those without cirrhosis after DAA therapy[45].

DAAs appear safe for patients with a history of treated HCC and are not associated with an increased risk for cancer recurrence except for cases with vascular invasion, where aggressive HCC recurrence was reported[67]. For HCC patient candidates for a liver transplant, decisions regarding the timing of DAA treatment depend on organ availability and region wait times and should be individualized[68]. Moreover, Fouad *et al*[69] suggested that anti-HCV therapy in HCC patients should be postponed until further research for safety and effectiveness is carried out.

### *Hepatitis C virus and hepatitis B virus co-infection*

Hepatitis B virus (HBV) and HCV are the major causes of liver disease worldwide. The administration of compulsory HBV vaccination is effective in providing long-term protection against infection, even with low seroprotection rate, proved by the presence of high anamnestic response rate after being given a HBV challenging dose[70,71]. However, poly-transfused vaccinated individuals, either with or without HCV infection, are at risk of HBV infection. In these patients, HBV-DNA was detected even among HBsAg negative patients (occult HBV infection)[72,73]. The co-infection with both viruses

increases the rates of cirrhosis and HCC[74,75]. When both HBV and HCV are present in the same cell, reciprocal inhibition of one viral genome by the other virus takes place and leads to the dominance of one virus over the other. The dominant virus replicates more actively and inhibits the replication of the non-dominant virus. However, co-dominance may occur if there is nearly equal replication of both HBV and HCV[76]. In 2018, EASL recommended that hepatitis C patients should be tested for HB surface (HBs) antigen, HB core antibody (anti-HBc), and HBs antibody (anti-HBs) prior to starting DAA-based treatment. In HBs antigen positive patients, concurrent HBV nucleoside/nucleotide analogue therapy is indicated. For anti-HBc positive patients with negative HBsAg, serum alanine transaminase (ALT) levels should be monitored and both HBs antigen and HBV DNA should be tested, if ALT levels rise or do not return to normal during or after anti-HCV therapy. In anti-HBs and anti-HBc antibodies positive patients, monitoring of serum ALT levels is indicated[51].

Co-infected patients may experience HBV reactivation after the cure of their HCV by PEG-IFN or DAA-based therapy and anti-HBV therapy should be started if clinically indicated[77]. The US FDA warned of the higher risk and earlier onset of HBV reactivation with DAA treatment[78]. This can be expected since HCV DAAs have no direct or immunomodulatory effect on the replication of HBV. Close monitoring of HBV infection status is settled by all guidelines with the implementation of IFN-free regimens. A retrospective study revealed that only 9 out of 62290 patients treated with DAAs had HBV reactivation. Eight patients were known to be HBsAg positive, and one patient was known to be isolated anti-HBc-positive. Seventeen other patients had a small increase in HBV DNA levels that did not qualify as HBV reactivation[79].

## EXTRAHEPATIC MANIFESTATIONS OF CHRONIC HEPATITIS C VIRUS INFECTION

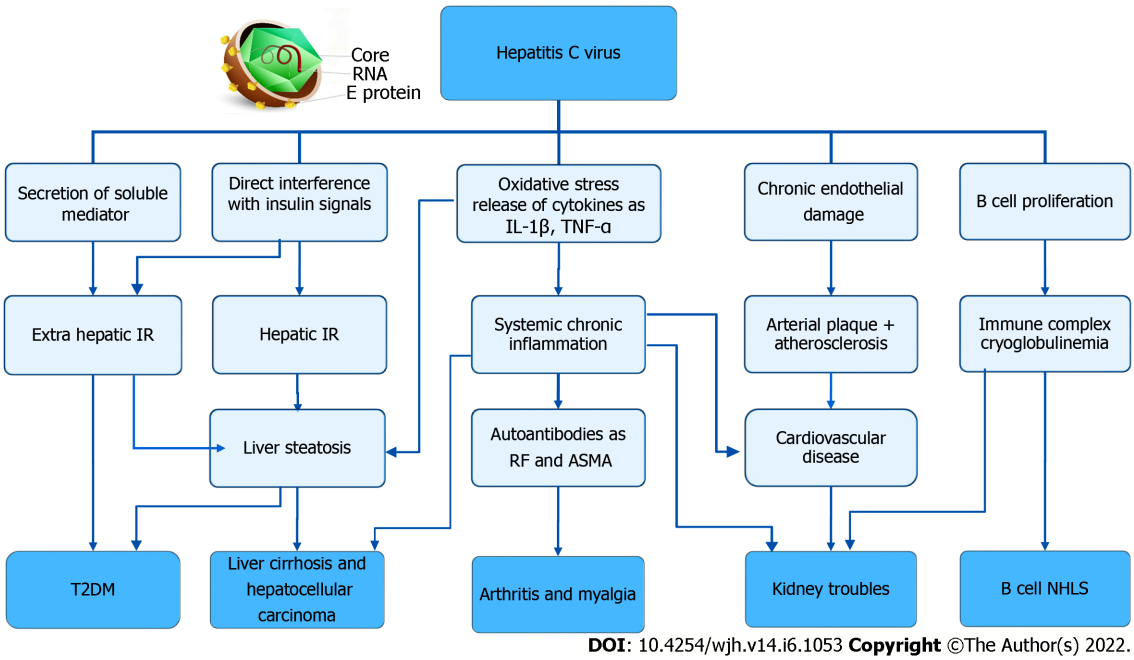
HCV can cause extrahepatic diseases that lead to an increase in the overall mortality. Figure 1 shows the pathophysiology of HCV infection in hepatic and extrahepatic diseases. The following extrahepatic diseases are related to HCV infection:

### *Mixed cryoglobulinemia and cryoglobulinemic vasculitis*

Chronic HCV infection is a common cause of mixed cryoglobulinemic vasculitis (MCV). In about 40%-60% of patients with chronic HCV infection, circulating mixed cryoglobulins are detected. However, overt cryoglobulinemia vasculitis (CV) is observed in only 5%-10% of patients[80,81]. As shown in Figure 1, the pathogenesis of MCV involves viral-induced activation of B cell clones which generate pathogenic IgM with rheumatoid factor (RF) activity. Monoclonal IgM and polyclonal IgG bind together and recognize hepatitis C nucleocapsid and core antigens. The resulting circulating immune complexes deposit in vascular beds of small-to-medium vessels, enhancing complement activation, leukocyte recruitment, and vasculitis[82]. The clinical manifestations of the disease are variable, ranging from mild symptoms such as purpura, arthralgia, and fatigue to more serious life-threatening complications resulting from neurologic and renal involvement[83].

Treatment of HCV-MCV is challenging. The main goal is SVR in order to down-regulate the B-cell arm of autoimmunity that is triggered by the virus. DAA regimens are now the drug of choice for HCV-associated MCV. The combination of PEG-IFNα with RBV has been abandoned for their side-effects, including the immune-stimulatory effects[84]. With DAA therapy, the rate of SVR after 12 months of therapy was the same for HCV patients with and without mixed cryoglobulinemia (MC). However, MCV may persist or reappear in some patients after SVR[85]. Moreover, new onset cases of cryoglobulinemic glomerulonephritis were also reported[86]. After DAA therapy for HCV associated MC, 64% to 96% of the patients improved clinically; however, the immunological response (defined by marked reduction or disappearance of circulating cryoglobulins and normalization of the levels of RF and C4) was only from 48% to 89%[87].

Artemova *et al*[88] reported complete disappearance of cryoglobulinemia among 48% of HCV-CV patients and a decrease in cryoglobulins among 17% of them. Response rates of HCV-CV after DAA treatment vary according to the organ involvement. A higher response rate (75%-100%) was attained for cutaneous and musculoskeletal presentations, while lower response rates (30%-70%) were attained in peripheral nerve and renal involvement[89]. The lag in immunologic and/or clinical response behind the viral clearance may be due to delay in the clearance of cryoglobulins from the circulation after successful antiviral therapy or to the persistence of the RF-producing memory B-cell clones for at least 24 wk[90]. Occult HCV infection is another possible explanation especially in case of cryoglobulinemic glomerulonephritis[91]. Abdelhamid *et al*[92] suggested that some forms of alteration in the immune system can be responsible for the persistent HCV-related immune disease after viral clearance. The recommended drug for patients with persistent or recurrent MCV after SVR is Rituximab, which is a B-cell depleting monoclonal antibody. In rapidly progressing or fulminant cases or severe exacerbation of vasculitis causing life-threatening complications, plasmapheresis is added to remove the circulating cryoglobulins[93].




**DOI**: 10.4254/wjh.v14.i6.1053 **Copyright** ©The Author(s) 2022.

**Figure 1 Pathophysiology of hepatitis C virus infection in hepatic and extrahepatic diseases.** IR: Insulin resistance; T2DM: Type 2 Diabetes Mellitus; IL-1β: Interleukin-1 beta; TNF-α: Tumor necrosis factor-alpha; RF: Rheumatoid factor; ASMA: Anti-smooth muscle antibody; B cell NHLs: B-cell non-Hodgkin lymphomas.

### *Thrombocytopenia*

Thrombocytopenia is a common complication in chronic HCV infection, causing an increased risk of bleeding[94,95]. The prevalence and severity of thrombocytopenia increase with the progression of liver disease and the development of hepatocellular damage and hepatic fibrosis[96]. Its prevalence is 6% in chronic liver disease patients, while it is 24% in chronic HCV infected patients and increases to 78% in cirrhotic patients[94-97]. The pathophysiology of thrombocytopenia in chronic HCV is multifactorial and largely related to the severity of hepatic infection. It includes splenomegaly and the related hypersplenism causing platelet sequestration, auto-immunogenicity, impaired production of thrombopoietin due to advanced fibrosis, possible direct effect of HCV as direct bone marrow suppression, and therapeutic adverse effects[94-96].

In the IFN era, starting or maintaining IFN therapy was a great challenge in the treatment of chronic HCV patients with thrombocytopenia[96]. IFN causes a further decrease in the platelet count in up to 13% of patients[98]. On the other hand, DAA treatment achieve an over 95% SVR at 24 wk among HCV infected thrombocytopenic patients with advanced fibrosis and cirrhosis. Moreover, the platelet count showed statistically significant improvement[99,100]. Chen *et al*[97] found that 99.6% of chronic HCV infected patients with thrombocytopenia receiving DAA treatment achieved a SVR and thrombocytopenia improved significantly in 41.7% of them. Another study reported a highly effective and safe DAA regimen, with improvement of platelet count in 73% of thrombocytopenic patients, especially in mild to moderate stages of hepatic fibrosis[95].

### *Hepatitis C virus and glomerulopathies*

HCV infection is associated with several glomerulopathies including membranoproliferative glomerulonephritis (MPGN). It is associated with MCV in 80%-95% of the cases. Other HCV glomerular diseases include membranous nephropathy, proliferative glomerulonephritis, focal segmental glomerulosclerosis, fibrillary glomerulonephritis, IgA nephropathy, immunotactoid glomerulopathy, and renal thrombotic microangiopathy. HCV infection also increases the risk of chronic kidney disease (CKD). The association between chronic HCV infection and CKD is more significant with high HCV viral load and HCV genotype 2[84,101,102].

In MPGN associated with MCV, the developed immune complex deposits in the mesangium, capillaries, and urinary space of glomeruli, which can be manifested as nephrotic and nephritic syndromes[91,103,104]. Furthermore, HCV can cause kidney damage through direct cytopathic effect by viral invasion of the renal parenchyma (mesangial, endothelial, and tubular cells of the kidney) and through nephrotoxicity of drugs used for its treatment. Additionally, non-immunological pathways as oxidative stress or pro-inflammatory cytokines help the development of renal disease by vascular injury as shown in Figure 1. In addition, HCV infected patients may have an increased risk of insulin resistance, which develops during the inflammation process and exacerbates renal damage[105-108]. In very few cases, viral NS3 was found in the glomerular deposits, capillary walls, and the mesangium[84].

Previously, HCV kidney manifestations were treated with PEG-IFN plus RBV; however, these drugs presented low efficacy, low SVR (< 50%), and severe side effects as acute renal failure, graft failure, and hemolytic anemia[109,110]. DAA therapy improves glomerular filtration rate, decreases proteinuria and hematuria, and shortens treatment duration to only 8-12 wk without significant side effects[111-113]. Delays in initiation of DAA therapy could have deleterious effects[114-116]. In patients with an estimated glomerular filtration rate (eGFR) > 30 ml/min/1.73 $m^2$, the use of SOF with SIM with or without ribavirin decreased proteinuria and improved eGFR[117,118]. There are different approved regimens for patients having an eGFR < 30 ml/min/1.73 $m^2$ or those on dialysis: (1) OBV + PTV + Ritonavir (RITV) + DSV; (2) RBV + Elbasvir + Grazoprevir; and (3) GLE + PIB. The side effects were only mild general symptoms like fatigue, insomnia, dizziness, and headache[119-121]. Furthermore, DCV and ASV are important options, especially for patients with renal impairment since both DCV and ASV have minimal renal excretion[122].

### *Hepatitis C virus and type 2 diabetes mellitus*

HCV infected patients have impaired glucose metabolism with hyperinsulinemia due to decreased insulin catabolism or insulin resistance (IR). Up to 60%-80% of HCV cases have glucose intolerance, 20% of them develop type 2 diabetes (T2DM), and up to 41%-70% of them have IR. T2DM which develops as a complication of HCV infection is known as hepatogenous diabetes[123-125].

HCV can induce IR through direct and indirect ways as shown in Figure 1. The viral core protein can directly interfere with intracellular insulin signaling by inhibiting the expression of insulin receptor substrate (IRS)-1 and IRS-2. HCV replicates in the pancreatic β-cells, causing impairment of their function involved in glucose metabolism. In addition, HCV infection can indirectly induce IR due to oxidative stress, liver steatosis, release of inflammatory cytokines such as TNF-α, interleukin (IL)-1, IL-6, and leptin, phosphorylation of the insulin-1 receptor substrate and protein kinase B, and up-regulation of gluconeogenic genes such as glucose 6 phosphatase and phosphoenolpyruvate carboxy kinase[84,126,127].

SVR with PEG-IFN and RBV is associated with decreased IR after 24 wk of therapy[128,129]. DAAs improve the IR by 90%. Treatment with DAA regimens has ameliorated hyperglycemia, recovered pancreatic beta-cell function, and reduced cytokine production[130-132]. Furthermore, the eradication of HCV by DAAs such as SOF-based regimen led to the improvement in hemoglobin A1c percentage[133,134].

### *Hepatitis C virus and rheumatological manifestations*

The rheumatologic and musculoskeletal manifestations are the most common EHMs, affecting 40-80% of HCV infected patients. MC is one of the causes of HCV associated rheumatologic manifestations (RM)[135]. Cryoglobulins were found to be deposited in small vessels of joints[136]. These manifestations are numerous and diverse, including fatigue, arthritis or arthralgia, myalgia, polyarthralgia, fibromyalgia, poly/dermato-myositis, sicca syndrome, and non-inflammatory musculoskeletal pain. The articular involvement is usually bilateral, symmetrical, and non-deforming, and it usually targets small joints such as the metacarpophalangeal joints, the proximal interphalangeal joints, wrists, and fingers. The knee, ankles, and back may be also affected[137,138]. RF is usually positive in these cases but anti-cyclic citrullinated peptide antibodies are negative and can be used to differentiate HCV arthropathy from early rheumatoid arthritis[139].

Sicca syndrome has been reported in 20 to 30% of patients with HCV infection. This may be due to the presence of the virus in the human salivary glands, where it can replicate. It is characterized by high RF titers, higher-frequency cryoglobulins, low antinuclear antibodies (ANA), hypocomplementemia, and a lower frequency of anti-Ro/SSA and anti-La/SSB autoantibodies[84]. Myalgia is a common finding in HCV infected patients, and it occurs in about 15% of cases. The mechanism of RM is possibly related to direct action of the virus as it was detected in muscle fibers. Figure 1 shows that these RM are mostly mediated by immunological mechanisms rather than being related to the infection of extrahepatic tissues. HCV envelope E2 protein binds with CD81 expressed on the membrane of B-cells, forming a complex that decreases the threshold for activation of B-cells and also causes reduction of its apoptosis. These lead to aberrant activation of B-lymphocytes as well as their prolonged survival, therefore increasing the production of antibodies (including the auto-antibodies) and systemic inflammation[140]. Tissue damages, either directly by viruses or as a result of immune aggressions against infected cells, result in the release of a large number of tissue antigens. Additionally, it has been previously postulated that similarities between HCV antigens and host antigens are partly responsible for the development of ANA and anti-smooth muscle antibodies (ASMA)[141]. RF was detected in 70% of patients, followed by ANA (20 to 40%), anticardiolipin antibodies (15%), antithyroid antibodies (12%), and ASMA (7%)[140].

IFN-based regimens for HCV infection lead to exacerbation of rheumatic diseases and worsening of preexisting autoimmune disorders or even developing a new one[142]. DAAs reduce the viral load and therefore decrease the production of antibodies. The eradication of HCV with DAAs supports improving the articular manifestations[141]. SOF and DCV with or without ribavirin combination therapy are an effective and safe treatment with minimal side effects for eradication of HCV infection and amelioration of HCV related RM[143,144].

### *Hepatitis C virus and cardiovascular diseases*

Chronic HCV infection has a significant, direct or indirect impact on the increased risk of cardiovascular diseases (CVD)[84]. HCV infection is associated with a 27% increase in risk of CVD and cerebrovascular atherosclerotic diseases including stroke events compared with uninfected controls[145,146]. The risk of death from cerebrovascular causes has been correlated with HCV RNA levels[147]. A meta-analysis of nine case-control studies showed a two-fold higher risk of carotid plaques in HCV infected individuals compared with uninfected controls[148]. Moreover, a higher prevalence of anti-HCV antibodies was detected in patients with cardiomyopathies and myocarditis than in the general population. The negative strand of HCV-RNA was detected in cardiac tissue, suggesting replication of the virus. These two findings indicate a direct association between HCV and cardiac injury, with CVD and heart failure seen in patients with HCV infection[145,149]. The effect of HCV infection on the risk of cardiovascular events was greater among older patients with hypertension or diabetes[146].

HCV infection leads to the development of atherosclerosis through different mechanisms as shown in Figure 1. A direct involvement of HCV in the induction of atherosclerosis, as the virus lives and replicates in thrombotic tissue, causes a chronic inflammatory reaction that participates in thrombus growth and instability[150]. Endothelial cells express HCV entry receptors which support viral replication. Moreover, HCV causes endothelial dysfunction through promoting migration and proliferation of smooth muscle cells from the tunica media to the intimal surface. HCV alters endothelial permeability, causes cell apoptosis and so, produces endothelial dysfunction[151]. Indirect mechanisms of atherosclerosis have been proposed, such as chronic low-grade systemic inflammation and activation of T helper cells with the release of pro-atherogenic cytokines and chemokines (*e.g.*, IL-1, IL-6, and TNF)[152,153]. These in turn induce soluble vascular adhesion molecule 1 at the endothelial level, which has been found to be associated with endothelial dysfunction as well as the risk of CVD. HCV also interferes with glucose and lipid metabolism, leading to IR, diabetes, and liver steatosis, which are known factors that induce atherosclerosis[151]. A high TNF-α/ adiponectin ratio was found in HCV infected patients that is related to the development of IR and atherosclerosis[154].

Clearance of HCV by DAAs is associated with an improvement in atherosclerosis and metabolic and immunological conditions that promote the development of CVD[152]. Several studies reported the association between the achievement of SVR by DAAs and a significant reduction of the risk of acute coronary syndrome, CVD, and heart failure[84,155,156]. In pre-diabetic patients, Sasso *et al*[157] conducted a prospective multicenter study on prediabetic HCV positive cohort. They concluded that HCV eradication by DAAs allows a significant reduction of major CVD in the pre-diabetic population, regardless of the severity of liver disease and CV risk factors (age and hypercholesterolemia). This positive effect is mainly due to an improvement of serum markers of endothelial dysfunction and glucose metabolism[158,159].

### *Hepatitis C virus and non-Hodgkin lymphoma*

A positive association was present between HCV and B-cell non-Hodgkin lymphoma (NHL) and it was detected among 5-15% of HCV patients. NHL includes marginal zone lymphoma, diffuse large B-cell lymphoma, lymphoplasmacytic lymphoma, follicular lymphoma, Burkitt's lymphoma, non-Hodgkin T-cell lymphoma, and primary cutaneous T-cell lymphoma[160-162].

There are several HCV mechanisms determining neoplastic lymphoproliferative diseases. Bcell receptors are continuously stimulated by HCV viral antigens, leading to consecutive Bcell proliferation. HCV replication inside Bcells produces HCVderived viral proteins that induce genetic damage in the Bcells. The HCV envelope protein E3 binds to CD81 on the surface of Blymphocytes and forms a complex with CD19 and CD21, which in turn stimulates intracellular proliferative signals. When HCV enters B-cells, it causes oxidative stress that might result in mutations and defective DNA repair. Moreover, HCV infections are associated with increased frequencies of *BCL6* and *p53* gene mutations in Bcells. Accordingly, HCVrelated lymphomagenesis may be attributed to either chronic viral antigen stimulation or genetic mutations that lead to the clonal expansion and malignant transformation of Bcells[163-165]. In addition, MC is considered as a B-cell benign lymphoproliferative disorder frequently induced by HCV infection[166]. Figure 1 shows the pathophysiology of HCV infection in the occurrence of NHL.

DAA regimens in combination or after the completion of immunochemotherapy should be recommended. DAA treatment has been reported to reduce the frequency of the malignant B-cells in peripheral blood of patients affected by HCV-related lymphoproliferative disorders[167-169]. Moreover, DAAs might have a lower anti-lymphoma activity than IFN[84,170].

### *Hepatitis C virus and neuro-psychiatric manifestations*

Up to 50% of patients with chronic HCV infection has neuropsychiatric symptoms. Among the reported psychiatric symptoms in chronic HCV patients are brain fog, depression, anxiety, weakness, and fatigue. These alterations lead to impaired quality of life[171]. The term HCV-associated neurocognitive disorder is used to refer to fundamental cognitive deficits unrelated to the severity of liver disease, viral load, and genotype and therefore distinct from the potentially reversible complications seen in patients with minimal hepatic encephalopathy (MHE)[172].

Chronic HCV patients exhibit prevalent involvement of the frontal lobe, which is responsible for alterations of executive functions[173] and the posterior regions of the cerebral cortex, particularly the occipital and parietal lobes[174]. Therefore, they exhibit difficulties in problem solving, monitoring one's own behavior, self-control, cognitive flexibility, working memory, volition, sustained attention, and logical reasoning, in addition to verbal learning and verbal recall[175,176]. On the other hand, cognitive domains related to posterior brain regions, primarily involved in visuospatial, visual perceptual abilities, and constructive practice regions are mainly altered in patients with MHE[172]. Moreover, T2DM as an EHM in HCV infected patients may be associated with cognitive impairments [177]. Conversely, some studies did not confirm the association between chronic HCV infection and neurologic disorders[178]. Direct neuroinvasion changes in metabolic pathways and cerebral and systemic inflammation have been proposed as pathogenetic mechanisms[179]. Central fatigue and depression may share the same neurobiological causal pathways triggered by HCV infection[180]. Lower levels of dopamine were found in the ascending reticular activating and limbic systems among fatigued patients with HCV infection possibly from cytokine-induced reduction in tetrahydrobiopterin, which is an enzyme involved in the dopamine synthesis[181,182]. Impaired serotonin transmission implicated in depression has also been correlated with increased fatigue[183].

The prevalence of depression in HCV patients ranges from 20 to 50%, compared to a 10% prevalence in the general population[184]. In the US National Health and Nutrition Examination Survey, a cross-sectional study involving 10231 patients suffering from various liver diseases, only HCV was found to be independently associated with depression. The presence and severity of depression were independent of cirrhotic status, viral load, degree of hepatic inflammation, and use of IFN[185]. They suggested the presence of another exclusive HCV-mediated mechanism contributing to depression. Depression among people with HCV infection may be partially attributed to social and occupational limitations that may precede the infection, often causing viral acquisition, *e.g.*, through intravenous drug use. Awareness of infection with subsequent poor acceptance and social stigma contributes to depressive symptoms independent of socioeconomic status or educational level[186]. Associated HCV complications such as cirrhosis, ascites, and encephalopathy, and comorbidities such as IR, RM, and CVD lead to limitation in physical function, and thus create higher physical load leading to increased depression[187].

The use of IFN was poorly tolerated as it was associated with neuropsychiatric disorders and impaired health related quality of life (HRQOL) in up to 70% of patients[172]. These disorders induced depression during and at the end of IFN treatment[188]. On the other hand, DAA regimens showed no significant psychiatric side effects and patients experience an improved HRQOL while on treatment, regardless of the stage of liver disease[189-192]. Viral clearance attained by DAAs improves fatigue, physical function, mental health, cognitive functions, and quality of life[193-196]. Several studies reported a significant reduction in the choline/creatine and myo-inositol/creatine ratios as well as an increase in cognitive functions in HCV infected patients who reached SVR compared to untreated patients or patients who did not achieve SVR[173,195]. It is suggested that the persistence of some cognitive symptoms at the end of therapy with DAAs can be attributed to compartmentalization of virus in the central nervous system that may represent a potential source of its reactivation[182,197].

Mazzaro *et al*[84] recommended starting DAAs as early as possible in the natural history of HCV infection. Early therapeutic approach not only will cure many of the EHMs that are still in a reversible stage, but it also can prevent those that develop due to delayed treatment.

## CONCLUSION

HCV is a common cause of liver disease and is associated with a variety of EHMs. Among these manifestations are the rheumatologic diseases, T2DM, IR, several glomerulopathies, cardiovascular diseases, neuropsychiatric, and cognitive disorders.

DAA regimens are considered pan-genotypic as they achieve a SVR of > 85% at 12 wk through all the major HCV genotypes as well as a very high percentage of SVR even in advanced fibrosis and cirrhosis. DAAs improved the symptoms of EHMs and reduced the risk of complications. The risk factors for DAA failure include advanced liver fibrosis/cirrhosis and the presence of RAS in the region targeted by the received DAAs. The effectiveness of GLE/PIB is reduced in HCV genotype 3 with baseline RAS like A30K, Y93H, and P53del. Baseline RAS testing is recommended for HCV genotype 3 infected patients with liver cirrhosis, as those without a baseline Y93H RAS in NS5A are eligible for SOF/VEL therapy. While, those with baseline Y93H RAS could be treated with SOF/VEL/VOX or SOF/VEL plus RBV. Moreover, EASL recommended the identification of baseline RAS for HCV genotype 1a. No RAS was reported in the NS5B region. The higher rate of HCC after DAA therapy may be explained by the fact that DAA regimens are offered to patients with advanced liver fibrosis and compensated or decompensated cirrhosis, where IFN was contraindicated. In addition, the change in the growth of pre-existing subclinical undetectable HCC upon DAA treatment might be a cause. Furthermore, after DAA therapy, the T cell-dependent immune response is much weaker upon HCV clearance, and the down-regulation of TNF-α or the elevated neutrophil to lymphocyte ratio might increase the risk of HCC.

DAAs appear to be safe for patients with a history of treated HCC except for cases with vascular invasion. DAAs can result in reactivation of HBV in HCV co-infected patients.

Concerning EHMs, DAAs are now the drug of choice for HCV-associated MCV, and they can achieve clinical and immunological responses for cutaneous and musculoskeletal manifestations, and peripheral nerve and renal involvement. DAAs have rapid and high effectiveness in thrombocytopenia. They also improve IR by 90%, increased glomerular filtration rate, and decrease proteinuria, hematuria, articular manifestations, and lymphoproliferative disorders. Moreover, HCV clearance by DAAs allows a significant improvement in atherosclerosis and metabolic and immunological conditions with a reduction of major cardiovascular events, regardless of the severity of liver disease. DAA treatment also improves physical function, fatigue, and HRQOL greatly during and at the end of treatment as well as at SVR. Viral clearance attained by DAAs improves also cognitive functions in patients with subtle cognitive defects independent of their liver condition. Early therapeutic approach with DAAs not only will cure many of the EHMs that are still in a reversible stage, but it can also prevent those develop due to delayed treatment.

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## FOOTNOTES

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**Country/Territory of origin:** Egypt

**ORCID number:** Iman Ibrahim Salama 0000-0001-8901-4625; Hala M Raslan 0000-0002-7571-5241; Ghada A Abdel-Latif 0000-0001-7165-0594; Somaia I Salama 0000-0001-8013-2342; Samia M Sami 0000-0003-1832-2932; Fatma A Shaaban 0000-0003-4129-2727; Aida M Abdelmohsen 0000-0003-4918-8967; Walaa A Fouad 0000-0001-9687-8245.

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*MINIREVIEWS*

# Second-line treatment of advanced hepatocellular carcinoma: Time for more individualized treatment options?

Senthil Rajappa, Kun-Ming Rau, Palanki Satya Dattatreya, Anant Ramaswamy, Philana Fernandes, Aarohan Pruthi, Rebecca Cheng, Mariusz Lukanowski, Yi-Hsiang Huang

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**Senthil Rajappa,** Department of Medical Oncology, Basavatarakam Indo-American Cancer Hospital and Research Institute, Hyderabad 500034, Telangana, India

**Kun-Ming Rau,** College of Medicine, I-Shou University, Kaohsiung 822, Taiwan

**Palanki Satya Dattatreya,** Department of Oncology, Omega Hospital, Hyderabad 500034, India

**Anant Ramaswamy,** Department of Oncology, Tata Memorial Hospital, Mumbai 400012, India

**Philana Fernandes,** Global Scientific Communications, Eli Lilly and Company Ltd, Cork 48006, Cork, Ireland

**Aarohan Pruthi,** Medical Affairs, Eli Lilly India, Haryana 122001, India

**Rebecca Cheng,** Medical Affairs, Eli Lilly Taiwan, Taipei 10543, Taiwan

**Mariusz Lukanowski,** Medical Affairs, Eli Lilly, Herlev 2370, Denmark

**Yi-Hsiang Huang,** Division of Gastroenterology and Hepatology, Department of Medicine, Taipei Veterans General Hospital, Institute of Clinical Medicine, National Yang-Ming Yang Ming Chiao Tung University School of Medicine, Taipei 112, Taiwan

**Corresponding author:** Yi-Hsiang Huang, Doctor, MD, PhD, Professor, Division of Gastroenterology and Hepatology, Department of Medicine, Taipei Veterans General Hospital, Institute of Clinical Medicine, National Yang-Ming Yang Ming Chiao Tung University School of Medicine, No. 201, Sec 2, Shi-Pai Road, Taipei 112, Taiwan. yhhuang@vghtpe.gov.tw

## Abstract

Hepatocellular carcinoma (HCC) is the most frequently diagnosed primary tumor of the liver and is usually detected as advanced disease. It is an aggressive disease that often progresses rapidly when it fails to respond to treatment. As such, patients have limited opportunities to try different subsequent-line treatment regimens. In the last 5 years, the number of agents and/or regimens available for the treatment of advanced HCC has significantly increased, which has made treatment choices for this patient population increasingly complex. In the second-line setting, several phase III trials of regorafenib (RESORCE), ramucirumab (REACH/REACH-2), and cabozantinib (CELESTIAL) have demonstrated clinically meaningful survival benefits in patients with the disease. However, the median overall survival of patients with advanced HCC remains unchanged at

approximately 12 mo from the start of systemic second-line therapy, with a limited duration of response. Evidence from the REACH/REACH-2 trials demonstrated for the first time that baseline alpha-fetoprotein (AFP) levels can be used as an identification factor to select those who are likely to benefit the most from ramucirumab treatment. Ramucirumab is both well tolerated and efficacious and has a clinically acceptable safety profile. Therefore, it should be considered an option for patients with AFP levels ≥ 400 ng/mL.

**Key Words:** Hepatocellular carcinoma; Alpha-fetoprotein; Prognostic factor; Ramucirumab; Second-line treatment; Survival

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**Core Tip:** Hepatocellular carcinoma (HCC) is the most frequently diagnosed primary tumor of the liver and is usually detected as advanced disease. Identifying any predictive or prognostic factors prior to and during systemic treatment of HCC is critical in determining optimal treatment patterns. Here, we summarize the contributions of the most recently developed treatment options in HCC beyond first line to improve outcomes for these patients.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequently diagnosed primary liver tumor, the sixth most common neoplasm overall, and the cause of 8.3% of all cancer-related deaths worldwide in 2020[1]. In total, 80% of patients with HCC are diagnosed in developing countries, with the largest burden in Asia, predominantly due to hepatitis B virus (HBV) infection[2,3].

Treatment of HCC is largely influenced by disease stage and usually based on the Barcelona Clinic Liver Cancer model, which accounts for factors used to predict prognosis such as tumor burden, liver function, and performance status[4]. Curative treatment options, such as liver transplant, surgical resection and radiofrequency ablation, are restricted to patients with early-stage HCC. Transarterial therapies, including conventional transarterial chemoembolization, prolong survival for patients with liver-localized disease for whom surgery is not an option[5,6]. However, not all patients with non-resectable HCC are able to benefit from transarterial chemoembolization, especially for patients with multiple and large tumors[7]. Worldwide, the majority of patients with HCC present with advanced disease and are candidates for systemic therapy opposed to liver-directed approaches[8].

Sorafenib was the first effective first-line treatment approved for advanced HCC after it improved overall survival (OS) in two double-blind, randomized clinical trials (RCTs)[9]. The relative risk of death was reduced by 30% [hazard ratio (HR) = 0.69, 95% confidence interval (CI): 0.55-0.87] compared with best supportive care (BSC) in the larger SHARP study[10,11]. Sorafenib is currently a standard systemic therapy indicated in patients with no chronic liver disease (Child-Turcotte-Pugh class A) and in specific patients with Child-Turcotte-Pugh class B disease with advanced tumors (Barcelona Clinic Liver Cancer stage C) or tumors that have progressed after locoregional therapy. In 2018, REFLECT, a phase III non-inferiority trial, demonstrated that envatinib was non-inferior for OS and significantly increased progression-free survival (PFS) relative to sorafenib. Additionally, time to progression (TTP) and objective response rate (ORR) were significantly increased with envatinib[12]. Lenvatinib was subsequentially granted approval for the treatment of patients with advanced or unresectable HCC who have received no prior systemic therapy. Recently, the United States Food and Drug Administration (FDA) granted approval of atezolizumab in combination with bevacizumab for the treatment of patients with unresectable or metastatic HCC who have not received prior systemic therapy. The approval was based upon findings from the phase III Imbrave150 clinical trial, which was the first to demonstrate an improved OS and PFS for immunotherapy *vs* sorafenib in patients with advanced HCC[13]. A 12-mo follow-up demonstrated a median OS of 19.2 mo with atezolizumab plus bevacizumab *vs* 13.4 mo with sorafenib (HR = 0.66; 95%CI: 0.52-0.85; $P$ = 0.0009). At 18 mo, the survival rate was 52% with atezolizumab plus bevacizumab and 40% with sorafenib, which is the longest survival recorded in a front-line phase III study in patients with advanced HCC[13].

There is an unmet need for second- and later-line therapies for patients who experience disease progression or demonstrate intolerance to first-line treatment. In the last 5 years, the number of agents/regimens available for the treatment of advanced HCC have increased significantly, making treatment choices complex for this patient population. HCC is an aggressive disease, often progressing rapidly when it fails to respond to treatment, giving patients limited opportunities to try different treatment regimens. Therefore, identifying any predictive or prognostic factors before and during systemic treatment is critical to the determination of optimal treatment patterns.

It is well accepted that the development of HCC is age-dependent. Given the increasing average life expectancy worldwide, treatment of elderly patients with HCC is becoming a significant global health issue. The likelihood of comorbidities such as diabetes, renal failure, and pulmonary and cardiovascular diseases means that the optimal treatment strategy is often difficult to define in such patients. Consequently, there is not only a risk of overtreatment in those with inherent fragility, causing severe toxicities, but also a risk of elderly but otherwise fit patients being undertreated. Furthermore, data on the treatment and management of elderly patients with HCC are lacking, and where data are available, the heterogeneous definitions of elderly make it difficult to interpret the data.

Serum alpha-fetoprotein (AFP) concentrations ≥ 400 ng/mL in patients with HCC have consistently been associated with worse outcomes including larger tumors, bilobar involvement, portal vein invasion, poorly differentiated histology, and decreased median survival[14,15]. Conversely, AFP response, defined as a ≥ 20% decrease in AFP levels, either from baseline or over an 8-wk period[16,17], has been associated with improved survival in patients with HCC treated with locoregional therapies such as chemotherapy, ablation, or surgery[18]. AFP response, *i.e.*, changes in AFP at treatment discontinuation, relative to baseline can predict the survival of patients with advanced HCC treated with sorafenib with or without transarterial chemoembolization[19]. Given that roughly half of all patients with advanced HCC have AFP concentrations ≥ 400 ng/mL[20,21], well-tolerated effective treatments are much needed in this population.

HCC incidence and mortality rates vary according to ethnicity, which are mainly attributed to differences in the prevalence of major risk factors such as HBV infection and disparities in access to high-quality medical care. The HCC incidence and mortality rate are particularly high in East and Southeast Asia. In patients with HCC, serum AFP levels can range from normal (0-20 ng/mL) to > 100000 ng/mL[22,23]. Several retrospective reports have noted that AFP levels appear to differ among ethnic groups[24,25], with Asian populations consistently being associated with elevated AFP levels when diagnosed with HCC. For example, the median baseline AFP for Asian patients in the pooled analysis of REACH-2 and REACH was more than twice that for non-Asian patients, with a median of 7107 ng/mL *vs* 2801 ng/mL for ramucirumab-treated patients[26]. In Sri Lanka, 23% of patients with HCC had AFP levels > 400 ng/mL[27], whereas 36% of Middle Eastern patients with HCC had levels > 200 ng/mL[28], and increased (20-200 ng/mL) levels have been reported repeatedly in Chinese patients with HCC[29-31].

In this narrative review, we summarize the efficacy and safety of second-line treatments for patients with HCC and important subgroups of patients with HCC, using OS, PFS, and tolerability data from phase III HCC RCTs. Our aim was to evaluate the contributions of second-line treatment options in the improvement of patient outcomes and highlight the importance of ramucirumab in this context.

## CURRENT SECOND-LINE OPTIONS FOR PATIENTS WITH HCC: TYROSINE KINASE INHIBITORS

### *Regorafenib*

Regorafenib is an oral multikinase inhibitor that blocks the signaling pathways involved in tumor angiogenesis [vascular endothelial growth factor (VEGFreceptors) 1-3 and tyrosine kinase, endothelial], oncogenesis [proto-oncogene c-KIT, rearranged during transfection (RET), Raf-1 proto-oncogene, serine/threonine kinase, and B-Raf proto-oncogene, serine/threonine kinase], metastasis, and tumor immunity[32]. Although sorafenib and regorafenib block similar kinases, regorafenib has a broader inhibitory profile and greater pharmacological activity.

**RESORCE:** In Regorafenib after Sorafenib in Patients with HCC (RESORCE), a randomized, double-blind, placebo-controlled, phase III trial, patients who had tolerated sorafenib treatment but had documented radiographic progression received regorafenib[21]. Tolerance was defined as receiving sorafenib ≥ 400 mg daily for ≥ 20 of a total of 28 d before discontinuation of treatment. Patients were excluded if they had discontinued sorafenib for toxicity reasons, probably because regorafenib has multikinase inhibitory activity similar to that of sorafenib. In the pivotal sorafenib SHARP study, 44% of the patients treated with sorafenib required dose adaptations because they experienced adverse events (AEs)[11]. In RESORCE, patients were randomized to receive once-daily oral regorafenib 160 mg or placebo for the first 21 d of 28-d cycles. Regorafenib improved OS, with a median survival of 10.6 mo (95%CI: 9.1-12.1) compared with 7.8 mo (95%CI: 6.3-8.8) with placebo (HR = 0.63; 95%CI: 0.50-0.79; one-sided $P < 0.0001$); this improvement in OS with regorafenib was maintained in all pre-planned subgroup

analyses. An OR was achieved in 40 (11%) regorafenib-treated patients compared with 8 (4%) placebo-treated patients. Median PFS by Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 was 3.4 mo (95%CI: 2.9-4.2) with regorafenib and 1.5 mo (95%CI: 1.4-1.5) with placebo (HR = 0.43; 95%CI: 0.35-0.52; one-sided $P < 0.0001$). Median TTP by RECIST 1.1 was 3.9 mo (95%CI: 2.9-4.2) with regorafenib and 1.5 mo (95%CI: 1.4-1.6) with placebo (HR = 0.41; 95%CI: 0.34-0.51). The most frequent clinically relevant grade 3 or 4 AEs in the regorafenib and placebo groups were hypertension [$n = 57$ (15%) *vs* $n = 9$ (5%)], palmar–plantar erythrodysesthesia [also known as hand foot skin reaction (HFSR)] [$n = 47$ (13%) *vs* $n = 1$ (1%)], fatigue [$n = 34$ (9%) *vs* $n = 9$ (5%)] and diarrhea [$n = 12$ (3%) *vs* $n = 0$ (0%)]. The most common AEs leading to discontinuation more frequently with regorafenib than with placebo were increased aspartate aminotransferase concentrations (8 of 374 patients receiving regorafenib *vs* 3 of 193 patients receiving placebo), HFSR (7 of 374 *vs* none), and increased alanine aminotransferase (4 of 374 *vs* none).

Patient-reported outcomes are an important component of assessing the benefits of treatment in advanced HCC. Health-related quality of life (HRQoL) derived from the Functional Assessment of Cancer Therapy - Hepatobiliary (FACT-Hep) questionnaire is considered a predictor of survival for patients with HCC and also contributes prognostic data to the Eastern Cooperative Oncology Group performance status. Although the FACT-Hep result for regorafenib *vs* placebo was statistically significant, it did not meet the threshold for clinical significance[21]. There were no clinically meaningful differences in HRQoL between regorafenib- and placebo-treated patients with the EQ-5D index or the EQ-5D visual analogue scale, and FACT-General scores were similar between the treatment groups[21,33].

The findings of the RESORCE trial led to the first approval of a drug as a second-line treatment for patients with HCC following sorafenib in the first line. Further exploratory analyses of the RESORCE trial demonstrated that regorafenib improved clinical outcomes in patients regardless of the speed of their disease progression or their last sorafenib dose, suggesting that sequencing therapy in this manner may extend patient survival[34].

In a separate retrospective analysis, Japanese patients who received lenvatinib as first-line, sorafenib as second-line, and regorafenib as third-line treatment demonstrated a greater PFS, ORR, and disease control rate (DCR) of 3.8 mo, 17.6%, and 41.2%, respectively, compared with 1.8 mo, 1.8%, and 20.8% in patients receiving sorafenib as second-line systemic therapy only[35]. Further clinical trials are warranted to assess the potential of regorafenib as a post-treatment therapy following lenvatinib.

Of the 1142 patients treated with regorafenib in randomized placebo-controlled trials, 40% were aged ≥ 65 years and 10% were aged ≥ 75 years. Although efficacy was similar between those aged ≥ 65 years or ≥ 75 years and younger patients, the frequency of grade 3 hypertension (18% *vs* 9%) was higher in patients aged ≥ 65 years than in younger patients. Additionally, 1 patient aged ≥ 65 years experienced a grade 4 hypertension event, whereas none were reported in younger patients[36].

*Post hoc* analyses from the RESORCE trial demonstrated higher AFP response rates with regorafenib than with placebo (46% *vs* 11%); the median OS was 13.8 mo (95%CI: 11.8-16.5) in AFP responders *vs* 8.9 mo (95%CI: 8.0-9.7) in non-responders (HR = 0.57; 95%CI: 0.40-0.82)[37]. However, AFP response in the RESORCE trial was associated with an increased rate of grade 3 HFSR in the regorafenib-treated group [37].

**REFINE:** Regorafenib Observational Study in HCC (REFINE; NCT03289273) is a large ongoing multicentric observational study evaluating regorafenib in the real world. Interim analyses suggest that regorafenib performs as expected from RESORCE findings in a real-world setting, with the most common treatment-emergent AEs (TEAEs) similar to those reported in RESORCE.

### *Cabozantinib*

Cabozantinib, an orally bioavailable inhibitor of tyrosine kinases including the mesenchymal-epithelial transition receptor tyrosine kinase, AXL receptor tyrosine kinase, RET, FMS-like tyrosine kinase 3, and VEGF receptors (VEGFRs), was evaluated in a phase II randomized discontinuation study with 9 patient cohorts classified by tumor type, including HCC[38]. Favorable clinical outcomes in patients with HCC were observed including objective tumor responses, disease stabilization, and decreased AFP levels.

**CELESTIAL:** The subsequent phase III RCT (CELESTIAL) showed positive survival results for cabozantinib, extending OS from 8 mo with placebo to 10.2 mo (HR = 0.76; 95%CI: 0.63-0.93; $P = 0.005$) and PFS from 1.9-5.2 mo (HR = 0.44; 95%CI: 0.36-0.52; $P < 0.001$)[39]. The ORR among patients in the cabozantinib group was 4% (18 of 470 patients experienced a partial response), which significantly differed from the ORR of < 1% (1 in 470 patients experienced a partial response) in the placebo group ($P = 0.009$). The grade 3 or 4 TEAEs occurring more frequently with cabozantinib compared with placebo were HFSR (17% *vs* 0%), hypertension (16% *vs* 2%), increased aspartate aminotransferase level (12% *vs* 7%), fatigue (10% *vs* 4%) and diarrhea (10% *vs* 2%). These were also the most frequent AEs of any grade that led to dose reductions among patients in the cabozantinib group.

*Post hoc* subgroup analyses of the CELESTIAL trial demonstrated that elderly patients aged > 65 years derived survival benefit from cabozantinib treatment, with an OS of 11.1 mo for cabozantinib *vs* 8.3 mo for placebo (HR = 0.74; 95%CI: 0.56-0.97) and PFS of 5.4 mo *vs* 2.0 mo (HR = 0.46; 95%CI: 0.35-0.59). Although the proportion of patients with grade 3 or 4 AEs did not differ by age, patients aged < 65 years

**Table 1 Summary of survival data from phase III randomized controlled trials of second- or later-line treatments in patients with advanced hepatocellular carcinoma**

| Ref. | Study design | Treatment arms | *n* | Patient population | Key findings |
|---|---|---|---|---|---|
| Zhu *et al*[20], REACH | Randomized, placebo-controlled, double-blind, multicenter, phase III trial | Ramucirumab or placebo | 565 | Patients with advanced HCC with previous progression or intolerance to sorafenib | Ramucirumab *vs* placebo. Median OS: 9.2 mo (95%CI: 8.0-10.6) *vs* 7.6 mo (95%CI: 6.0-9.3), HR = 0.87 (95%CI: 0.72-1.05) $P$ = 0.14. Median PFS: 2.8 mo (95%CI: 2.7-3.9) *vs* 2.1 mo (95%CI: 1.6-2.7), HR = 0.63 (95%CI: 0.52-0.75) $P$ < 0.0001 |
| Zhu *et al*[44], REACH-2 | Randomized, placebo-controlled, double-blind, multicenter, phase III trial | Ramucirumab or placebo | 292 | Patients with advanced HCC with previous progression or intolerance to sorafenib, AFP ≥ 400 ng/mL | Ramucirumab *vs* placebo. Median OS (7.6 mo follow-up): 8.5 mo (95%CI: 7.0-10.6) *vs* 7.3 mo (95%CI: 5.4-9.1), HR = 0.710 (95%CI: 0.531-0.949) $P$ = 0.0199. Median PFS: 2.8 mo (95%CI: 2.8-4.1) *vs* 1.6 mo (95%CI: 1.5-2.7), HR = 0.452 (95%CI: 0.339-0.603) $P$ < 0.0001 |
| Bruix *et al*[33], RESORCE | Randomized, double-blind, parallel-group, phase III trial | BSC + regorafenib or placebo | 573 | Patients with advanced HCC with previous progression or intolerance to sorafenib | BSC + regorafenib *vs* placebo. Median OS: 10.6 mo (95%CI: 9.1-12.1) *vs* 7.8 mo (95%CI: 6.3-8.8), HR = 0.63 (95%CI: 0.50-0.79) one-sided $P$ < 0.0001. Median PFS (RESIST 1.1): 3.4 mo (95%CI: 2.9-4.2) *vs* 1.5 mo (95%CI: 1.4-1.5), HR = 0.43 (95%CI: 0.35-0.52) $P$ < 0.0001 |
| Abou-Alfa *et al*[39], CELESTIAL | Randomized, double-blind, placebo-controlled, phase III trial | Cabozantinib or placebo | 773 | Patients with advanced HCC with previous progression or intolerance to sorafenib | Cabozantinib *vs* placebo. Median OS: 10.2 mo (95%CI: 9.1-12.0) *vs* 8.0 mo (95%CI: 6.8-9.4), HR = 0.76 (95%CI: 0.63-0.92) $P$ = 0.005. Median PFS: 5.2 mo (95%CI: 4.0-5.5) *vs* 1.9 mo (95%CI: 1.9-1.9), HR = 0.44 (95%CI: 0.36-0.52) $P$ < 0.001 |

AFP: Alpha-fetoprotein; BSC: Best supportive care; CI: Confidence interval; HCC: Hepatocellular carcinoma; HR: Hazard ratio; OS: Overall survival; PFS: Progression-free survival.

had lower AE-related discontinuation rates in the cabozantinib arm than those aged ≥ 65 years (11% *vs* 22%)[40].

A *post hoc* analysis of the CELESTIAL trial assessed QoL with cabozantinib compared with placebo [41]. During the initial treatment period, cabozantinib was associated with lower EQ-5D scores than was placebo, and following this early deterioration, differences between EQ-5D scores for cabozantinib and placebo were numerically smaller but did not reach statistical significance. *Post hoc* analyses of the CELESTIAL trial demonstrated that cabozantinib-treated patients with an AFP response had an OS increase of 7 mo relative to patients without an AFP response (16.1 *vs* 9.1; HR = 0.61; 95%CI: 0.45-0.84) and an increase of 3.3 mo in median PFS (7.3 *vs* 4.0; HR = 0.55; 95%CI: 0.41-0.74)[42].

## CURRENT SECOND-LINE OPTIONS FOR PATIENTS WITH HCC: RAMUCIRUMAB

Ramucirumab is a fully human immunoglobulin G1 monoclonal antibody that binds to and selectively inhibits VEGFR2 by preventing the binding of VEGFR ligands VEGF-A, VEGF-C, and VEGF-D. In doing so, ramucirumab inhibits a number of angiogenic pathways involved in tumor development and progression.

### *REACH-2*

Significantly higher microvessel density and VEGF tissue expression have been reported in patients with HCC who have high AFP serum levels, and the cross-talk between AFP and VEGF signaling cascades have been elucidated by *in vitro* studies[43]. The pivotal phase III trial, REACH-2, randomized patients with advanced HCC (who progressed on or were intolerant to sorafenib) and elevated baseline AFP levels (≥ 400 ng/mL) to ramucirumab ($n$ = 197) or placebo ($n$ = 95)[44].

The REACH-2 trial results demonstrated that ramucirumab reduced the risk of death by 29% in patients with HCC, with a median OS of 8.5 mo *vs* 7.3 mo for the placebo group (HR = 0.71; 95%CI: 0.53-0.95; $P$ = 0.0199). Median PFS was significantly ($P$ < 0.0001) longer in the ramucirumab group (2.8 mo; 95%CI: 2.8-4.1) than in the placebo group (1.6 mo; 95%CI: 1.5-2.7), with an HR of 0.45 (95%CI: 0.34-0.60). Although the proportion of patients with an OR did not differ significantly between treatment arms [9 of 197 (5%) *vs* 1 of 95 (1%); $P$ = 0.1697], the proportion of patients with disease control was significantly higher in the ramucirumab group than in the placebo group (59.9%; 95%CI: 53.1-66.7 *vs* 38.9%; 95%CI: 29.1-48.8; $P$ = 0.0006). Overall, the drug was well tolerated. Hypertension and hyponatremia were the sole grade 3 or higher TEAEs that occurred in ≥ 5% of patients, with greater occurrence in the ramucirumab group than in the placebo group. Conversely, aspartate aminotransferase concentrations were higher in the placebo group (5%) than in the ramucirumab group (3%). TEAEs resulting in treatment discontinuation were more frequent in the ramucirumab group than in the placebo group

(11% *vs* 4%).

***Post hoc* analysis from REACH-2 (AFP response):** In REACH-2, AFP response was significantly higher ($P < 0.0001$) with ramucirumab (42%) than with placebo (10.5%). OS for patients with and without an AFP response was 13.5 mo *vs* 6.7 mo (HR = 0.470; $P < 0.0001$)[17]. Furthermore, of the 11 patients who experienced complete normalization of their AFP levels, 8 had received ramucirumab. OS for these patients was significantly longer than for patients who experienced an AFP response without complete normalization of AFP level ($n = 111$) (25.6 mo *vs* 10.6 mo, HR = 0.147; $P = 0.0019$).

### REACH

The efficacy and safety of ramucirumab were evaluated in REACH, a phase III RCT[20]. In this trial, second-line treatment with ramucirumab failed to demonstrate an improvement in OS for patients with advanced HCC compared with placebo in an unselected population; however, pre-planned subgroup analysis showed that patients with elevated AFP values (≥ 400 ng/mL) benefited from ramucirumab treatment, with such patients experiencing improved outcomes in the ramucirumab arm: Longer median OS (7.8 mo; 95%CI: 5.8-9.3 *vs* 4.2 mo; 95%CI: 3.7-4.8) and PFS (7.8 mo; 95%CI: 5.8-9.3 *vs* 4.2 mo; 95%CI: 3.7-4.8; HR = 0.70; 95%CI: 0.53-0.92) *vs* the placebo arm. A Cox model with baseline AFP fitted as a continuous variable was used to evaluate the interaction between the treatment effect of ramucirumab on survival and baseline AFP concentrations. Results suggested that ramucirumab had an increased efficacy with increasing values of baseline AFP. This finding ultimately led to the development of the aforementioned REACH-2 study. A summary of survival data from phase III randomized controlled trials of second- or later-line treatments in patients with advanced HCC are presented in Table 1.

***Post hoc* analysis from REACH (AFP response):** Patients with an AFP response in REACH demonstrated significantly longer median OS than patients without an AFP response (13.6 mo *vs* 6.2 mo; HR = 0.46; 95%CI: 0.34-0.62; $P < 0.0001$), irrespective of treatment arm[45]. However, patients in the ramucirumab arm showed an observed benefit in delaying time to AFP progression; 3.5 mo with ramucirumab (95%CI: 2.8-4.5; $n = 283$) and 2.6 mo with placebo (95%CI: 1.6-2.8; $n = 282$; HR = 0.613; $P < 0.0001$).

**REACH and REACH-2 pooled analyses:** As both REACH and REACH-2 were international trials with similar objectives, eligibility criteria and protocols, data from both trials were combined and pooled for analyses of a larger patient population[44]. This provided greater statistical power, and treatment effects were measured with greater precision for subgroup analyses. The pooled analysis included 542 patients (ramucirumab, $n = 316$; placebo, $n = 226$) with baseline AFP concentrations ≥ 400 ng/mL. Pooled patients in the ramucirumab arm demonstrated a significantly ($P = 0.0002$) longer median OS than those in the placebo arm (8.1 mo; 95%CI: 6.9-9.3 *vs* 5.0 mo; 95%CI: 4.3-6.1; HR = 0.694; 95%CI: 0.571-0.842), which was consistent with the HRs and OS reported in the individual studies.

Improvements in PFS and the proportions of patients achieving responses or disease control in the pooled analysis were also consistent with those in each study. Both the frequency and the type of TEAEs observed in REACH-2 were also reported in the combined population[44]. These AEs are likely on-target effects from VEGFR2 inhibition. A major factor that differentiates ramucirumab from the multi-kinase inhibitors is that it does not seem to cause HFSR, so this may fulfil the need for a second-line treatment for patients with elevated AFP levels for whom first-line therapy failed because of significant HFSR.

Safety and efficacy was assessed in three prespecified age groups (< 65, ≥ 65 to < 75 and ≥ 75 years) in the pooled data of patients participating in REACH and REACH-2 with AFP ≥ 400 ng/mL in a *post hoc* subgroup analysis[46]. Ramucirumab improved median OS in all three age subgroups relative to placebo [< 65 years: 8.18 mo *vs* 4.76 mo (HR = 0.753; 95%CI: 0.581-0.975); ≥ 65 years to < 75 years: 7.62 mo *vs* 5.22 mo (HR = 0.602; 95%CI: 0.419-0.866); ≥ 75 years: 8.87 mo *vs* 6.31 mo (HR = 0.709; 95%CI: 0.420-1.199)]. Additionally, ramucirumab improved PFS relative to placebo in all three age subgroups [< 65 years: 2.73 mo *vs* 1.45 mo (HR = 0.613; 95%CI: 0.472-0.796); ≥ 65 years to < 75 years: 2.78 mo *vs* 1.84 mo (HR = 0.563; 95%CI: 0.396-0.802); ≥ 75 years: 4.17 mo *vs* 1.64 mo (HR = 0.480; 95%CI: 0.282-0.817)]. The safety profile, including the incidence of grade 3 or higher AEs, was similar between age subgroups < 65 years and ≥ 65 years to < 75 years. However, the frequency of grade 3 or higher TEAEs (hypertension and fatigue) was higher for ramucirumab (62%) than placebo (39%) in the ≥ 75 years subgroup but was similar in the two younger subgroups (54% and 60%). Proteinuria (4.1%) was the most common TEAE resulting in dose adjustment in the ramucirumab arm in patients aged < 65 years, and hypertension was most common in the two older subgroups (7.5% and 5.8%). *Post hoc* analysis indicated that AEs of interest, selected based on the known safety profile of ramucirumab, were similar across all age subgroups.

The Functional Hepatobiliary Symptom Index (FHSI-8) is a patient-administered 5-point Likert-type scale questionnaire focusing on the type and frequency of symptoms experienced by patients with hepatobiliary malignancies. Recent qualitative research supports its validity in patients with HCC and AFP ≥ 400 ng/mL[47]. The FHSI-8 questionnaire comprises eight symptoms: Lack of energy, nausea, pain, weight loss, back pain, fatigue, jaundice and stomach pain or discomfort. These patient-reported outcomes for HRQoL were assessed by age (< 65 years, ≥ 65 years to < 75 years, and ≥ 75 years) in the

**Table 2 Randomized controlled trials in hepatocellular carcinoma: Subgroup analyses[37,40,42,17,48,49]**

| | | | | | |
|---|---|---|---|---|---|
| **Ramucirumab (REACH, REACH-2 or AFP ≥ 400 ng/mL pooled population)** | | | | | |
| Patient Reported Outcomes | Pooled population REACH + REACH-2 | Ramucirumab or placebo | 542 | AFP ≥ 400 ng/mL | Ramucirumab *vs* placebo. TtD in FHSI-8 Total Score: 3.3 mo *vs* 1.9 mo, HR = 0.725; *P* = 0.0152 |
| Age | Pooled population REACH + REACH-2 | Ramucirumab or placebo | 542 | AFP ≥ 400 ng/mL | Ramucirumab *vs* placebo. < 65 yr: 8.18 mo *vs* 4.76 mo, HR = 0.716 (95%CI: 0.556-0.922). ≥ 65 to < 75 yr: 7.62 mo *vs* 5.22 mo, HR = 0.593 (95%CI: 0.413-0.851). ≥ 75 yr: 8.87 mo *vs* 6.31 mo, HR = 0.641 (95%CI: 0.390-1.054) |
| AFP dynamics | REACH-2 | Ramucirumab or placebo | 292 | AFP ≥ 400 ng/mL | Ramucirumab *vs* placebo. Time to AFP progression: 2.4 mo *vs* 1.4 mo, HR = 0.422 (95%CI: 0.309-0.576) *P* ≤ 0.0001. Time to radiographic progression: 3.0 mo *vs* 1.6 mo, HR = 0.427 (95%CI: 0.313-0.582) *P* ≤ 0.0001. AFP response: ≥ 20% decrease anytime post-baseline from baseline (% of patients): 42 *vs* 11 *P* ≤ 0.0001. ≥ 20% increase anytime post-baseline from baseline (% of patients): 62 *vs* 79, *P* = 0.0043 |
| **Regorafenib (RESORCE)** | | | | | |
| AFP response | RESORCE | Regorafenib or placebo | 232 | baseline AFP ≥ 20 ng/mL and an AFP measurement at the start of cycle 3 | Regorafenib *vs* placebo. Median OS: 13.8 mo *vs* 8.9 mo, HR = 0.57 (95%CI: 0.40-0.82) |
| **Cabozantinib (CELESTIAL)** | | | | | |
| Age subgroup | CELESTIAL | Cabozantinib or placebo | 707 | Subgroups based on age (< 65 yr and ≥ 65 yr) | Cabozatinib *vs* placebo. Median OS: < 65 yr: 9.6 mo *vs* 7.7 mo (HR = 0.81, 95%CI: 0.62-1.05); ≥ 65 yr: 11.1 mo *vs* 8.3 mo (HR = 0.74, 95%CI: 0.56-0.97). Median PFS: < 65 yr: 5.0 mo *vs* 1.9 mo (HR = 0.45, 95%CI: 0.35-0.57); ≥ 65 yr: 5.4 mo *vs* 2.0 mo (HR = 0.46, 95%CI: 0.35-0.59) |
| AFP | CELESTIAL | Cabozantinib or placebo | | Baseline AFP < 400 ng/mL | Cabozatinib *vs* placebo. Median OS: 13.9 mo *vs* 10.3 mo (HR = 0.81, 95%CI: 0.62-1.04). Median PFS: 5.5 mo *vs* 1.9 mo (HR = 0.47, 95%CI: 0.37-0.60) |
| | | | | Baseline AFP ≥ 400 ng/mL | Cabozatinib *vs* placebo. Median OS: 8.5 mo *vs* 5.2 mo (HR = 0.71, 95%CI: 0.54-0.94). Median PFS: 3.9 mo *vs* 1.9 mo (HR = 0.42, 95%CI: 0.32-0.55) |

AFP: Alpha-fetoprotein; CI: Confidence interval; FHSI: Functional Hepatobiliary Symptom Index; HR: Hazard ratio; OS: Overall survival; PFS: Progression-free survival.

pooled REACH/REACH-2 dataset[46,48,49]. Treatment with ramucirumab resulted in a delay in the deterioration of symptoms as measured by FHSI-8 compared with placebo across all subgroups, although this was not significant. Median time to deterioration was also numerically longer with ramucirumab than with placebo in all three age subgroups. Together, these results support the use of ramucirumab for the treatment of HCC with elevated AFP after prior sorafenib treatment, irrespective of age.

A limitation of the design of both REACH trials was that it excluded patients who received first-line systemic treatment with any drug except for sorafenib, as this was the only therapy associated with an OS benefit at the time. To address this limitation, an ongoing global open-label expansion cohort of REACH-2 is evaluating ramucirumab in patients with advanced HCC and baseline AFP ≥ 400 ng/mL following a non-sorafenib-based systemic therapy[50]. Recently, final results from an expansion cohort of REACH-2 were presented at the 2022 American Society of Clinical Oncology Gastrointestinal Cancers Symposium. Of 47 patients, 51% with second- to third-line or more advanced HCC were classed as Eastern Cooperative Oncology Group performance status 1 at baseline, with a median AFP of 3236 ng/mL. The majority of patients had received lenvatinib ($n$ = 20) as a prior systemic regimen, followed by checkpoint inhibitor (CPI) monotherapy ($n$ = 11), CPI plus an antiangiogenic ($n$ = 15), and CPI plus another CPI ($n$ = 4). Grade 3 or higher TEAEs were reported in 57% ($n$ = 27) of patients, 23% ($n$ = 11) of which were classified as treatment related. The most frequent grade 3 or higher AEs occurring in ≥ 5% of patients were hypertension (11%), followed by proteinuria, hyponatremia and increased aspartate aminotransferase (6% each). Two deaths associated with treatment-related AEs were reported during treatment or within 30 d following treatment discontinuation. The median OS, PFS, and TTP were 8.7 mo (95%CI: 4.6-12.2), 1.7 mo (95%CI: 1.5-4.1) and 2.8 mo (95%CI: 1.5-4.2), respectively. The ORR was 10.6% (95%CI: 1.8-19.5; $n$ = 5), with a median duration of response (DOR) of 8.3 mo (95%CI: 2.4-not reached)[51]. These results indicate that the safety and efficacy of ramucirumab following a non-sorafenib-based systemic therapy was consistent with results of the REACH-2 study in patients following prior sorafenib treatment. Table 2 summarizes subgroup analyses of randomized controlled trials in HCC.

## CURRENT SECOND-LINE OPTIONS FOR PATIENTS WITH HCC: IMMUNE CPIS

Immune CPIs are revolutionizing the treatment of HCC, and immunotherapy biomarker development to identify patients with the best potential response has necessarily become a research priority. Whilst persistent HBV and hepatitis C virus infection can contribute to chronic inflammatory conditions in the liver, the immunosuppressive properties of these infections, as well as the inherent unique immunobiology of the liver, are well documented, meaning that HCC is generally not regarded as an immunogenic tumor. Nevertheless, immunotherapy has been explored as both first- and second-line options for patients with advanced HCC.

### *Nivolumab*

Antibodies that disrupt programmed cell death-1 (PD-1) immune checkpoint signaling have the potential to restore the antitumor activity of otherwise suppressed effector T cells. Nivolumab, a fully human immunoglobulin G4 monoclonal antibody, was evaluated for its potential to treat patients with HCC in the second-line setting in the phase I/II dose-escalation and expansion study CheckMate 040, an open-label, non-comparative trial carried out in the United States[52]. In this study, nivolumab treatment resulted in substantial tumor reductions and an ORR of 15% (95%CI: 6-28) in patients with advanced HCC in the dose-escalation phase, with responses occurring early in treatment. The DCR, median TTP and median DOR were 58% (95%CI: 43-72), 3.4 mo (95%CI: 1.6-6.9) and 17 mo (95%CI: 6-24), respectively. OS at both 6 and 9 mo was 66% (95%CI: 51-78). Patients in the dose-escalation phase demonstrated a median OS of 15.0 mo (95%CI: 9.6-20.2), and the median DOR in both phases of the study suggested that nivolumab might offer durable responses hitherto unseen in patients with HCC. Overall, these results were encouraging in the metastatic setting in patients who were previously treated with sorafenib.

Given the favorable ORR and the improved 9-mo OS rates in CheckMate 040[53], the United States FDA granted nivolumab accelerated approval as a second-line treatment option in the United States despite the study lacking a randomized control arm[53], a major limitation of the study. In the subsequent phase III CheckMate 459 trial, nivolumab failed to significantly improve OS *vs* sorafenib in patients without previous systemic treatment[54].

A randomized cohort expansion phase of the CheckMate 040 study demonstrated that a combination approach may have merit: Nivolumab in combination with ipilimumab resulted in clinically meaningful responses, with an ORR of 31%, DCR of 49%, 24-mo OS of 40% and a more than 2-fold increase in ORR compared with nivolumab monotherapy (31% *vs* 14%)[55]. Although these findings led to FDA approval of the combination of nivolumab plus ipilimumab in a second-line setting for the treatment of advanced HCC, the FDA Oncologic Drug Advisory Committee recently voted 5:4 against the continued accelerated approval of nivolumab[56].

### *Pembrolizumab*

Pembrolizumab, a humanized monoclonal anti-PD1 antibody, showed promising clinical efficacy and manageable safety in patients with advanced HCC in a non-randomized, open-label phase II trial (KEYNOTE-224)[57]. Following these results, accelerated approval of pembrolizumab was granted in November 2018 for patients with HCC who received prior treatment with sorafenib. The randomized, double-blind, placebo-controlled, phase III trial (KEYNOTE-240) evaluated the efficacy and safety of pembrolizumab plus BSC *vs* placebo plus BSC in the second line setting[58,59]. Although PFS and OS were numerically improved *vs* placebo, KEYNOTE-240 did not meet its prespecified statistical dual endpoints of improvements in PFS and OS. Programmed cell death ligand 1 expression in immune and tumor cells in patients enrolled in KEYNOTE-224 was positively associated with response to anti-PD-1 therapy with pembrolizumab[57]. A similar observation in patients enrolled in KEYNOTE-240 is yet to be confirmed. KEYNOTE-394 is another ongoing trial in the same setting, and results are anticipated soon. At the recent Oncologic Drug Advisory Committee meeting, continuing the accelerated approval for pembrolizumab in sorafenib-pre-treated patients with HCC was unanimously sanctioned[60].

Recent real-world evidence from Taiwan demonstrated that patients who received nivolumab or pembrolizumab as second-line therapy for unresectable HCC achieved an ORR of 24.4%, indicating that a certain subset of patients may benefit from immunotherapy following sorafenib failure[61]. In this study, a novel 10-10 rule (baseline AFP level ≥ 10 ng/mL and 10% reduction within 4 wk of treatment) was proposed to predict survival following immunotherapy in patients with unresectable HCC.

## CONCLUSION

Drug-related AEs, complications due to liver disease, the safety profile of the candidate therapy and the patient's QoL all aid in the identification of a suitable second-line drug for patients with advanced HCC after first-line treatment. The role of immune CPIs is somewhat unclear in second-line HCC treatment. Despite being granted accelerated approval by the FDA in the second line setting after failure of sorafenib, both nivolumab and pembrolizumab were recently removed from the European Society for

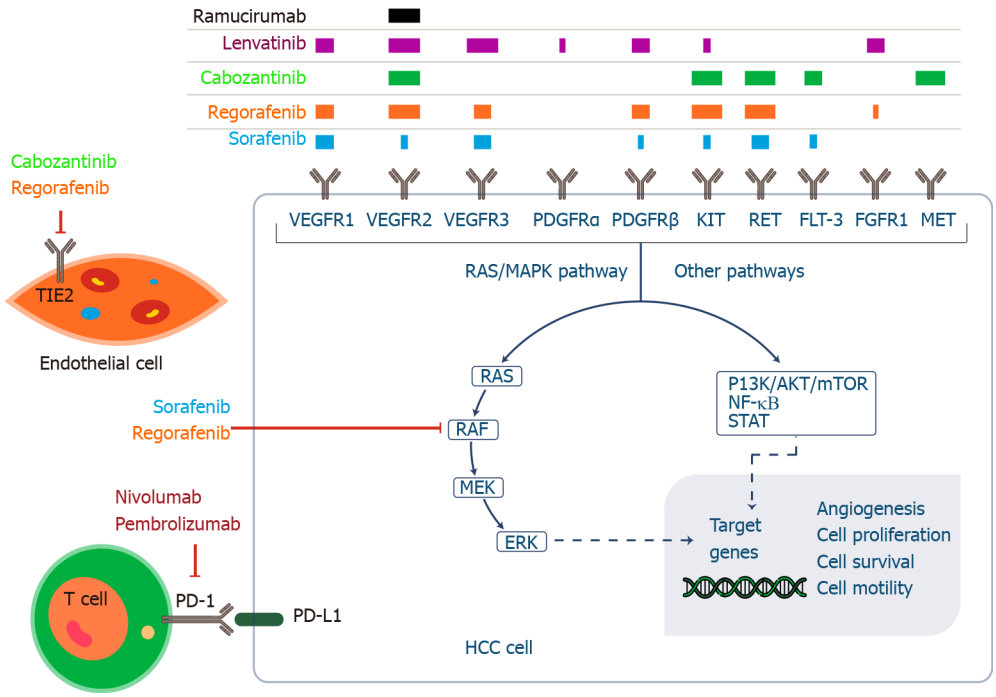


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**Figure 1 Graphical abstract.** ERK: Extracellular receptor kinase; HCC: Hepatocellular carcinoma; FGFR: Fibroblast growth factor receptor; FLT-3: Cytokine Flt3 ligand; KIT: Tyrosine-protein kinase; MEK: Mitogen-activated protein kinase; MET: Mesenchymal epithelial transition factor; mTOR: Mammalian target of rapamycin; NF-kB: Nuclear factor kappa B; PD-1: Programmed cell death 1; PDGFR: Platelet-derived growth factor receptors; PD-L1: Programmed death ligand 1; RAF: Rapidly accelerated fibrosarcoma; RAS: Rat sarcoma virus; RET: Rearranged during transfection; STAT: Signal transducer and activator of transcription; VEGFR: Vascular endothelial growth factor.

Medical Oncology treatment guidelines because of their failure to demonstrate an improvement in OS and PFS as single agents.

Two tyrosine kinase inhibitors (TKIs), cabozantinib and regorafenib, and one monoclonal antibody, ramucirumab, have been approved for use after sorafenib by the FDA, the European Medicines Agency, and the Japanese Regulatory Agency in the second-line setting for the treatment of patients with advanced HCC. However, regorafenib is only suitable for patients who demonstrated prior tolerance to sorafenib. For sorafenib-intolerant patients, cabozantinib and ramucirumab remain viable treatment options. Treatment choice is also often based on several other factors, including comorbidities and the drug safety profile. For example, in patients with prior HFSR with sorafenib, the risk of recurrence with cabozantinib or regorafenib makes them less rational choices.

Research efforts to identify subgroups of patients with HCC who will benefit from specific therapies are ongoing. Ramucirumab has a very different mechanism of action to the TKIs by virtue of being a monoclonal antibody with a very high specificity for VEGFR2. Data from REACH and REACH-2 support the clinical relevance of this difference, given the contrasting toxicity profile of ramucirumab compared with the TKIs. This may contribute to the tolerability of ramucirumab in a variety of traditionally hard-to-treat patient subpopulations such as the elderly and patients who do not tolerate or whose disease progresses on sorafenib.

It is well documented that elevated AFP serum levels are associated with a poor prognosis in patients with HCC, and - given that almost half of patients have AFP concentrations ≥ 400 ng/mL following sorafenib treatment - efficacious and well-tolerated options are needed for such patients. Evidence from the REACH-2/REACH trials demonstrated for the first time that baseline AFP levels can be used as an identification factor to select patients who are likely to reap the greatest benefits from ramucirumab treatment. In the face of multiple second-line options for patients with advanced HCC, the onus is on the physician to make a judicious choice. Ramucirumab has been shown to be both well-tolerated and efficacious for patients with baseline AFP ≥ 400 ng/mL and to have a clinically acceptable safety profile. Graphical abstract is shown in Figure 1.

## FOOTNOTES

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**Country/Territory of origin:** Ireland

**ORCID number:** Senthil Rajappa 0000-0002-5597-756X; Kun-Ming Rau 0000-0001-6090-4341; Palanki Satya Dattatreya 0000-0001-6090-4342; Anant Ramaswamy 0000-0002-8169-4264; Philana Fernandes 0000-0002-7978-7066; Aarohan Pruthi 0000-0001-9796-8672; Rebecca Cheng 0000-0003-0987-2430; Mariusz Lukanowski 0000-0001-7460-3363; Yi-Hsiang Huang 0000-0001-5241-5425.

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*MINIREVIEWS*

# Metabolic-associated fatty liver disease from childhood to adulthood: State of art and future directions

Francesca Lanzaro, Stefano Guarino, Elisabetta D'Addio, Alessandra Salvatori, Josè Alberto D'Anna, Pierluigi Marzuillo, Emanuele Miraglia del Giudice, Anna Di Sessa

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**Francesca Lanzaro, Stefano Guarino, Elisabetta D'Addio, Alessandra Salvatori, Josè Alberto D'Anna, Pierluigi Marzuillo, Emanuele Miraglia del Giudice, Anna Di Sessa,** Department of Woman, Child, and General and Specialized Surgery, University of Campania "Luigi Vanvitelli", Naples 80138, Italy

**Corresponding author:** Pierluigi Marzuillo, MD, PhD, Assistant Professor, Department of Woman, Child, and General and Specialized Surgery, University of Campania "Luigi Vanvitelli", Via de Crecchio, 2, Naples 80138, Italy. pierluigi.marzuillo@gmail.com

## Abstract

In 2020, an international group of experts proposed to replace the term of nonalcoholic fatty liver disease with metabolic-associated fatty liver disease (MAFLD). This recent proposal reflects the close association of fatty liver with metabolic derangements, as demonstrated by previous robust data. Several factors [including genetics, inflammation, metabolic abnormalities, insulin resistance (IR), obesity, prenatal determinants, and gut–liver axis] have been found to be involved in MAFLD pathophysiology, but this tangled puzzle remains to be clearly understood. In particular, IR has been recognized as a key player in metabolic impairments development in children with fatty liver. On this ground, MAFLD definition focuses on the pathophysiological basis of the disease, by emphasizing the crucial role of metabolic impairments in this condition. Although primarily developed for adults, MAFLD diagnostic criteria have been recently updated with an age-appropriate definition for sex and age percentiles, because of the increasing attention to cardiometabolic risk in childhood. To date, accumulating evidence is available on the feasibility of MAFLD definition in clinical practice, but some data are still conflicting in highly selected populations. Considering the growing prevalence worldwide of fatty liver and its close relationship with metabolic dysfunction both in children and adults with subsequent increased cardiovascular risk, early strategies for MAFLD identification, treatment and prevention are needed. Novel therapeutic insights for MAFLD based on promising innovative biological techniques are also emerging. We aimed to summarize the most recent evidence in this intriguing research area both in children and adults.

**Key Words:** Metabolic; dysfunction; Fatty; Liver; Pathophysiology; Cardiovascular; Risk; Adults; Children

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**Core tip:** Recently, experts have proposed to rename nonalcoholic fatty liver disease as metabolic-associated fatty liver disease (MAFLD), by emphasizing the close association of fatty liver with the metabolic milieu. Given that, a growing number of studies have tested the effectiveness of the new definition in adults and children, although evidence in this latter population is still limited. However, expanding knowledge about MAFLD and its pathophysiology is crucial for a better identification of subjects at greater metabolic risk.

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## INTRODUCTION

As proposed by an international consensus in 2020[1], the nomenclature of nonalcoholic fatty liver disease (NAFLD) has been updated to metabolic-associated fatty liver disease (MAFLD). MAFLD diagnosis is based on histological (biopsy), imaging or blood biomarker evidence of hepatic steatosis, and on the presence of any condition among: (1) Overweight/obesity; (2) diabetes mellitus; or (3) evidence of metabolic dysregulation[1], commonly defined as ≥ 2 of these characteristics: (1) Waist circumference ≥ 102 cm in Caucasian male subjects and 88 cm in women (or ≥ 90/80 cm in Asian individuals); (2) blood pressure ≥ 130/85 mmHg or specific drug treatment; (3) triglyceride level ≥ 1.70 mmol/L or specific drug treatment; (4) high-density lipoprotein (HDL)-cholesterol < 1.0 mmol/L for men and < 1.3 mmol/L for women; (5) prediabetes (*i.e.*, fasting glucose levels 5.6–6.9 mmol/L, or 2-h post-load glucose levels 7.8–11.0 mmol/L or hemoglobin A1c 5.7%–6.4%; (6) homeostasis model assessment-insulin resistance (HOMA-IR) score ≥ 2.5; and (7) high-sensitive C-reactive protein (hs-CRP) level > 2 mg/L.

Numerous different factors such as inflammation, sex, age, ethnicity, diet, microbiota, hormones, and genetics have been pathogenically linked to NAFLD[2-4], but current knowledge about MAFLD pathophysiology is still limited[5-6].

During the past decades, research focused on the strong association between insulin resistance (IR) and NAFLD[7]. In particular, previous data have largely supported the role of NAFLD as a hepatic manifestation of systemic metabolic disorders[2,3]. Based on these premises, the new nomenclature aims to strengthened the close association of fatty liver with metabolic dysfunction[2,8-12] to identify early subjects at higher risk of long-term metabolic consequences.

As noted for obesity and its related consequences [*e.g.*, metabolic syndrome (MetS) and Type 2 diabetes (T2D)[13-15]], a key pathogenic role has been described for the low-grade systemic inflammation in modulating fibrosis development and the overall course of the hepatic disease. As a result, an inflammatory biomarker such as hs-CRP, has been considered as a MAFLD diagnostic criterion. However, it should be kept in mind that further specific diagnostic criteria for MetS define this peculiar cluster of metabolic abnormalities, according to age group[16,18]. In fact, the MetS definition provided for adults and children aged ≥ 10 years by the International Diabetes Federation (IDF)[16,17] was further integrated for subjects aged 2–11 years (Table 1). The comparison between MetS and MAFLD criteria (Tables 2 and 3) allows identification of MetS subjects with fatty liver as MAFLD patients. Although both conditions allow identification of subjects at higher cardiometabolic risk, the inclusion of fatty liver as a MAFLD criterion enhances the multifactorial pathophysiology of the disease and its close relationship with metabolic derangements[16-20]. Given the overall emphasis of this latter association in MAFLD definition (from normal weight to obesity), the new term includes a wide phenotypical range from metabolically unhealthy normal weight to metabolically unhealthy. Nevertheless, an accurate definition of metabolic health is still lacking, especially in patients with obesity[21].

An increasing number of studies have explored metabolically healthy obesity (MHO) and metabolically unhealthy obesity (MUO) in adult and pediatric cohorts[22-24]. MUO individuals have a higher cardiovascular risk than their metabolically healthy counterparts. However, MHO also might predispose over time to an increased risk of cardiometabolic derangements[25-27]. In light of this, a detailed clinical assessment of the cardiometabolic risk in children (including evaluation of anthropometric measures such as weight, height, waist, and hip circumferences according to age- and gender-specific percentiles and Acanthosis nigricans detection as a clinical marker of IR) represents a crucial first step for the evaluation of these patients.

**Table 1 Comparison between metabolic associated fatty liver disease and non-alcoholic fatty liver disease diagnostic criteria**

| MAFLD criteria[1] | NAFLD criteria[62] |
|---|---|
| Histological (biopsy), imaging or blood biomarker evidence of hepatic steatosis and the presence of one of these criteria: | Presence of steatosis in > 5% of hepatocytes detected by biopsy |
| (1) Overweight/obesity | -The proton density fat fraction (providing a rough estimation of the volume fraction of fatty material in the liver) > 5.6% assessed by proton magnetic resonance spectroscopy |
| (2) Diabetes mellitus | |
| (3) Evidence of metabolic dysregulation defined as the presence of ≥ 2 of the following conditions: | |
| (a) Waist circumference ≥ 102 cm in Caucasian men and 88 cm in women (or ≥ 90/80 cm in Asian men and women); | |
| (b) Blood pressure ≥ 130/85 mmHg or specific drug treatment; (c) triglyceride ≥ 1.70 mmol/L or specific drug treatment; | |
| (d) High-density lipoprotein cholesterol < 1.0 mmol/L for men and < 1.3 mmol/L for women; | |
| (e) Prediabetes (*i.e.*, fasting glucose levels 5.6–6.9 mmol/L, or 2-h postload glucose levels 7.8–11.0 mmol/L or hemoglobin A1c 5.7%–6.4%; | |
| (f) Homeostasis model assessment-insulin resistance score ≥ 2.5; | |
| and (g) High sensitive C-reactive protein > 2 mg/L | |
| | -Quantitative fat/water selective magnetic resonance imaging |
| | Exclusion of both secondary causes and a daily alcohol consumption ≥ 30 g for men and 20 g for women |

MAFLD: Metabolic associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

Adipose distribution pattern is considered to have a critical influence on MAFLD development, as demonstrated by the positive correlation of amount of visceral adipose tissue with liver inflammation and fibrosis[4].

To date, the clinical feasibility of MAFLD definition has been mostly studied in adults, but a similar growing interest is also emerging in children. Therefore, we aimed to provide a comprehensive overview by summarizing the most recent evidence on the tangled puzzle of MAFLD in adults and children.

## PATHOPHYSIOLOGY

Fatty liver pathophysiology includes a well-known spectrum of determinants such as inflammation, IR, genetics and environment[4,28,29]. Genetic determinants commonly implied in NAFLD susceptibility (such as *PNPLA3*[30-32], *TM6SF2*[33], *MBOAT7*[34-36] and *HSD17B13*[37-42] genes) have been also linked to MAFLD pathogenesis[43-45] (Table 2). In particular, the effect of the *PNPLA3* I148M polymorphism as a key genetic factor for NAFLD susceptibility across different ethnicities has been largely recognized both in adults and children[45]. Similarly, robust data have also supported the role of the *TM6SF2* gene in hepatic steatosis development both in adults and children[46-48]. Noteworthy, a pleiotropic effect has been described for both genes because of their extrahepatic role in affecting also kidney function in children with obesity[49,50] and adult with T2D and fatty liver[51]. In addition, robust evidence showed that the downregulation of the *MBOAT7* gene predisposed to fatty liver development both in children and adults[34,52,53]. In contrast, the *HSD17B13* variant has been recognized as a protective factor against liver injury and its progression[38,54,55]. As described for other well-known single nucleotide polymorphisms related to fatty liver, this variant has been found also to influence kidney function[56].

Minor genetic variants affecting IR, oxidative stress and inflammation pathways have been found to be related to fatty liver development[45,57]. In particular, a significant association between the rs17618244 G>A variant in the *KLB* gene and hepatic fibrosis has been described, and this gene is a central player in obesity and lipid and glucose metabolism, as demonstrated by its association with lobular inflammation and cirrhosis in patients clustered according to obesity degree[57].

MAFLD genetic susceptibility is still poorly explored[58,59]. Liu *et al*[59] confirmed the role of the *HSD17B13* region in a cohort of 427 Han Chinese adults as a genetic factor predisposing to MAFLD-related fibrosis and of modulated *PNPLA3* rs738409 polymorphism on fatty liver development[58].

**Table 2 Main findings of the studies on MAFLD genetics**

| Gene | Study design | Population | Gene pathophysiology | Main findings |
|---|---|---|---|---|
| *b-Klotho (KLB) gene* | Panera *et al*[57], Hospital-based retrospective cohort study | 1111 adult Italian MAFLD patients from the Metabolic Liver Diseases outpatient service at Fondazione IRCCS Ca'Granda of Milan between January 1999 and December 2019. Patients were stratified according to obesity status:<br>-BMI > 35: 708 subjects<br>-BMI ≤ 35: 403 subjects<br>Inclusion criteria were liver biopsy or severe obesity and availability of DNA samples | The *rs17618244 G>A* variant in the *b-Klotho (KLB)* gene encodes for a transmembrane protein which complexes with Fibroblast Growth Factor Receptors to bind the hormones FGF21 and FGF19. Both genes play an important role in lipid and glucose metabolism and in obesity | *KLB rs17618244* variant was linked to hepatic fibrosis<br>KLB A allele was associated with lobular inflammation and cirrhosis in patients stratified for obesity status; Hepatic KLB mut expression seemed to be linked to proliferative rate improvement and pro-fibrogenic genes induction |
| *Hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13)* gene | Liu *et al*[59], Cross-sectional analysis | 427 Han Chinese from the PERSONS cohort with biopsy confirmed MAFLD;<br>Aged ≥ 18 yr | *Hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13)* gene encodes a hepatic lipid droplet protein | Data confirmed that the *HSD17B13* region is a susceptibility locus for MAFLD-related fibrosis<br>An effect of modulated PNPLA3 rs738409 on hepatic steatosis was reported<br>Significant differences in levels of fasting glucose, triglycerides, and high-density lipoprotein cholesterol among subject with *HSD17B13*- rs72613567 (TA allele) genotypes were observed, but no differences in biochemical parameters among the rs6531975 (A allele) genotypes were found; The minor TA allele was linked to an increased risk of fibrosis, while the minor A allele had a protective effect against liver damage |
| Membrane-bound O-acyltransferase domain-containing protein 7 (MBOAT7) | (1) Meroni *et al*[52], Review: 21 studies: -6 case control studies; -10 case only; -2 metanalysis; -2 GWAS; -1 cohort studies | (1) Age: -4 pediatric studies; -17 adult studies; Ethnicity: -14 Caucasian; -5 multiethnic; -2 Asian | The MBOAT7 codifies for an enzyme highly expressed in hepatocytes, hepatic stellate cells and hepatic sinusoidal cells; It has been involved in fatty acid metabolism and in hepatic both inflammation and fibrosis | (1) In patients with MAFLD, MBOAT7 might affect liver damage<br>Downregulation of liver expression of MBOAT7 induces changes in phosphoinositide composition pattern with subsequent modified membrane lipid composition and lipid mediator profiles<br>Hyperinsulinemia, is a cofactor for MBOAT impairment; MBOAT7 dysfunction may influence liver disease progression to steatohepatitis and fibrosis and chronic hyperinsulinemia to steatosis development |
| | (2) Ismaiel *et al*[53], Review: 22 studies: -7 case control studies; -3 case only; -5 metanalysis; -7 cohort studies | (2) A total of 22 studies: -4 pediatric studies with ultrasound (US) diagnosis of fatty liver; -18 adult studies: 17 with fatty liver diagnosis with liver biopsy/ imaging and 1 with US | | (2) Except for Asian population, studies on European, Hispanic, and African American adults with MAFLD evaluating the rs641738 variant reported a downregulation of the MBOAT7 expression, which increased MAFLD severity, liver fat, NASH progression, advanced fibrosis, and HCC<br>No association with coronary artery disease was found. In children with obesity this variant was associated with increased plasma ALT levels |

MAFLD: Metabolic associated fatty liver disease; FGFR: Fibroblast growth factor receptor; ALT: Alanine transaminase; MBOAT7: Membrane-bound O-acetyltransferase domain-containing protein 7; US: Ultrasound; GWAS: genome-

wide association study.

Recent evidence supports an inverse allelic effect of the association of *HSD17B13* variants on liver damage: in particular, hepatic fibrosis risk has been found to be increased by the minor allele TA of the rs72613567 variant, while a protective role against liver damage for the minor A allele of the rs6531975 variant has been demonstrated[59].

## EVIDENCE ON MAFLD: FROM ADULTHOOD TO CHILDHOOD

As the renaming of the liver condition, the clinical usefulness of MAFLD definition has been tested in several studies[60-64] (Table 2). Lin *et al*[60] first compared MAFLD and NAFLD criteria in a large cohort of 13 083 subjects grouped as MAFLD (31.24%), NAFLD (33.23%) and non-metabolic-dysfunction-associated NAFLD (non-MD-NAFLD) (4.74%) (*e.g.*, subjects with NAFLD but not covered by MAFLD criteria)[61-62]. Authors found that patients with fatty liver were older, more likely to be male, and have worse cardiometabolic and hepatic profile independently of the used criteria[60].

Compared to NAFLD, MAFLD subjects were older (48.39 ± 15.20 years) and presented with higher body mass index (BMI), liver enzymes, and noninvasive liver fibrosis scores. In addition, an increased percentage of metabolic comorbidities (including diabetes, IR and hypertension) was reported in these patients[60]. Patients in the non-MD-NAFLD group were the youngest and presented with a better metabolic profile than those belonging to the MAFLD and NAFLD groups. In this framework, a more accurate identification of patients at higher risk of negative metabolic consequences seemed to be achieved by MAFLD criteria[60].

Conversely, no significant differences for the main clinical and biochemical variables between NAFLD and MAFLD were found in a large cohort of 780 adult patients with biopsy-proven fatty liver diagnosis [55]. Taking into account the alcohol consumption in MAFLD definition, patients with MAFLD with significant alcohol intake showed a worse hepatic profile (characterized by higher steatosis degree and transaminase levels) compared to those with MAFLD only[55].

The usefulness of MAFLD definition has been also examined by Sun *et al*[65] in a highly selected population such as patients with chronic kidney disease (CKD). Authors demonstrated a better performance of MAFLD diagnostic criteria than NAFLD in identifying patients with CKD[65], as previously found[64]. Of note, a strong and independent relationship of MAFLD and MAFLD with increased liver fibrosis scores with CKD and abnormal albuminuria was described[65].

Recently, differences between NAFLD and MAFLD criteria were tested in a 2-year follow-up Italian study conducted in 221 patients receiving a new diagnosis of celiac disease (CD) as a high-risk condition for fatty liver[66]. Compared to NAFLD, MAFLD definition allowed a better identification of CD patients at risk of disease progression and the coexistence of fibrosis seemed to enhance the occurrence of adverse outcomes in these patients[66].

Yamamura *et al*[67] compared the diagnostic accuracy of MAFLD and NAFLD in identifying individuals with significant hepatic fibrosis and clarified the influence of mild alcohol consumption (< 20 g/d) on the degree of the hepatic disease in a large cohort of 765 subjects clustered in two groups as

**Table 3 Metabolic syndrome criteria in adults and children**

| | Abdominal obesity | Hypertension | Dyslipidemia | Fasting glucose |
|---|---|---|---|---|
| IDF central obesity + 2 of 4 criteria in adult patients and children aged >10 yr[87-89] | 10–15 yr old waist circumference (WC) ≥ 90$^{th}$ percentile for age and sex | Systolic BP ≥ 130 mmHg or diastolic BP ≥ 85 mmHg | TG ≥ 150 mg/dL or specific treatment HDL < 40 mg/dL (male), HDL < 50 mg/dL (female) | ≥ 100 mg/dL or diagnosis of type 2 diabetes mellitus |
| | >15 yr old WC ≥ 94 cm (male) b WC ≥ 80 cm (female) | | | |
| Panel: IDEFICS definition of metabolic syndrome in children aged 2–11 yr[90][1] | 10–15 yr old WC ≥ 90$^{th}$ percentile for age and sex | Blood pressure: systolic ≥ 90$^{th}$ percentile or diastolic ≥ 90$^{th}$ percentile | TG: ≥ 90$^{th}$ percentile or HDL cholesterol: ≤ 10$^{th}$ percentile | Insulin ≥ 90$^{th}$ percentile or fasting glucose ≥ 90$^{th}$ percentile |
| | > 15 yr old adults criteria | | | |

[1]Children would require close monitoring if three or more of these risk factors exceed the 90$^{th}$ percentile (or ≤ 10$^{th}$ percentile for HDL cholesterol), and an intervention if three or more of these risk factors exceed the 95$^{th}$ percentile (or ≤ 5$^{th}$ percentile for HDL cholesterol).
BP: Blood pressure; HDL: High-density lipoprotein; IDEFICS: Identification and prevention of dietary- and lifestyle-induced health effects in children and infants; IDF: International diabetes federation; TG: Triglycerides; WC: Waist circumference.

NAFLD and MAFLD. Compared to NAFLD, MAFLD criteria provided careful detection of hepatic fibrosis, as reflected by the strong relationship between certain hepatic fibrosis markers and liver stiffness in patients diagnosed with MAFLD[67]. Given that, dysmetabolic patients at high risk of adverse hepatic outcomes were better identified through MAFLD than NAFLD criteria[12,21].

As the well-known relevance of alcohol intake on hepatic fibrosis risk development was not included in MAFLD definition, the authors also examined its influence on fatty liver severity[67]. Patients with MAFLD and alcohol intake of 1–59 g/d were more likely to be male and to have higher fasting blood glucose, serum liver enzymes, creatinine, and uric acid levels than those with MAFLD and no alcohol consumption[67]. Of note, there is no evidence on the potential negative effect of alcohol intake on renal damage risk in MAFLD individuals[67]. Authors concluded that MAFLD presence was an independent risk factor for significant fibrosis (defined by FIB-4 index ≥ 1.3 and liver stiffness ≥ 6.6 kPa using Shear wave elastography), and both MAFLD and mild alcohol intake were associated with increased prevalence of significant fibrosis (25.0% *vs* 15.5%)[67].

Further data examining the role of alcohol intake in this context[60] demonstrated a better metabolic profile but increased transaminase levels in subjects with MAFLD having a greater alcohol intake compared to those with no alcohol consumption. However, no consensus has been reached on the effect of alcohol in MAFLD, but some noninvasive fibrosis scores have been positively associated with MAFLD and alcohol intake[60].

Despite accumulating data on the impact of MAFLD on liver disease severity[60,65,67], its influence on the potential malignant transformation into hepatocellular cancer has been not evaluated.

Unlike adults, pediatric MAFLD data are limited. Because of the widespread distribution of this hepatic condition in childhood, recent epidemiological data reported a worrying increase of pediatric MAFLD prevalence[68-70].

MAFLD definition has been tested first in adult subjects; therefore, its clinical utility in a pediatric setting is still under investigation, since the fatty liver etiology at this stage[71-73] and the obesity status [21]. A recent Italian study investigated the usefulness of MAFLD criteria in 954 children with obesity [21]. The authors grouped their cohort as subjects with (1) obesity only; (2) obesity and NAFLD; and (3) obesity, NAFLD and metabolic dysregulation. The latter group was significantly older and showed higher BMI, systolic blood pressure, diastolic blood pressure, waist/hip ratio, HOMA-IR, triglyceride levels, baseline and 2-h oral glucose tolerance test glycemia, and transaminase levels. A higher prevalence of carriers of the *PNPLA3* rare allele was reported in this group compared with others. Taken together, these findings suggest a worse cardiometabolic profile in subjects with obesity, fatty liver, and metabolic dysregulation than in those belonging to other groups. As a preliminary study, MAFLD diagnosis based on metabolic dysregulation in children with obesity seemed more accurate for cardiometabolic risk stratification in a high-risk population such as children with obesity[21]. *PNPLA3* gene seems to play a role in a wider metabolic milieu beyond NAFLD[21], as previously found in a similar pediatric cohort[50,74].

More recently, an international panel[75] has proposed an age-appropriate MAFLD definition based on sex and age percentiles. Diagnostic criteria for pediatric MAFLD are based on the presence of hepatic steatosis (detected either by liver histology, imaging, blood biomarkers or blood scores) in addition to one of the following conditions: excess adiposity, T2D or prediabetes, or evidence of metabolic dysregulation (defined by the presence of at least two metabolic risk conditions according to sex and age percentiles such as hypertension, increased waist circumference, hypertriglyceridemia, low serum HDL cholesterol levels, triglyceride-to-HDL ratio ≥ 2.25, and impaired fasting glucose)[75].

Contrary to the adult findings, the natural history of fatty liver in children is still not fully understood but its increase has been mainly linked to obesity[75]. Pediatric fatty liver usually does not occur in children < 3 years and is rare in those aged < 10 years. To date, it has been demonstrated that the entire spectrum of liver disease severity (from simple steatosis to steatohepatitis, fibrosis, and end-stage cirrhosis) might occur also in pediatric patients diagnosed with fatty liver, and that the progression is strongly related to IR severity[75]. As a consequence, the occurrence of severe complications (including liver transplantation) at this early age has also been reported. The pivotal role of primary care for early detection of pediatric fatty liver is widely recognized, and lifestyle modifications are the only valid treatment for the disease[75]. Therefore, redefinition of pediatric MAFLD represents a crucial step for global management improvement, including risk stratification and multidisciplinary care.

## MAFLD: NEW INSIGHTS AND FUTURE DIRECTIONS

The tangled and multifactorial physiopathology of MAFLD (including inflammation, sex, age, ethnicity, diet and microbiota, hormones, and genetics) is still poorly defined. Despite the centrality of metabolic dysfunction, diagnosing fatty liver is also essential for MAFLD definition. Liver biopsy represents the common diagnostic gold standard for hepatic fat content assessment, but its invasiveness has limited its clinical utility in children[76,77]. A growing number of studies has evaluated different noninvasive biomarkers for MAFLD diagnosis, by identifying novel attractive therapeutic options for the management of the disease[78-81]. In this context, investigation of the gut–liver axis has attracted scientific attention[81-84]. Considering the relevance of the intestinal barrier in multiple biological mechanisms and the crucial influence of the immune system (located in the liver, intestine and adipose tissue)[84], this term strengthens the association of the liver with the gut barrier.

The association of gut–liver axis changes with MAFLD pathophysiology have recently been explored [78], by pointing out the role of inflammation and release of chemokines and cytokines by liver-infiltrating macrophages as key factors for progressive forms of fatty liver[78].

Dysbiosis and gut barrier changes have both been linked to inflammation and metabolic abnormalities in MAFLD. Remarkably, a peculiar association of microbiome alterations with carbo-hydrates, lipids and amino acids metabolism in MAFLD has also been described[81], but no consensus has been reached in this field. Nevertheless, promising preclinical studies[81] have enriched the spectrum of potential MAFLD therapeutic tools such as fecal microbiota transplantation[82-84]. A similar study on MAFLD adults[84] investigated microbiota-derived metabolites as potential noninvasive biomarkers for MAFLD, by identifying certain metabolites [*e.g.*, phosphatidylcholine (PC), lysoPC, plasma eicosanoic acid or fatty acid 20:1 (FA20:1), PCaaC24:0, xanthine, and triglycerides] as early microbiota-related products involved in liver disease progression[84]. In addition, a significant association of the *PNPLA3* gene with plasma monounsaturated fatty acid FA(20:1) or eicosanoic acid was also demonstrated.

Notably, serum mi-RNA-122 (as the major hepatic mi-RNA involved in metabolic diseases) is significantly related to MAFLD progression in subjects with obesity and MAFLD[80]; therefore suggesting their potential prognostic utility for liver disease progression[80].

Although preliminary, some promising evidence supports the identification of novel potential therapeutic targets for MAFLD[85-88]. In particular, a significant decrease in MAFLD prevalence has been reported in normal-weight adolescents treated with a low-dose combination of spironolactone, pioglitazone and metformin (SPIOMET)[86-90] than those with classical hormone therapy, by underlining the role of SPIOMET treatment as a promising new pathophysiological approach in MAFLD patients[88]. Due to the relevant cardiometabolic burden of MAFLD and the absence of effective pharmacological agents both in children and adults, further studies are needed to identify specific noninvasive markers able to improve the management of MAFLD patients[75]. Several novel therapeutic targets based on molecular pathways are under investigation[78,84], but there are no current licensed MAFLD treatments[75].

## CONCLUSIONS

The natural history of pediatric MAFLD remains to be defined, but mounting evidence from adults supports a significant increased cardiovascular risk in view of the concomitant occurrence of metabolic impairments with liver disease. Therefore, better knowledge of the intricate MAFLD pathophysiology might pave the way for new therapeutic approaches to improve the management of these patients at greater cardiometabolic risk.

## FOOTNOTES

**Author contributions:** Lanzaro F wrote the first draft of the manuscript; Miraglia del Giudice E, Di Sessa A, and

Marzuillo P conceived the manuscript; Di Sessa A, Miraglia del Giudice E, and Marzuillo P supervised the manuscript drafting; D'Addio E, Salvatori A, Guarino S, and D'Anna JA reviewed the literature data; Guarino S and Lanzaro F prepared the tables; all author contributed important intellectual content during manuscript drafting or revision.

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**Country/Territory of origin:** Italy

**ORCID number:** Francesca Lanzaro 0000-0002-0976-6833; Stefano Guarino 0000-0002-0551-5236; Elisabetta D'addio 0000-002-2325-5255; Alessandra Salvatori 0000-0002-6289-2249; Josè Alberto D'anna 0000-0002-9922-0163; Pierluigi Marzuillo 0000-0003-4682-0170; Emanuele Miraglia del Giudice 0000-0002-1492-076X; Anna Di Sessa 0000-0002-5877-3757.

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*MINIREVIEWS*

# Liver dysfunction during COVID-19 pandemic: Contributing role of associated factors in disease progression and severity

Tarun Sahu, Babita Pande, Manasa PL, Henu Kumar Verma

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**Tarun Sahu, Babita Pande,** Department of Physiology, All India Institute of Medical Sciences, Raipur 492001, Chhattisgarh, India

**Manasa PL,** Center for Basic Sciences, Pt. Ravishankar Shukla University, Raipur 492001, Chhattisgarh, India

**Henu Kumar Verma,** Department of Immunopathology, Institute of Lungs Health and Immunity, Munich 85764, Bavaria, Germany

**Corresponding author:** Henu Kumar Verma, PhD, Research Scientist, Senior Researcher, Department of Immunopathology, Institute of Lungs Health and Immunity, Ingolstädter Landstrasse 1, Munich 85764, Bavaria, Germany. henu.verma@yahoo.com

## Abstract

In December 2019, a new strain of coronavirus was discovered in China, and the World Health Organization declared it a pandemic in March 2020. The majority of people with coronavirus disease 19 (COVID-19) exhibit no or only mild symptoms such as fever, cough, anosmia, and headache. Meanwhile, approximately 15% develop a severe lung infection over the course of 10 d, resulting in respiratory failure, which can lead to multi-organ failure, coagulopathy, and death. Since the beginning of the pandemic, it appears that there has been consideration that pre-existing chronic liver disease may predispose to deprived consequences in conjunction with COVID-19. Furthermore, extensive liver damage has been linked to immune dysfunction and coagulopathy, which leads to a more severe COVID-19 outcome. Besides that, people with COVID-19 frequently have abnormal liver function, with more significant elevations in alanine aminotransferase and aspartate aminotransferase in patients with severe COVID-19 compared to those with mild/moderate disease. This review focuses on the pathogenesis of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in the liver, as well as the use of liver chemistry as a prognostic tool during COVID-19. We also evaluate the findings for viral infection of hepatocytes, and look into the potential mechanisms behind SARS-CoV-2-related liver damage.

**Key Words:** SARS-CoV-2; COVID-19; Liver function; Hepatic injury; Viral infection

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**Core Tip:** Understanding the hepatic consequences of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, as well as its molecular mechanism, has advanced significantly. Since the start of the pandemic, it appears that there has been thought that pre-existing chronic liver disease may predispose to deprived outcomes when combined with coronavirus disease 19 (COVID-19). Evidence suggests that COVID-19 patients have abnormal liver function more frequently, with more significant elevations in alanine aminotransferase and aspartate aminotransferase in severe COVID-19 patients than those with mild/moderate disease. In this review, we focus on the pathogenesis of SARS-CoV-2 infection in the liver, as well as the use of liver chemistry as a prognostic tool during COVID-19.

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## INTRODUCTION

A new strain of coronavirus was discovered in China in December 2019 which was declared as a pandemic in March 2020 by the World Health Organization[1,2]. It was initially noted by the large number of pneumonia cases that suddenly appeared amongst local citizens of Wuhan region. The majority of those with coronavirus disease 19 (COVID-19) show no indications or just moderate manifestations which include fever, cough, anosmia, and headache. Meanwhile, about 15% develop severe lung infection over the course of 10 d, resulting in respiratory failure, that can result in multi-organ failure, coagulopathy, and ultimately death[3,4].

A systematic review and meta-analysis by Li *et al*[5] involving 281461 individuals with COVID-19 revealed that 23% of them developed severe lung infection, out of which 5.6% died. A similar study by Tan *et al*[6] with 16561 patients from 17 countries also reported that 15% developed severe lung infection and the mortality rate was between 23.4% and 33.0%. A recent population-based cohort study from England revealed that individuals with pre-existing respiratory diseases are more vulnerable to COVID-19 related admissions to intensive care unit (ICU) and death. A total of 8256161 individuals were screened for pre-existing lung disease like asthma, chronic obstructive pulmonary disease, and bronchiectasis, out of which 14479 (0.2%) were admitted in hospital with COVID-19 and among which 1542 were upgraded to ICU, while 5956 died from COVID-19[7]. Similar reports are also published from South Korea that 7669 individuals with pre-existing lung disease were admitted due to COVID-19 and 251 (3.2%) died[8].

Since the beginning of the pandemic, there seems to be consideration that pre-existing chronic liver disease (CLD) may dispose to deprived consequences along with COVID-19, especially because interconnecting possible causes for COVID-19 and CLD, such as old age, overweight, and diabetes. Furthermore, extensive liver damage is linked to immune dysfunction and coagulopathy, which leads to a more severe outcome of COVID-19[9,10]. In addition, individuals with COVID-19 often have abnormal liver function, with more substantial elevation in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in severe COVID-19 than in mild/moderate COVID-19[11]. However, there are many unanswered questions that need to answer. In this context, this review deeply emphasizes on the pathogenesis of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in the liver, as well as the application of liver chemistry as a prognostic tool during COVID-19. We also evaluate the findings for viral infection of hepatocytes and look into the potential mechanisms behind SARS-CoV-2-related liver damage. Lastly, we discuss the management of this disease and therapeutic strategies for liver damage due to COVID-19.

## MECHANISM OF LIVER INJURY DURING COVID-19

### *Mechanism of infection*

Fan and colleagues[12] found that patients without a history of liver disease also had abnormal liver test parameters, indicating direct entry of the SARS-CoV-2 virus into the liver *via* interaction with angiotensin converting enzyme (ACE) 2 receptors. These receptors are also expressed in liver cells, lungs, intestines, and many other different tissues of the human body[13,14]. Evidence also demonstrates that SARS-CoV-2 spike proteins bind to ACE2 receptors in cholangiocytes rather than hepatocytes, which may cause liver damage[15]. However, Chai and colleagues found significantly low (0.31%) ACE2 expression in hepatocytes compared to 20 times higher expression in bile duct cells as per single-cell sequencing. Further, for transmission to the liver, the SARS-CoV-2 virus could use the gut-

liver route through the hepatic reticular system[16].

### Systemic inflammatory response syndrome and cytokine storms

The inflammatory cytokine storms have been considered responsible for liver injury of COVID patients. There is increased levels of interleukins (ILs) like IL-2 and IL-6 in the serum of COVID-19 patients that have been linked with poor clinical outcomes (Figure 1). In addition to the secretion of tumor necrosis factor-α, IL-2, IL7, IL-18, IL-4, and IL-10 increased pro-inflammatory cells (CCR4+CCR6+ Th17), which was more prominent in ICU admitted patients compared to non-ICU admitted ones[17-19]. These inflammatory cytokine storms cause systemic inflammatory response syndrome, acute respiratory distress syndrome, ischemia, and ultimately cell destruction and necrosis of the liver and multiple organ damage[18].

### Ischemia and hypoxia reperfusion injury

It is well known that most patients with severe symptoms of COVID-19 have hypoxia and severe cases require oxygen. The inefficient lung function and other multi-organ damage can cause hypoxia and ischemia along with shock. Ischemia and hypoxia promote lipid accumulation, glycogen consumption, and adenosine triphosphate depletion in hepatocytes, suppressing cell survival signal transduction, which leads to liver cell death. Also, respiratory distress syndrome accelerated reactive oxygen species (ROS) generation and oxidative stress. ROS and lipid peroxidation products can activate redox-sensitive transcription factors, causing the release of a variety of pro-inflammatory factors that harm the liver. These alterations can aggravate the ischemia of hepatocytes and affect the excretion of toxic metabolites that eventually induce liver injury[18,19].

### Antibody-dependent enhancement

To inhibit the viral infection, antibodies are used that specifically block the binding of viral protein and cell surface receptors. However, during certain viral infections, specifically viruses with many antigenic epitopes, there is enhanced affinity of binding of virus proteins to host cell receptors. This mechanism is known as antibody-dependent enhancement (ADE). Certain viruses like corona virus (SARS-CoV) through this pathway use the antiviral antibodies to enter into host cells like macrophages, granulocytes, and monocytes, and also for replication inside these cells through interaction with Fc and/or complement receptors[20,21]. This causes an increase in infection and disease progression with worsening outcomes. It has been suspected that SARS-CoV-2 may use ADE property to infect immune cells through a non-ACE2-dependent pathway, leading to liver injury[18]. However, further research is warranted to confirm the ADE mediated replication of the SARS-CoV-2 and the underlying mechanism. Further, ADE is considered an important aspect for the development and application of vaccines against SARS-CoV-2 since the virus may use ADE mechanism to amplify the infection and increase the severity of disease[22,23].

### Hepatotropism of SARS-COV-2

Due to the challenges in obtaining tissue samples from COVID-19 persons and the need for research laboratory confinement facilities, the tissue repositories for SARS-CoV-2 replication have yet to be thoroughly understood. To obtain cell entrance, the viral protein binds ACE2, and TMPRSS2 and FURIN are also necessary for infection; hence, the activation of these receptors revealed initial signs for probable hepatic susceptible cells. According to RNA sequencing in healthy hepatocytes, cholangiocytes (alveolar type 2 cells) had the most significant gene expression levels for ACE2, followed by sinusoidal endothelial cells and liver cells[24,25]. TMPRSS2 and FURIN also have diverse gene expression patterns across various hepatic cells. Cell lines generated from hepatocellular carcinoma can sustain the whole viral life cycle[26], although replication in hepatic cells is still to be revealed. Zhao and colleagues created human liver ductal organoids that express ACE2 and TMPRSS2, which could mimic SARS-CoV-2 infection, indicating that the bile duct epithelium might facilitate pseudo-particle entrance[27].

The impact of liver damage and underlying liver disease on SARS-CoV-2 hepatotropism is unknown, and no research has mainly looked at the histological abnormalities seen in COVID-19 patients with pre-existing CLD. However, before COVID-19, studies indicated that in individuals with hepatitis C virus-related cirrhosis, ACE2 expression is 30 times higher than that in healthy people's livers[28]. Furthermore, hepatic mRNA expression of *ACE2* and *TMPRSS2* was elevated in non-infected individuals with obesity and non-alcoholic hepatic steatosis, but not with only steatosis[29]. SARS-CoV-2 hepatotropism might be exacerbated by liver damage and inflammation by altering viral receptor expression, with *ACE2* being recognized as an interferon-inducible gene in human respiratory epithelial tissue[30]. Nevertheless, this discovery should be considered carefully since the upregulation might be due to the shortened isoform of ACE2, known as deltaACE2, instead of the viral receptor protein itself [31]. An *in vitro* study revealed that high-density lipoprotein scavenger receptor B type 1 (SR-B1) facilitates ACE2-dependent coronavirus adhesion[32], which is quite similar to hepatitis C virus infection[33].

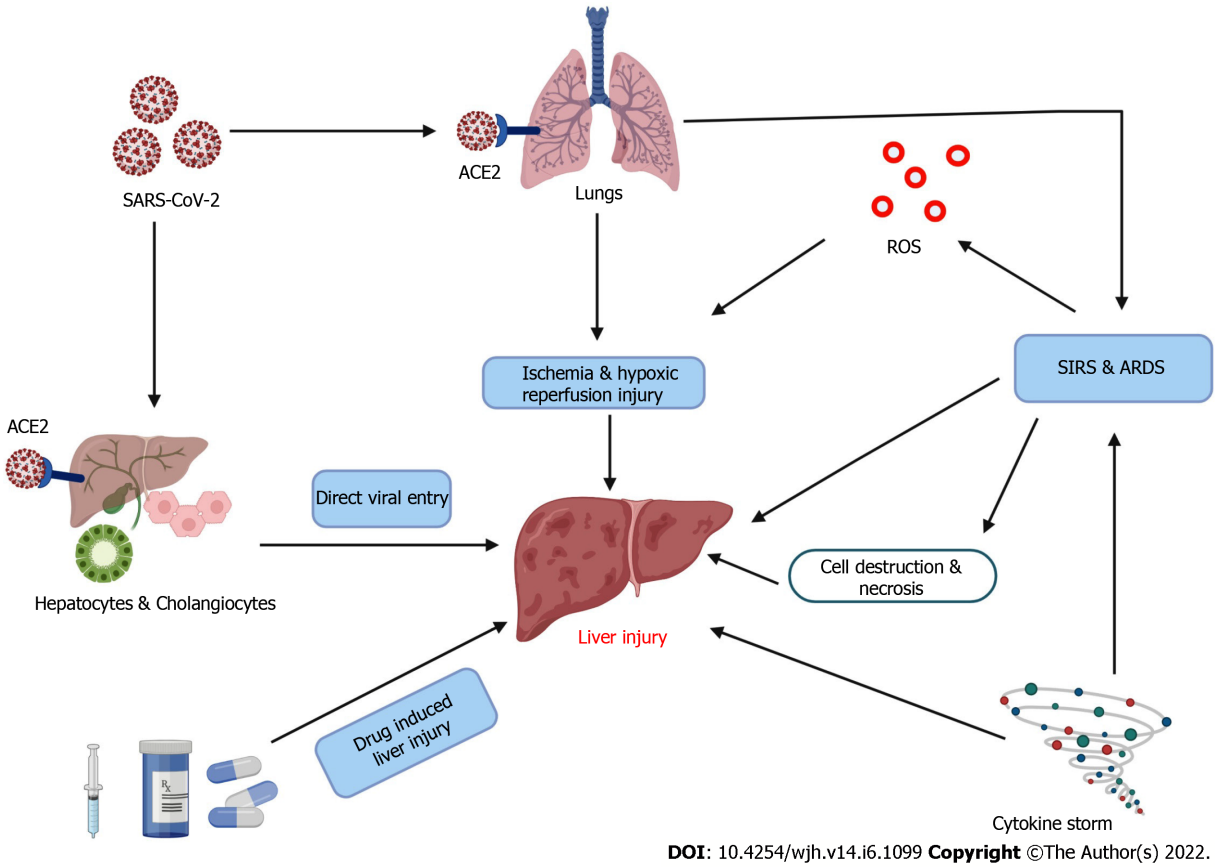


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**Figure 1 Schematic representation of possible causes of liver injury.** ACE2: Angiotensin converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ROS: Reactive oxygen species.

### *Clinical manifestations and pathological alterations in hepatic dysfunction and abnormality in COVID-19 patients*

COVID patients suffer from respiratory problems as a primary consequence of SARS-CoV-2 infection. The last two years of extensive research has revealed different respiratory problems, including multi-organ damage due to COVID. Initial studies in COVID patients from China showed the prevalence of functional abnormality of the liver and liver damage in many hospitalized patients[34-37]. The liver dysfunction and damage have been observed at the biochemical and histological levels.

The abnormal liver test results are essential biomarkers for evaluating liver abnormalities as a function of COVID severity[38]. The liver function abnormalities are confirmed through the increased concentrations of certain liver enzymes in serum such as ALT > 40 U/L, AST > 40 U/L, gamma-glutamyl transferase (GGT) > 49 U/L, alkaline phosphatase (ALP) > 135 U/L, and total bilirubin > 17.1 μmol/L[39,40]. Most of the studies showed increased concentrations of enzymes ALT and AST in SARS-CoV-2 infected patients[12,17,35]. Fan *et al*[12] in their retrospective, single-center study observed abnormal liver functions in one third which are characterized by abnormal liver tests like increased levels of enzymes, *i.e.*, the "cholangiocyte-related enzymes" such as gamma-glutamyl transferase & alkaline phosphatase, and total bilirubin in addition to alanine and aspartate aminotransferases (Table 1). In their multicentric study, Ding and colleagues found that the concentrations of AST and direct bilirubin both at the time of initial observation and peak were independently associated with COVID-19 patients' mortality[37]. Besides, LDH, prealbumin, albumin, ALP, GGT, and total and direct bilirubin in COVID patients have been suggested for diagnosis and progression of disease severity[41]. Liver damage in patients with COVID-19 has also been attributed to hypoxemia and reperfusion or passive congestion[42].

Xu and colleagues[43] reported liver damage through liver biopsy of a dead COVID patient manifested as moderate micro-vesicular steatosis and mild lobular and portal activity. This damage could be due to direct infection of SARS-CoV-2 or hepatotoxicity caused by drugs used to treat the patients[43]. Ji and colleagues also examined the postmortem liver biopsy showing microvascular steatosis over T cell activation. They suggested that COVID-19 associated liver injury may be immune-mediated rather than direct cytopathic damage[16]. Philips and colleagues' evidence regarding COVID-19-associated liver damage obtained through liver biopsy, such as hepatocyte apoptosis, binuclear or occasionally multinuclear syncytial hepatocytes, mild focal lobular or portal inflammation, and altered hepatocyte mitochondrial features, substantiated that SARS-CoV-2 may not be involved in liver damage

**Table 1 Studies of abnormal liver biochemistries in patients with coronavirus disease 19**

| Ref. | Patients (*n*) | Findings | Conclusion |
|---|---|---|---|
| Wang *et al*[11], 2020 | 105 | 56.2% of the patients had abnormal ALT, AST, and total bilirubin throughout the course of the disease | Patients with COVID-19 often have abnormal liver function indices |
| Fan *et al* [12], 2020 | 148 | 37.2% had abnormal liver function at hospital admission; 14.5% out of these patients had high fever; patients with abnormal liver function had longer mean hospital stays (15.09 ± 4.79 d) than patients with normal liver function (12.76 ± 4.14 d) | More than one third of SARS-CoV-2 infected patients admitted to hospitals had elevated liver function parameters, which are linked to a prolonged hospital stay |
| Ding *et al*[37], 2021 | 2,073 | Out of 2073 patients, 61.8% showed abnormal liver chemistries during hospitalization, and 14.3% had liver injury | COVID-19-related mortality is predicted by abnormal levels of AST and D-Bil during admission. Infection with HBV does not raise the risk of poor COVID-19-related outcomes in patients |
| Cai *et al* [38], 2020 | 417 | 76.3% had abnormal liver test results and 21.5% had liver injury during hospitalization.ALT, AST, total bilirubin, and gamma-glutamyl transferase levels rose to more than 3 × the upper limit of normal, respectively | The negative effects on liver damage are mostly due to certain drugs taken during hospitalization |
| Fan *et al* [41], 2021 | 288 | Except for AST, the levels of total bilirubin and ALP in normal and severe patients varied within the normal range, with an increasing trend in critical patients | In critical patients, COVID-19 can induce significant hepatic dysfunction, necessitating early monitoring and management. Because of their connection with disease severity in COVID-19, LDH, ALP, GGT, total bilirubin, prealbumin, and albumin may be useful for assessing and predicting disease prognosis |

COVID-19: Coronavirus disease 19; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltransferase; D-BiL: Direct bilirubin.

or impaired liver function, which indicated liver involvement in severe systemic inflammatory diseases, fatty liver disease, sepsis, or multi-organ dysfunction[44].

The impact of COVID has been classified into mild or non-severe and severe according to the manifestation of symptoms. The mild symptoms are fever, cough, expectoration, shortness of breath, muscles ache, and other upper respiratory tract symptoms, and without abnormalities, or with mild changes on chest radiography, such as multiple small patchy shadows and interstitial changes, primarily in the outer zone of the lung and under the pleura. Severe COVID symptoms include significantly increased respiration rate (≥ 30 times/min) or hypoxia with oxygen saturation at resting state ≤ 93%; or partial pressure of oxygen/fraction of inspired oxygen ($PaO_2$) /FiO2) ≤ 300 mmHg; or respiratory/another organ failure with emergency ICU monitoring and treatment, or shock[34,39].

## CONTRIBUTING ROLE OF COVID ASSOCIATED FACTORS IN DISEASE PROGRESSION AND SEVERITY

### *Liver functional test abnormalities*

Clinical evidence demonstrated that elevated serum levels of the liver enzymes ALT and AST were associated with adverse outcomes such as shock and ICU admission, and mechanical ventilation, as well as disease progression symptoms such as the development of severe pneumonia[39,45,46]. Fan and colleagues reported longer hospital stays of around average 15 d in COVID patients with abnormal liver functions. Increased levels of liver enzymes increased hospitalization compared to COVID patients with normal liver functions. The liver test abnormalities have been stated as a predictor of disease progression and severity of COVID symptoms[47]. According to Leo and colleagues, the underlying events for abnormal liver function test results are due to "cytokine storm, a sinusoidal thrombotic event associated to SARS-CoV-2 infection coagulopathy, liver damage induced by hypoxia or could be secondary to alterations of blood outflow and inflow that may occur when positive end-expiratory pressure is applied"[48]. Recently, in a retrospective study, Kalal and colleagues from India also documented elder age (median 50 years), longer hospital stays, and higher values for liver function tests in severe patients than in non-severe (mild and moderate) patients (median age 37 years)[49]. A large retrospective cohort study from China documented the abnormal AST and direct bilirubin levels in COVID patients at hospital admission were associated with COVID-19-related mortality[37]. Therefore, the liver function tests for monitoring AST and direct bilirubin have been suggested as imperative to halt the disease progression.

### *Drug hepatotoxicity*

During the first phase of the COVID-19 pandemic, the primary therapeutic procedure was to treat patients with antibacterial drugs such as moxifloxacin, cephalosporins, antiviral drugs oseltamivir and acyclovir, and antipyretic drugs such as acetaminophen to alleviate COVID-19 symptoms[12]. The benefits like improved outcomes in decreasing diarrhea, fever, worsening of chest radiographs, and worsening of viral load by treating severe acute respiratory syndrome (SARS) patients with lopinavir/ritonavir for 3 wk were earlier validated[50]. However, the side effects of lopinavir and ritonavir administered during treatment of patients with SARS-CoV-2 viral infection have been noted[51], with even four times increased risk of liver damage, which can be attributed to a higher dose of this medication[12,39]. Yip and colleagues also recorded treatment with lopinavir-ritonavir, with or without ribavirin, interferon beta, or corticosteroids was independently linked with heightened ALT/AST serum concentrations[45].

It has been speculated that the overdose of a combination of lopinavir and ritonavir could trigger the hepatic endoplasmic reticulum stress cascade, stimulate inflammatory reactions, activate hepatocyte apoptosis through the caspase mechanism, suppress hepatocyte growth, and amplify the liver damage oxidative stress[18]. In a mouse model, the role of ritonavir in hepatotoxicity has been shown to occur through CYP3A4-dependent pathways, which are modulated by pregnane X receptor. This transcription factor is concerned with ritonavir bioactivation, oxidative stress, endoplasmic reticulum stress, destruction of membrane integrity, and interruption of the internal and external Ca2+ homeostasis of liver cells, and causes death[18,52]. It has been studied that SARS-CoV-2 replication can be inhibited by human immunodeficiency virus (HIV) protease inhibitors; however, progression of liver damage has been observed in patients undergoing hormones and HIV protease inhibitor therapy. In a retrospective study in China, Shen and colleagues also reported traditional Chinese medicines, herbal and dietary supplements, and antituberculosis drugs as the main causes of drug-induced liver injury[18,53]. Fan and colleagues also reported that administration with ACE inhibitors/angiotensin II receptor blockers (ARBs) did not produce any side effect in COVID-19 patients, even in those with hypertension as co-morbidities. Thus, the prolonged and higher dose of antibiotics, non-steroidal anti-inflammatory drugs, herbal medications, and interferon used to treat COVID-19 patients seems to link with the progression of disease with severe outcomes like liver failure.

### *Pre-existing liver problem and other co-morbidities/factors*

Most of the COVID-19 patients reported fever as a symptom of infection, for which they used antipyretic and analgesic drugs. Therefore, overdose or prolonged use of these drugs has been linked to liver damage. Due to their hepatotoxic properties, the use of drugs like lopinavir/ritonavir could be fatal to patients with pre-existing liver problems or weak immune systems like old age, children, and patients with co-morbidities[12,51].

Aged patients with co-morbidities such as hypertension, diabetes, cardiovascular disorders *etc.* showed severe outcomes compared to other age groups or those without co-morbidities[54]. In addition to age, clinical features like inflammation and hypoxia at the time of admission to hospital and drug treatment have been associated with COVID-19 related development and progression of liver damage [48]. COVID-19 patients with hypertension who received ACE inhibitors and ARBs at the time of hospitalization developed higher though insignificant liver abnormalities than patients without a history of hypertension[12]. Liver tissues of patients with chronic liver diseases such as existing cirrhosis, dysplasia, non-alcoholic steatohepatitis, and simple steatosis showed elevated levels of ACE2 than normal liver tissues[55]. Patients with alcohol-related liver disease have been suspected as a high-risk group for COVID-19 related severity because these patients, due to drinking habits, are less likely to maintain social isolation and follow regular therapy[56]. Ji and colleagues also noticed patients with non-alcoholic fatty liver disease with a high body mass index are at greater risk for progression and severity of COVID-19 outcomes and reported longer viral shedding time. The respective researchers hypothesized that in patients with non-alcoholic fatty liver disease, "the polarization status of hepatic macrophages might be skewed from inflammation-promoting M1 macrophages to inflammation-suppressing M2 macrophages, which results in progression of COVID-19"[16].

The virulence of SARS-CoV-2 has imposed challenges for organ transplants. Since the immune system of the recipient is already dwindling due to chronic illness, these patients are susceptible to infection. Both recipient and donor of liver transplant are at risk of SARS-CoV-2 infection before liver transplantation, increasing the hospital stay and treatment duration that further aggravating the infection in patients undergoing liver transplantation[57]. Thus, strict guidelines for the diagnosis of donor and maintenance of isolation and sterilized environment during operation and postoperative patient care should be assured.

## DISEASE MANAGEMENT, PREVENTIVE MEASURES, AND TREATMENT

SARS-CoV-2 is a novel coronavirus that causes COVID-19. Retrospective studies and clinical trials that we mentioned in the previous section of this review demonstrated that the drug hepatocytotoxicity due

to inappropriate dose and long-term use causes aggravated immune response and systemic inflammatory reaction due to invading virus or viral reactivation of existing liver disease, and the adverse clinical outcome & disease progression and co-morbidities[16,46,47]. Therefore, frequent monitoring conditions of COVID-19 patients with pre-existing liver disease or who developed liver abnormalities during treatment is needed. Evaluating liver enzymes during in-hospital stay or post-COVID-19 duration at different time intervals in months could help track liver health and effective treatment. Still, there is a possibility of managing the disease with combined efforts among individual, societal, and health care sectors, the research community, and the government level. Detailed strategy is mentioned in Figure 2.

### Disease management of patients with liver disease

We confirmed from previous research that SARS-CoV-2 infection causes mainly liver damage and abnormal liver functions, which manifest as hypoxia, systemic inflammatory reactions, and medication; thus, regulating these associated factors, such as oxygen supplementation, mechanical ventilation, and renal replacement therapy for cytokine storm, could reduce liver injury[18,42]. If hepatotoxicity due to drug use is suspected, patients must be admitted to intensive care for any severe symptoms, and emergency precautionary measures must be taken. Patients with chronic liver diseases such as cirrhosis and non-alcoholic fatty liver disease treated with immunosuppressive drugs should be prioritized for COVID-19 testing and hospitalization. Patients showing signs of chronic viral hepatitis (HCV and HBV) should be treated according to prescribed guidelines. In case of urgent liver transplantation, the donor and recipient should be tested for COVID before operation and postoperative precautionary measures should be taken to prevent any infection. If post-transplant patients are detected with SARS-CoV-2 infection, they should be administered with a reduced dose of immunosuppressive agents according to a degree of severity along with vaccination[18,58].

### Dietary management for health

Probiotics, vitamins, and minerals are essentially dietary supplements that are helpful to improve overall health condition and deficiency of proper nutrition weakens the immune system. Insufficient intake of vitamin A and serum retinol, vitamin C, and selenium have been linked with the severity of liver fibrosis in patients with non-alcoholic fatty liver disease[59]. As a result, it has been speculated that taking probiotics, vitamins such as fat-soluble vitamins A, D, and E and water-soluble vitamin C, and minerals such as zinc, magnesium, and copper during COVID-19 could boost and maintain liver health. Sivandzadeh and colleagues have highlighted the anti-inflammatory properties of probiotics and micronutrients, along with the antioxidant and immunomodulatory properties of vitamins that could reduce the TNF-α, oxidative stress, and apoptosis of hepatocytes[60].

### Research in liver health

COVID-19 pandemic has arrived as a hurdle and interrupted research, clinical trials related to liver health, and drug development due to pandemic generated difficulties such as problem in the recruitment of patients, timely supply of research related aids, and maintenance of laboratories specifically during lockdown[61]. For example, liver cancer management has been affected due to COVID-19, and the more prominent among them is the cancellation of screening, diagnosis, and treatment[62]. The development of non-invasive tools, and proteomics, transcriptomics, lipidomics, and metabonomics based biomarkers for diagnosis and progression of liver disease could also helpful to lessen the COVID severity[63]. Research on development of software and algorithms for maintaining databases of patients' details for early diagnosis and treatment of liver disease such as liver fibrosis at the community level (https://clinicaltrials.gov/ct2/show/NCT04666402) could help to track patients with liver disease and their treatment & care at the time of COVID. Research and more clinical trials on regressing fibrosis and enhancing tissue regeneration using cell therapy could be the promising alternative to liver transplant that could increase the longevity of patients[64]. The clinical trials on role of vitamin C as an antioxidant to improve liver functions and the underlying mechanism on healing the non-alcoholic fatty liver disease suggest that it could be a compelling dietary supplement to reduce the COVID associated symptoms and the severity[65]. Drug development and clinical trials through nanotechnology for diseases like non-alcoholic fatty liver disease/metabolic-associated fatty liver disease[66], and other liver disease could ensure better health in patients with liver disease in this COVID pandemic era. Now, after global vaccination and following proper corona guidelines, the normal research activity has started, which could progress with the support of government and funding sources/sponsors.

### Future directions

Still, there is a lack of safe drugs that can effectively treat COVID patients precisely because of its umbrella effect at the multi-organ level. In a meta-analysis from Morocco, around 20 medicinal plants belonging to 19 genera and 14 botanical families and their products have been reported to prevent and treat COVID 19. The extracts of these plant species are rich in bioactive compounds like flavonoids, alkaloids, saponins, essential oil, *etc.* which are effective as antiviral, antibacterial, antifungal, anti-

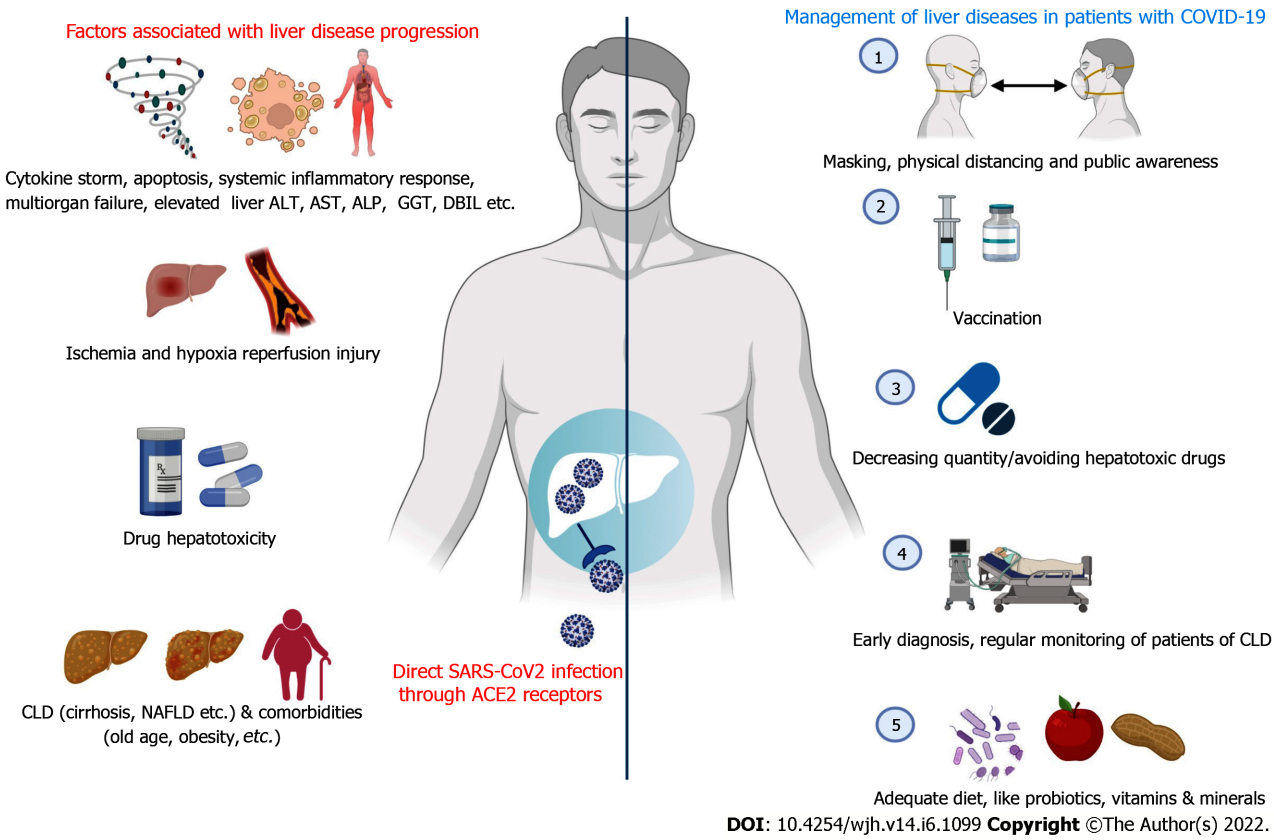


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**Figure 2 Associated factors for liver disease progression and its management strategies in patients with coronavirus disease 19.** CLD: Chronic liver disease; NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltransferase; DBIL: Direct bilirubin. Created with BioRender.com.

inflammatory, antioxidant, antipyretic antiseptic, antibiotic, and analgesic agents[67]. Certain plants such as *Tinospora cordifolia, Andrograhis paniculata, Cydonia oblonga, Zizyphus jujube*, and *Cordia myxa* are also beneficial for symptomatic management of COVID-19[68]. Through randomized controlled trials, the integrated therapy including treatment with both Western as well as herbal medicine has also been shown to promote faster recovery, and reduce symptoms and duration of treatment time in COVID-19 patients[69]. However, some traditional Chinese herbal medicines were suspected to impose side effects and increase the disease course[70]. The MACH-19 (Mushrooms and Chinese Herbs for COVID-19) trials are ongoing, and these herbs could be used as potential adjuvant to COVID-19 vaccines[71]. Thus, effective herbal medicines and more clinical trials to evaluate their potential to cure COVID-19 are required. The immunogenicity of vaccines developed against SARS-CoV-2 in patients still needs to explore. The vaccination of two doses has been widely carried out all over the world, so the post-vaccination symptoms are required to monitor in patients with liver diseases like chronic disease cirrhosis and non-fatty liver disease, and in liver transplant recipient whose immunity is compromised. Better pathogenesis knowledge helps to formulate specific therapy that may arrest viral replication. Therefore, the promotion of intensive research on liver health due to COVID-19 by the government and an approach for collaborative research among physicians, scientists, and academicians from different institutes is required. Public awareness regarding maintaining hygiene, the right lifestyle, self-care, following COVID-19 protocols, and vaccination could help to reduce community transmission and COVID-19 related causalities.

## CONCLUSION

A large number of studies, including retrospective and clinical trials conducted around the world, have supported the existence of liver dysfunction in COVID-19 patients. The main factors contributing to disease severity and progression are drug-associated hepatotoxicity and immune-mediated liver injury due to the possibility of direct cell cyto-toxicity. Through ACE2 receptor mediated SARS-CoV-2 infection of liver cells, viral reactivation might occur in cases of pre-existing liver dysfunction. As predictors of disease progression and liver damage, biochemical indicators of the liver have been proposed. However, reports on liver damage by direct infection with SARS-CoV-2 and safer drug

rendering protection to the liver are insufficient. Therefore, further research on different aspects such as the mechanism of liver injury, drug-related toxicity, different stages of disease progression, link of liver dysfunction with co-morbidities, immune state of patients, along with effective drug (specifically exploiting the indigenous herbal medicine knowledge) and vaccine development is need of the time. Individuals, particularly patients with liver disease, may take preventive measures such as masking, physical distancing, avoiding self-medication without a physician's prescription, avoiding alcohol consumption, exercise, a balanced diet, adequate sleep, and a healthy lifestyle to reduce their chances of contracting SARS-CoV-2 infection.

## FOOTNOTES

**Author contributions:** Verma HK designed the review; Sahu T, Manasa PL, and Pande B performed the literature search and collected and assembled the data; HKV and BP analyzed the obtained articles; Sahu T, Manasa PL, Pande B, and Verma HK wrote the manuscript and revised it critically; all authors have read and agreed to the published version of the manuscript.

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**Country/Territory of origin:** Germany

**ORCID number:** Tarun Sahu 0000-0002-2721-7432; Babita Pande 0000-0002-0545-6002; Manasa PL 0000-0001-9079-6353; Henu Kumar Verma 0000-0003-1130-8783.

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*MINIREVIEWS*

# Understanding fatigue in primary biliary cholangitis: From pathophysiology to treatment perspectives

Erica Nicola Lynch, Claudia Campani, Tommaso Innocenti, Gabriele Dragoni, Maria Rosa Biagini, Paolo Forte, Andrea Galli

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**Erica Nicola Lynch, Claudia Campani, Tommaso Innocenti, Gabriele Dragoni, Maria Rosa Biagini, Andrea Galli,** Gastroenterology Research Unit, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence 50134, Italy

**Claudia Campani,** Department of Experimental and Clinical Medicine, University of Florence, Florence 50134, Italy

**Gabriele Dragoni,** Department of Medical Biotechnologies, University of Siena, Siena 53100, Italy

**Paolo Forte,** Division of Gastroenterology, University Hospital "Careggi", Florence 50134, Italy

**Corresponding author:** Andrea Galli, MD, PhD, Professor, Gastroenterology Research Unit, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Viale GB Morgagni 50, Florence 50134, Italy. andrea.galli@unifi.it

## Abstract

Fatigue is considered one of the most frequent and debilitating symptoms in primary biliary cholangitis (PBC), affecting over 50% of PBC patients. One in five patients with PBC suffer from severe fatigue, which significantly impairs quality of life. Fatigue is made up of a central and a peripheral component, whose pathophysiology is still greatly unresolved. Central fatigue is characterised by a lack of self-motivation and can manifest both in physical and mental activities (lack of intention). Peripheral fatigue includes neuromuscular dysfunction and muscle weakness (lack of ability). Peripheral fatigue could be explained by an excessive deviation from aerobic to anaerobic metabolism leading to excessive lactic acid accumulation and therefore accelerated decline in muscle function and prolonged recovery time. As opposed to itching, and with the exception of end-stage liver disease, fatigue is not related to disease progression. The objective of this review is to outline current understanding regarding the pathophysiology of fatigue, the role of comorbidities and contributing factors, the main tools for fatigue assessment, the failed therapeutic options, and future treatment perspectives for this disabling symptom. Since fatigue is an extremely common and debilitating symptom and there is still no licensed therapy for fatigue in PBC patients, further research is warranted to understand its causative mechanisms and to find an effective treatment.

**Key Words:** Fatigue; Primary biliary cholangitis; Treatment; Pathophysiology; Central fatigue; Peripheral fatigue

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**Core Tip:** Fatigue is considered one of the most frequent and debilitating symptoms in primary biliary cholangitis, affecting over 50% of patients. The objective of this review is to outline current understanding regarding the pathophysiology of fatigue, the role of comorbidities and contributing factors, the main tools for fatigue assessment, the failed therapeutic options, and future treatment perspectives for this disabling symptom. Since fatigue is an extremely common and debilitating symptom and there is still no licensed therapy for fatigue in PBC patients, further research is warranted to understand its causative mechanisms and to find effective treatment.

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## INTRODUCTION

Fatigue is considered one the most frequent and debilitating symptoms in primary biliary cholangitis (PBC), affecting over 50% of patients with PBC[1]. As opposed to itching, and with the exception of end-stage liver disease, fatigue is not related to disease progression[1,2]. One in five patients with PBC suffer from severe fatigue, which significantly impairs quality of life[3]. The severity of chronic fatigue symptoms in PBC predicts liver-related mortality and liver transplantation outcome[4]. Latitude and sun exposure might influence PBC phenotype, including fatigue status[5].

The objective of this review is to outline current understanding regarding the pathophysiology of fatigue, the role of comorbidities and contributing factors, the main tools for fatigue assessment, the available treatments, and future therapeutic options for this disabling symptom.

## DEFINITION AND PATHOPHYSIOLOGY

Fatigue is defined as an overwhelming sense of tiredness, lack of energy, and a feeling of exhaustion[6]. Its pathophysiology in PBC is still unresolved. It can be considered as made up of two different entities: Peripheral and central fatigue. Peripheral fatigue includes neuromuscular dysfunction and muscle weakness (lack of ability)[7]. Central fatigue is characterised by a lack of self-motivation and can manifest both in physical and mental activities (lack of intention)[7].

### *Peripheral fatigue*

Anti-mitochondrial autoantibodies (AMA), which specifically target pyruvate dehydrogenase complex (PDC), are a hallmark of PBC[8]. In these patients, anti-PDC antibodies are mainly directed against the inner lipoyl domain of the PDC-E2 component, which has an alpha-lipoic acid covalently bound to a specific lysine residue, that is an absolute requirement for its enzymatic activity. The PDC-E2 component is loosely bound to the inner membrane of mitochondria. Immune reactivity against the lipoylated substrate of PDC-E2-also found in some bacteria and yeasts and mimicked by some xenobiotics-has been suggested to be the cause of PBC, as in patients with PBC, this antigen is aberrantly expressed on the surface of intrahepatic biliary epithelial cells[8]. On the other hand, as AMA highly inhibit mammalian PDC, and moderately and weakly inhibit yeast and bacteria PDC, loss of tolerance is most probably the underlying mechanism which induces PBC[9]. Since PDC is ubiquitous, a reason for the tissue specificity of epithelial damage which is found in PBC could be that secretory IgA anti-PDC, not IgG, is responsible for epithelial cell damage. IgA undergoing transcytosis across the intrahepatic biliary or salivary epithelium might bind to nascent PDC components while being transported to the mitochondria and may export them to the epithelial cell surface. Depletion of these critical proteins would result in chronic metabolic damage to the epithelial cell[9].

In fatigued PBC patients, there seems to be an excessive deviation from aerobic to anaerobic metabolism leading to excessive lactic acid accumulation and therefore accelerated decline in muscle function and prolonged recovery time. Various studies support this conclusion: Fatigued PBC patients

perform worse than non-fatigued patients on hand grip test with no association with liver disease severity[10]; when bioenergetics of muscle function was assessed using $_{31}$P magnetic resonance spectroscopy in PBC patients, non-PBC patients with chronic fatigue syndrome, patients with primary sclerosing cholangitis, and controls, only patients with PBC showed increased post-exercise muscle acidosis and prolonged adenosine diphosphate and phosphocreatine recovery time suggesting mitochondrial dysfunction[11]. pH recovery appeared to be related to fatigue severity[11]. How AMA can induce PDC depletion or dysfunction in muscles of patients with PBC remains uncertain.

It should also be noted that the reduction of AMA through B-cell depletion with rituximab did not have any effect on fatigue, suggesting the existence of other fatigue-inducing pathophysiologic mechanisms than antibody-mediated damage[12]. In addition, peripheral fatigue measured by twitch interpolation did not differ between PBC patients and controls, although patients with PBC were not differentially assessed based on fatigue symptoms[13]. Twitch interpolation can supposedly distinguish central from peripheral fatigue as it allows to assess whether all motor units have been recruited by the central nervous system or not[13]. In centrally fatigued patients, central activation is low, and a smaller number of motor units are stimulated[13].

### *Central fatigue*

Patients with PBC often report cognitive symptoms, such as memory impairment, and higher rates of sleep-wake disturbance with delayed sleep timing, worse sleep quality, and excessive daytime somnolence[14], seemingly unrelated to liver disease severity[13,15]. Evidence supporting the central origin of fatigue in PBC patients is mostly made up of small-scale studies and its pathophysiology is unknown. Treatment for excessive daytime somnolence with modafinil was ineffective[16]. Mosher *et al* [17] studied the resting-state functional connectivity (rsFC) of deep grey matter brain structures (putamen, thalamus, amygdala, and hippocampus) using resting-state functional magnetic resonance imaging in 20 non-cirrhotic PBC patients compared with 21 matched controls. PBC patients exhibited significant alterations in rsFC levels as compared to controls. Fatigue, itch, and verbal working memory performance were associated with alterations of deep grey matter rsFC, possibly reflecting chronic immune-mediated signalling from the liver to the brain in PBC patients[17]. In a study by McDonald *et al*[13], twitch interpolation and paired-pulse trans-cranial magnetic stimulation were used to study central nervous system function in PBC patients and its relationship to fatigue symptoms. PBC patients had significantly lower levels of central activation[13]. Interestingly, no differences were found between transplanted and non-transplanted patients. However, a volitional contribution could justify the results and could not be excluded; central activation might be reduced as a protective mechanism to avoid exhaustion (due to peripheral fatigue?).

Altered central neurotransmission has been a leading hypothesis to explain the development of fatigue in PBC patients, involving both serotonergic and noradrenaline pathways. Unfortunately, no specific treatment to stimulate the serotonin pathway (ondansetron, fluvoxamine, or fluoxetine) has brought positive results[18,20].

Large-scale clinical studies are warranted that assess whether fatigue in PBC patients is predominantly central or peripheral, or both, in order to concentrate future research in the right direction.

## ROLE OF COMORBIDITIES AND CONTRIBUTING FACTORS

There are many conditions and therapies which can cause fatigue or deteriorate existing weariness; in fatigued PCB patients, a complete assessment should be performed, and any detected condition should be addressed. Among these conditions, we can find autoimmune diseases such as hypothyroidism, anaemia, type II diabetes, nocturnal pruritus, autonomic dysfunction, dehydration, restless leg syndrome, and concurrent medications such as anti-hypertensive therapy. Depressive symptoms in PBC patients seem to be the consequence rather than the cause of fatigue, as the prevalence of a depressive disorder in patients with PBC does not seem to be higher than that in the general population[21].

A complete list of additive factors to fatigue burden is presented in Table 1.

## ASSESSMENT OF FATIGUE

Although fatigue is a ubiquitous symptom in medical practice, one single questionnaire might not fit the purpose of measuring fatigue in a specific group of patients. Each assessment tool should be validated not to compromise the quality of research. In a systematic review by Kim *et al*[22], the authors found that, between 1990 and 2019, patient reported outcomes in PBC had been mostly assessed with unlabelled, nonspecific versions of numeric ratings or Likert scales and that fatigue has been measured with over ten different instruments although ideally, the use of questionnaires should be standardised to allow comparison.

**Table 1 Conditions and drugs contributing to fatigue burden[1,2]**

| Conditions | Drugs |
|---|---|
| Addison disease; Anaemia; Autonomic dysfunction; Cancer; Chronic Lyme disease; Dehydration; Depression; Diabetes; Heart failure; Hypothyroidism; Infectious/inflammatory state; Myasthenia gravis; Multiple sclerosis; Obstructive sleep apnoea; Parkinson's disease; Pregnancy; Renal failure; Restless legs syndrome; Tuberculosis | Antibiotics; Antidepressants; Anti-hypertensive therapy; Muscle relaxants; Opioids; Sedative-hypnotics |

In a recent systematic review, Machado *et al*[6] evaluated existing fatigue scales commonly used to assess fatigue in patients with various medical conditions. Eleven fatigue scales were identified and analysed: Five were unidimensional [Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-F), Brief Fatigue Inventory, Fatigue Severity Scale (FSS), Numerical Rating Scale-Fatigue, and Visual Analog Scale-Fatigue (VAS-F)] and six multidimensional [Fatigue Impact Scale (FIS), Checklist Individual Strength (CIS), Chalder Fatigue Scale (CFS), Multidimensional Assessment of Fatigue, Multidimensional Fatigue Inventory Scale, and Piper Fatigue Scale][6]. Unidimensional scales can be useful to assess severity or as screening tools, whereas multidimensional scores are more informative and can evaluate affective, cognitive, somatic, and behavioural manifestations of fatigue.

FACIT-F and FSS can be used as screening tools as they present a cut-off point to differentiate patients with fatigue *vs* non-fatigue. Eight of the previously reported scales (FACIT, FSS, BFI, CIS, MAF, MFI, FIS, and CFS) are able to detect disease progression or response to treatment[6].

Of all the above-mentioned fatigue-specific scales, the Fatigue Impact Scale is the only one which has been validated in PBC[23,24]. It was initially validated in 1994 by Fisk *et al*[25] in patients with multiple sclerosis and mild hypertension and was then found to be highly acceptable and consistent also in patients with PBC. It takes approximately 5 to 8 minutes to be completed and has a coefficient of reproducibility of 13% of the mean (vs 33% for the VAS-F)[23]. FIS measures the impact of fatigue on 40 aspects of daily life over the previous month. Patients are required to grade from zero to four how impaired has each aspect been to give a maximum score of 160 (severely fatigued). FIS assesses the impact of fatigue on psycho-social, cognitive, and physical activities[23].

However, the PBC-40 is the only instrument which can claim to be truly representative of PBC-related fatigue as it was originally developed for PBC patients. Jacoby *et al* [5] developed the PBC-40 in 2005 to assess PBC patients' quality of life. It investigates the impact of the disease in six domains: Fatigue, pruritus, social, cognitive, and other symptoms. The patients rate 40 items on a five-point scale, with a higher score indicating a worse quality of life. It has also been adapted in shorter versions. As it includes a fatigue subscale with proven content validity, it is the ideal instrument for studies on PBC-related fatigue. None of the proposed questionnaires specifically differentiate between peripheral and central fatigue.

Fatigue can also be assessed by measuring objective (*e.g.*, brain imaging, serological, and physical performance measures), as well as patient reported outcomes, but no consensus has been reached with regard to objective or combined assessments in PBC[7]**.** In the literature, there are a certain number of objective methods to distinguish central from peripheral fatigue. Peripheral fatigue, or impairment of muscle excitation, is most commonly evaluated with electromyography[26]. Serum lactate and IL-6 have been identified as the most accurate and valid biomarkers to measure muscle fatigue, although they are influenced by workload conditions and timing of testing with respect to exercise[27]. Other non-invasive methods have been employed for the detection of peripheral fatigue (*e.g.*, acoustic myography) [28]. On the other hand, central fatigue can be assessed either with percutaneous nerve stimulation[29] or transcranial magnetic stimulation during maximal contractions[30]. If the stimulation evokes an extra-force, it means that not all muscle units have been recruited, suggesting that central fatigue is present[30].

## LIFESTYLE ADJUSTMENTS AND DEVELOPING COPING MECHANISMS

Patients need to be advised and supported to develop coping strategies while retaining ownership of the problem. Pacing strategies (using available energy to its best advantage) and timing strategies (fatigue is worse later in the day typically so arranging key tasks for earlier in the day can make them less demanding) are recommended[3]. Awareness and understanding from carers should be promoted [3].

## EFFECT OF MAIN DRUGS FOR PBC ON FATIGUE

Ursodeoxycholic acid (UDCA) at a daily dosage of 13-15 mg/kg is the first line treatment for PBC. Although UDCA slows liver disease progression, increases transplant-free survival, and reduces mortality, it does not improve fatigue[1,31]. In China, a phase IV trial (NCT03345589) is being conducted

to compare the efficacy of an intermediate dosage of UDCA of 18–22 mg/kg/day and the standard dose over 6 mo in achieving biochemical remission. Unfortunately, since Angulo *et al* found no symptom improvement with an UDCA dosage increase to 23-25/mg/kg/day, the same is to be expected with the intermediate dosage[32].

Obeticholic acid (OCA) is a semi-synthetic hydrophobic bile acid analogue which can be used in patients who experience an inadequate response or are intolerant to UDCA. It is administered at an initial dose of 5 mg which can be titrated to 10 mg according to tolerability at 6 mo[1]. Fatigue is not responsive to OCA therapy[33]. OCA is associated with a dose dependent exacerbation in pruritus which can impair sleep and worsen fatigue[33].

Fibrates are a readily available but unlicensed treatment option for patients with PBC[34]. In the BEZURSO trial, a multicentre, double-blind, placebo-controlled, phase III clinical trial, 100 patients with inadequate response to ursodeoxycholic acid were randomly assigned to receive benzafibrate at a daily dose of 400 mg or placebo. After a 24 mo follow-up, 15% of patients in the benzafibrate group *vs* 9% in the placebo group reported an improvement in fatigue so benzafibrate could be the first therapeutic drug for PBC which has an effect on fatigue[35]. However, in this trial, no validated metrics were used to assess fatigue, as it was only categorised as absent, intermittent, or continuous. Further studies are required to confirm these results.

Budesonide is a synthetic corticosteroid with a high first-pass metabolism in the liver, which was found to improve liver histology and biochemistry in PBC patients with interface hepatitis on biopsy [36]. A recent phase-III, double-blind, randomised trial comparing budesonide combined with UDCA and UDCA only did not detect any improvement in liver histology, nor was fatigue alleviated[37].

## FAILED THERAPEUTIC OPTIONS

Modafinil is an approved treatment for daytime somnolence due to narcolepsy, sleep apnoea, and fatigue related to shift work sleep disorder. A randomised, double-blind, placebo-controlled study was conducted to assess the efficacy of modafinil for the treatment of fatigue in PBC did not show a significant improvement *vs* placebo[16], despite positive results from an uncontrolled study. The use of modafinil should therefore be limited to patients with formally diagnosed sleep disorders.

As previously mentioned, rituximab, which could have influenced fatigue severity by reducing circulating anti-PDC antibodies, did not significantly reduce fatigue in a single-centre randomised controlled trial with 57 participants[38]. The anaerobic threshold improved, possibly due to an effect on muscle bioenergetics dysfunction, but this did not lead to reduced fatigue symptoms. Interestingly, in two small sample studies on the use of plasmapheresis in PBC, in one study all patients who suffered from fatigue (4/5) reported reduced symptoms after treatment[39] and in the second study on 13 patients, the reduction of the PBC-40 fatigue domain score was statistically significant (30 *vs* 38, $P$ = 0.004)[40].

Ondansetron (a 5HT1 A receptor antagonist) did not determine an improvement in fatigue in a crossover study[20], nor did the use of selective serotonin reuptake inhibitors (fluoxetine and fluvoxamine) show any effect on fatigue in randomised controlled trials[18,19].

The empirical use of antioxidant therapy (vitamins A, C and E, selenium, methionine, and ubiquinone) had no effect on fatigue scores in a randomised, placebo-controlled crossover trial[41].

## LIVER TRANSPLANTATION

In the last few decades, there has been a decrease in the need for liver transplantation (LT) in PBC, most probably due to the introduction of UDCA as standard therapy[42]. According to current guidelines, liver transplant for fatigue in PBC is not appropriate as fatigue persists after transplantation in most patients[1]. Montali *et al*[5] conducted a prospective study to assess the impact of LT on fatigue. Although fatigue scores were significantly lower after LT, nearly half of LT recipients reported ongoing fatigue (44% of the total cohort and 47% of patients with low Model for End Stage Liver Disease score). These results have been confirmed in later studies[43].

## FUTURE TREATMENT PERSPECTIVES

### *Seladelpar*

Seladelpar is a selective peroxisome proliferator activated receptor delta agonist which has recently been assessed in an open-label, uncontrolled phase 2 study in PBC patients[44]. After 1 year of treatment, PBC-40 fatigue scores improved in 55%-64% of patients. Patients also reported a decrease in itch and sleep disturbance. These results need to be confirmed in a placebo-controlled and randomised trial.

**Table 2 Failed therapeutic options and future therapeutic perspectives for fatigue in primary biliary cholangitis**

| Treatment for fatigue in PBC | Ref. |
|---|---|
| Failed therapeutic options | |
| Ursodeoxycholic acid | Angulo *et al*[32], 1999 |
| Obeticholic acid | Hirschfield *et al*[33], 2015 |
| Budesonide | Hirschfield *et al*[37], 2021 |
| Fluoxetine | Talwalkar *et al*[18], 2006 |
| Fluvoxamine | ter Borg *et al*[19], 2004 |
| Ondansetron | Theal *et al*[20], 2005 |
| Rituximab | Khanna *et al*[14], 2019 |
| Modafinil | Silveira *et al*[16], 2017 |
| Methotrexate | Combes *et al*[48], 2005 |
| Oral antioxidant supplementation | Prince *et al*[23], 2003 |
| Lifestyle changes | |
| Morning bright light treatment | Turco *et al*[15], 2018 |
| Home-based exercise programme | Freer *et al*[46], 2021 |
| Possible future therapeutic options | |
| Fibrates | Corpechot *et al*[35], 2018 |
| Plasmapheresis | Wunsch *et al*[40], 2021 |
| S-adenosyl-L-methionine | Wunsch *et al*[45], 2018 |
| Seladelpar | Kremer *et al*[44], 2022 |

PBC: Primary biliary cholangitis.

### *S-adenosyl-L-methionine*

S-adenosyl-L-methionine, added to UDCA, can improve cholestasis in non-cirrhotic PBC patients, probably due to its hepatoprotective effects[45]. In an open label clinical trial on 24 PBC patients, there was a significant improvement in fatigue, assessed with the PBC-40 questionnaire[45].

Although causative mechanisms of fatigue in PBC are still unknown, therapeutic approaches have been sought to alleviate this debilitating symptom.

### *Home-based exercise programme*

Since fatigue in patients with PBC could be caused by muscle bioenergetic abnormalities, as previously mentioned, Freer *et al*[46] have performed a phase 1, single-arm, open-label clinical trial evaluating a novel exercise programme in patients with PBC with moderate-severe fatigue. Thirty-one patients concluded the 12-week home-based exercise programme which consisted of individualised resistance, aerobic exercises, and telephone health calls, although the results have not yet been published[46]. Peripheral muscle excessive acidosis and delayed pH recovery which characterise PBC patients can be improved with repeated single exercise episodes[47]. This programme is of great interest as patients with PBC tend to lead a sedentary lifestyle due to fear of exacerbating fatigue, but muscle fatigability is increased when physical activity is reduced.

### *Morning bright light treatment*

PBC is associated with poor sleep quality and delayed sleep-wake profile which can worsen the burden of fatigue. For this reason, Turco *et al*[15] conducted a pilot study to assess the efficacy of a short course of morning bright light treatment on sleep-wake patterns of fifteen PBC patients, six healthy individuals, and seven cirrhotic patients[15]. In patients with PBC, 15 d of light therapy resulted in subjective sleep quality improvement and a reduction in daytime sleepiness. In addition, sleep onset and get-up time were significantly advanced. Unfortunately, fatigue was not formally assessed, although daytime dysfunction due to somnolence was reported as improved[15].

Failed therapeutic options and future therapeutic perspectives for fatigue in PBC are summarised in Table 2.

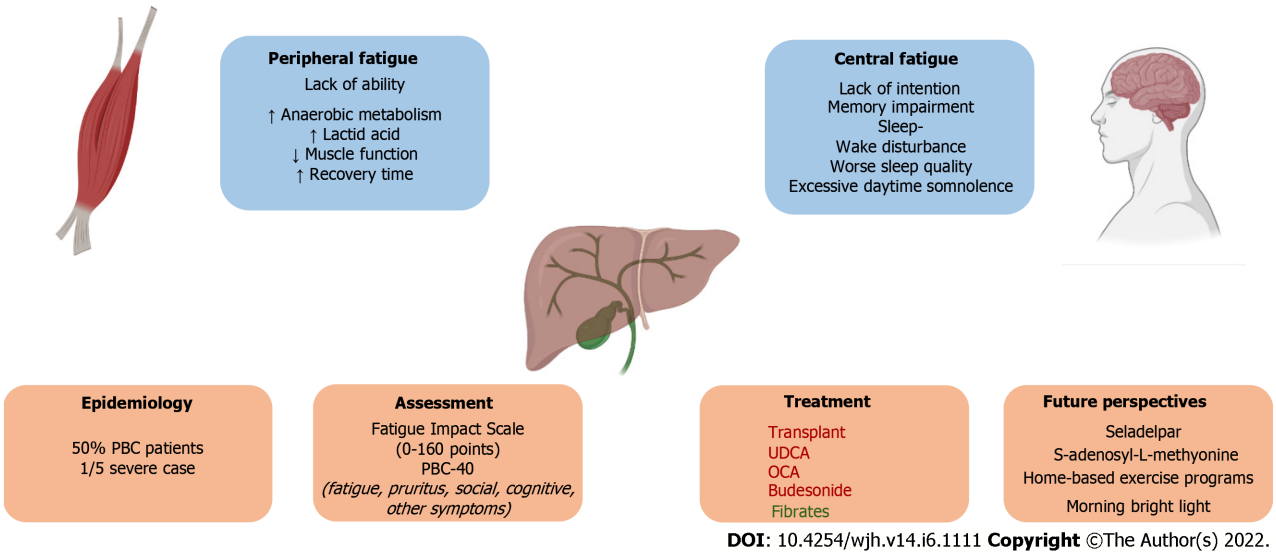


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**Figure 1 Fatigue in primary biliary cholangitis: Key concepts.** PBC: Primary biliary cholangitis; UDCA: Ursodeoxycholic acid; OCA: Obeticholic acid.

The key concepts presented in this review are illustrated in Figure 1.

## CONCLUSION

The pathophysiology of fatigue in patients with PBC is still unresolved and as yet, there is no licensed therapy for fatigue in PBC patients. Since fatigue is an extremely common and debilitating symptom, further research is warranted to understand its causative mechanisms and to find effective treatment.

## FOOTNOTES

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**Country/Territory of origin:** Italy

**ORCID number:** Erica Nicola Lynch 0000-0002-2638-2559; Claudia Campani 0000-0003-3842-782X; Tommaso Innocenti 0000-0002-2154-0490; Gabriele Dragoni 0000-0001-5752-5113; Maria Rosa Biagini 0000-0003-4840-6731; Paolo Forte 0000-0002-3107-5238; Andrea Galli 0000-0001-5416-6290.

**Corresponding Author's Membership in Professional Societies:** United European Gastroenterology; Associazione Italiana per lo Studio del Fegato; European Association for the Study of the Liver.

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*MINIREVIEWS*

# Fibrosis regression following hepatitis C antiviral therapy

Aisha Elsharkawy, Reham Samir, Mohamed El-Kassas

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**Aisha Elsharkawy, Reham Samir,** Department of Endemic Medicine and Hepatogastroenterology, Faculty of Medicine, Cairo University, Cairo 11566, Egypt

**Mohamed El-Kassas,** Department of Endemic Medicine, Faculty of Medicine, Helwan University, Cairo 11795, Egypt

**Corresponding author:** Mohamed El-Kassas, MD, MSc, PhD, Professor, Department of Endemic Medicine, Faculty of Medicine, Helwan University, Helwan, Cairo 11795, Egypt. m_elkassas@hq.helwan.edu.eg

## Abstract

Hepatitis C virus (HCV) infection is one of the most common causes of liver pathology. It is a major etiological factor of continuous liver injury by triggering an uncontrolled inflammatory response, causing liver fibrosis and cirrhosis. Liver fibrosis is a dynamic process that can be reversible upon timely cessation of the injurious agent, which in cases of HCV is represented by the sustained virological response (SVR) following antiviral therapies. Direct-acting antiviral therapy has recently revolutionized HCV therapy and minimized complications. Liver fibrosis can be assessed with variable invasive and non-invasive methods, with certain limitations. Despite the broad validation of the diagnostic and prognostic value of non-invasive modalities of assessment of liver fibrosis in patients with HCV, the proper interpretation of liver stiffness measurement in patients after SVR remains unclear. It is also still a debate whether this regression is caused by the resolution of liver injury following treatment of HCV, rather than true fibrosis regression. Regression of liver fibrosis can possess a positive impact on patient's quality of life reducing the incidence of complications. However, fibrosis regression does not abolish the risk of developing hepatocellular carcinoma, which mandates regular screening of patients with advanced fibrosis.

**Key Words:** Fibrosis regression; Hepatitis C virus; Direct-acting antivirals; Hepatocellular carcinoma; Liver fibrosis; Cirrhosis

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**Core Tip:** Hepatitis C virus (HCV) infection is one of the most common causes of hepatitis that results in continuous liver injury. Uncontrolled inflammatory responses result in liver fibrosis and cirrhosis. Liver fibrosis is a dynamic process that can be reversible upon timely cessation of the injurious agent. In cases of HCV, achievement of sustained virological response by antiviral therapies might be accompanied by regression of liver fibrosis and improvement of the patient's clinical profile. Assessment of liver fibrosis can be done with invasive and non-invasive methods, with certain limitations. Fibrosis regression can positively impact patients' quality of life, reducing complications.

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## INTRODUCTION

Hepatitis C virus (HCV) infection is a major causative agent incriminated in liver pathology. It commonly causes progressive liver disease that ranges from chronic inflammation to fibrosis and cirrhosis, with its complications including hepatocellular carcinoma (HCC). A long-term, persistent and uncontrolled inflammatory response is the hallmark of such diseases, leading to hepatic injury and more serious disease progression[1]. Chronic infection develops in around 85% of infected patients. According to the World Health Organization, about 71 million individuals worldwide are chronically infected with HCV, and mortality because of HCV-related hepatic complications approaches 0.39 million infected people annually. A major complication of HCV is liver fibrosis; and there is an ongoing need of better assessment of hepatic fibrosis with different accurate modalities[2]. Also, discovering an effective antiviral therapy was a major target for research, and fibrosis regression following treatment was a substantial challenge[3]. With the evolution of INF-free direct-acting antivirals (DAAs) has; the natural history of chronic hepatitis C has been modulated and now viral cure has become much more feasible[4].

## HCV AND LIVER FIBROSIS

Following infection with HCV, the immune responses in the liver are initiated by hepatocytes, Kupffer cells, hepatic stellate cells (HSCs) and immune cells (macrophages, mast cells, dendritic cells, and natural killer cells) recruited to the liver, causing spontaneous elimination of acute HCV infection. However, failure of the immune responses to eliminate the virus is documented in 70%-80% of cases during the acute phase, leading to chronic infection[5]. HSCs respond to a variety of extracellular signals that drive the fibrogenic response. Recent single-cell RNA sequencing studies have shown remarkable variability in HSCs and identified unique markers for different HSC subtypes[6].

Persistent HCV replication in hepatocytes triggers uncontrolled inflammation and production of excessive inflammatory cytokines, which exacerbates tissue damage (1) and stimulates the quiescent HSCs, leading to their activation and differentiation into myofibroblasts. Myofibroblasts are the main cells responsible for triggering fibrogenesis and the formation of various extracellular matrix (ECM) components in order to repair damaged tissues[7]. Because of increased cross-linking by tissue transglutaminases and resistance to proteolysis by metalloproteinases, the ensuing liver damage speeds up the thickening of septae, preventing the total regression of fibrosis. Furthermore, excessive ECM deposition leads to scar development, which may generally be corrected by fibrolysis. ECM deposition and breakdown alternate in the progression of liver fibrosis. When hepatic damage persists, fibrogenesis finally outpaces the liver's ability for scar clearance, and extracellular matrix accumulates[8]. However, liver fibrosis can be reversible following the resolution of HCV infection early. This potential reversibility decreases by the chronic persistent damage-causing fibrogenesis besides insufficient fibrinolysis even if HCV infection has resolved. At this point, fibrosis becomes irreversible and more progressive ending toward LC clearance of activated HSCs through apoptosis is a determining factor for liver fibrosis regression in chronic HCV patients[9]. Despite the remarkable efficacy of currently used direct-acting antivirals (DAAs) in eradicating HCV in over 95% of cases, this does not signify a cure from late-stage fibrosis or cirrhosis[10]. Infected individuals with HCV-associated fibrosis and viremia may need further therapy to effectively resolve liver damage caused by the virus.

## FIBROSIS REGRESSION FOLLOWING HCV TREATMENT: DOES FIBROSIS REALLY REGRESS?

The exact definition of fibrosis regression has not been properly established, but it shows a reduction in fibrosis content. However, this definition does not consider the other changes in liver architecture, including changes in nodule size, the extent of terminal venular collapse, elements of regeneration, or altered types or distributions of collagen and other ECM components, especially in cirrhotic liver. Neither is there a standardized definition of "clinically significant fibrosis regression." However, it is proposed that the concept is most often used to describe fibrosis regression that is adequate to improve clinical outcomes and decrease the risk of decompensation and consequences associated with portal hypertension. Studies that have characterized fibrosis regression following DAAs have only followed patients for 2-3 years, hindering the possibility of tracking long-term histologic changes after sustained virological response rates (SVR)[11]. However, most of these studies documented significant fibrosis regression following DAA therapy[12,13]. Tables 1 and 2 show review of different studies assessing fibrosis outcome and hepatic histological changes following DAA therapy[13-37]. Interestingly, fibrosis regression has been proved to continue as far as years following viral eradication; however, fibrosis regression was mainly documented shortly after end of treatment (EOT) partly due to lack of long term follow up. Several recent studies though could manage to follow HCV patients years following viral eradication to clarify this issue[11,18,23].

At the cellular level, variable mechanisms may explain the process of fibrosis regression. Most processes linked to fibrosis regression have been more described than the final destiny of activated HSCs following damage resolution, albeit mostly in animal models. In animal studies, three routes of HSC responses during regression have been identified: (1) Return to a state of inactivity; (2) Apoptosis/autophagy; and (3) Cellular senescence. HSCs may return from an active to a quiescent state [38]. The reversion or inactivation of HSCs reflects that the cells move to an inactivated state when liver injury resolves, yet they retain the ability to reawaken faster than fully dormant cells. Such data raise the possibility that reverted HSCs might contribute to the regression of fibrosis, but may promote a rapid progression of fibrosis and a severe recurrence of liver injury[39]. Aging, obesity, diabetes, and other variables have been linked to prolonged liver inflammation and fibrosis after hepatitis C SVR[18]. With the discovery of the Tcf21 transcription factor in mice, as well as additional transcription factors involved in HSC quiescence, such as GATA 4/6, LhX2, RAR, IRF 1/2, PPAR, ETS 1/2, GR, and NF1, the molecular basis for inactivation has recently been studied[40].

Fibrosis regresses, both in experimental and animal models. Eradication of the causative agent of liver fibrosis is the most appropriate way for the resolution of fibrosis by inducing remodeling of liver vascular architecture and regaining the normal lobular architecture. At some point, liver fibrosis may be reversed by removing the offending cause of liver disase. Regression of liver fibrosis was documented upon early management of cases of autoimmune hepatitis, or hepatitis B virus (HBV) infection[41]. The evolutions of variable inflammatory cascades, activated cells, and fibrogenic cytokines have been postulated as the driving force in liver fibrosis[11]. Similarly, fibrosis regression is associated with myofibroblast deactivation, collagenase enzyme activation, fibrillar cellular matrix disintegration, cell death (senescence and apoptosis of active stellate cells), and fibrous septa resorption[42]. Cirrhosis is a more complicated form of end-stage fibrosis that includes angiogenesis, necro-inflammatory alterations, innate immunity, oxidative stress, tissue hypoxia, and bacterial translocation[43]. The likelihood of fibrosis remission rather than cirrhosis remission is high. However, reversing liver fibrosis does not ensure that the problematic chemical will be removed. The age of an individual, genetic and epigenetic factors, rate of fibrosis progression (slow or rapid fibrosis), and disease-related factors like the etiology and staging of chronic liver disease are all factors that influence the fibrosis regression process. Other possible factors that may cause arrest in fibrosis regression or even cause progression such as liver steatosis with inflammation, alcohol use and diabetes mellitus working as contributing factors for liver injury and fibrosis[18,21]. According to Soliman *et al*[20], 2020; scores of fibrosis regression shows more regression in patients with lower degrees of steatosis and lower body mass index (BMI).

Interference must occur at a certain period for liver fibrosis to be reversible; otherwise, no regression is foreseeable. The "point of no return," or the moment at which the liver is sufficiently damaged that SVR will not reverse the illness, has yet to be defined. It is at this point that liver fibrosis progresses inexorably[20]. It's debatable whether progression and regression rates are connected, indicating that people who advance faster may also regress faster[11]. Despite the lack of evidence, most specialists feel that major regression is unlikely after severe architectural distortion, vascular collapse, and portal hypertension have occurred. This might be due to collagen's substantial structural cross-linking. As the collagen bands develop, the fibrotic bands are mostly fibrillar collagen. Some of these cross-links are permanent and cannot be destroyed by conventional collagenases, indicating that fibrosis development is unavoidable[44]. As a result, there is limited evidence that extensive areas of parenchymal extinction may be repopulated by regenerated hepatocytes, and vascular lesions in liver cirrhosis often remain with little evidence of full recovery to normal microcirculation in cirrhotic livers[45].

In the IFN era, before deciding for treatment choices, a liver biopsy was the gold standard for appropriate liver fibrosis staging. The majority of studies used paired liver samples to assess fibrosis

**Table 1 Studies illustrating outcome of liver fibrosis following hepatitis C virus clearance with direct-acting antivirals therapy**

| Ref. | Therapy | *n* | Follow-Up | Method of fibrosis assessment | Outcome |
|---|---|---|---|---|---|
| Knop *et al*[14], 2016 | DAA | 54 | 24 wk | TE, ARFI | - 88% achieved an improvement of LS values at FU24; - 46% showed reduction in liver stiffness higher than 30% at FU24 |
| Bachofner *et al* [15], 2016 | DAA | 392 | 18 mo | TE, FIB-4 and APRI scores, histopathological results were recorded if available | - Overall TE values of the included patients significantly decreased (regression of 32.4%); - Median FIB-4 and APRI values significantly decreased from 2.54 and 1.10 to 1.80 and 0.43 |
| Dolmazashvili *et al*[16], 2017 | INF-based/DAA | 304 | 24 wk | TE | - 65.1% achieved at least a 20% reduction in LS; - Overall proportion of patients with stage F4 decreased from 56.6% to 36.5% |
| Pietsch *et al*[17], 2018 | DAA | 143 | 24 and 96 wk | TE, FIB-4 and APRI | -A short-term reduction in LS until FU24 was seen in almost every patient regardless of stage of liver disease; -Further regression was seen in patients with early cirrhosis but not in individuals with cirrhosis and impaired liver function; -Progression of LS values occurred despite viral clearance in about one-sixth of the patients (17%) |
| Hedenstierna *et al*[18], 2018 | DAA | 269 | 7.7 yr | TE | - A majority improved their fibrosis stage after SVR; - 24% had persisting advanced fibrosis with a LS level of ≥ 9.5 kPa |
| Lledó *et al*[19], 2018 | DAA | 260 | SVR12 | TE | Significant fibrosis regression in 40% of the cohort patients more pronounced in patients with baseline advanced fibrosis and cirrhosis |
| Soliman *et al* [20], 2020 | DAA/ INF-based | 180 DAA/ 180 INF-based | at SVR12, 6 mo and 1 yr | TE | Reduction in fibrosis was reported in; 46.7% and 49.3% of patients with moderate fibrosis, and 89% and 78.7% of patients with advanced fibrosis after one yr of INF containing and INF free DAAs regimens respectively |
| Shiha *et al*[21], 2020 | DAA | 2326 | 12-45 mo | TE, and (FIB-4, FIB-5 and APRI) | - In cirrhotic patients, 21.8% showed reversal of cirrhosis, 27.4% showed fibrosis regression while 50.8% remained stationary; - About 26.5% of F3 patients showed reversal of fibrosis, 31.5% showed fibrosis regression and 30.6% remained stationary while 11.4% progressed to F4 |
| Hablass *et al* [22], 2021 | DAA | 137 | 12 mo | TE, and FIB-4 | In all patients, the FIB-4 and TE values after HCV elimination was significantly lower than its mean values at baseline |
| El-Kady *et al* [23], 2021 | DAA | 300 | 2 yr | TE | Both HCV treatment regimens showed improvement in liver fibrosis |
| Zakareya *et al* [13], 2021 | DAA | 655 | 1, 3, and 5 yr | TE | There was an overall significant regression of liver stiffness in all patients after sustained HCV eradication |
| Agwa *et al*[24], 2022 | DAA | 1230 | 12 and 48 wk | TE, FIB-4 | - 42.7% F4 patients improved and became (190) F3, (90) F2, and (220) F1; - 40 of 60 F3 patients improved and became (10) F2 and (30) F1; - 28.4% off the treated patients transited from significant fibrosis (≥ F3) to non-significant fibrosis (≤ F2) |
| Hassanin *et al* [25], 2022 | DAA | 162 | 48 wk | TIMP-1, FIB-4 and TE | Liver fibrosis was improved as evidenced by significant decline in serum level of TIMP-1, significant improvement in Fib-4 score, and significant decline in LSM |
| Thanapirom *et al* [26], 2022 | DAA | 89 | 1 yr | TE, SWE, and MRE | Viral eradication resulted in the reduction of LS values as evaluated by three elastography techniques |
| Rosato *et al*[27], 2022 | DAA | 516 | 24 mo | TE | - LS significantly decreased till SVR with a progressive reduction until 24 mo; - Only patients with steatosis and those who developed HCC did not experience a late improvement in LS |
| Yoo *et al*[28], 2022 | DAA | 112 | 48, 96 and 144 wk | TE, FIB-4 and APRI | LS value in patients achieving SVR signifcantly decreased over time (19.4 ± 12.9 kPa [baseline], 13.9 ± 9.1 kPa [48 wk], 11.7 ± 8.2 kPa [96 wk], 10.09 ± 6.23 [144 wk] |

DAA: Direct acting antiviral; INF: Interferon; TE: Transient elastography; ARFI: Acoustic Radiation Force Impulse; LSM: Liver stiffness measurement; SWE: Shear wave elastography; MRE: Magnetic resonance elastography; SVR: Sustained virological response; HCC: Hepatocellular carcinoma; TIMP-1: Tissue inhibitor of metalloproteinases-1.

changes after therapy. Several studies proved that fibrosis regression was documented in patients receiving IFN therapy by liver biopsy and non-invasive liver fibrosis parameters[32,46]. In the DAA era, dynamics of fibrosis regression following SVR have not been well identified, particularly because liver biopsy is infrequently performed. The quantity of fibrosis and its physical distribution, other underlying disorders, environmental or hereditary variables, and the variable elements that drive fibrosis advancement may all influence fibrosis regression. There have been no studies to discover genetic

**Table 2 Studies illustrating hepatic histological changes following hepatitis C virus clearance with direct acting antiviral therapy**

| Ref. | Therapy | *n* | Follow-up | Scores utilized | Outcome |
|---|---|---|---|---|---|
| Shiratori *et al* [29], 2000 | INF-based | 487 | Median of 3.7 yr apart (range, 1 to 10 yr) | Criteria of Desmet and colleagues (F0 to F4) and those of the French METAVIR Cooperative Study Group (A0 to A3) | - A mean reduction in fibrosis score of -0.60+/-0.07 at less than 3 yr of follow-up and -0.88+/-0.08 at 3 yr or more of follow-up; - Reversal of cirrhosis among 11 CHC patients; - Seven patients decreased their level of fibrosis from F4 to F2 and four from F4 to F3 |
| Poynard *et al* [30], 2002 | INF-based | 1030 | 20 mo mean duration between the biopsies | METAVIR scoring system | - Reversal and/or regression of cirrhosis occurred in 49% of patients where 15% regressed to stage 3, 16% reversed to stage 2, 15% reversed to stage 1 and 2% reversed to stage 0; - Fibrosis worsened in 15% |
| Maylin *et al* [31], 2008 | INF-based | 126 | 3.27 yr | METAVIR scoring system | - Fibrosis stage was improved in 56%, stable in 32%, progressed in 12%; - Regression of cirrhosis was observed in 64% patients; - No cirrhosis decompensation was observed, and 3 patients developed HCC |
| George *et al* [32], 2009 | INF-based | 49 | 5 yr | METAVIR scoring system | - 82% had a decrease in fibrosis score, and 92% had a decrease in combined inflammation score; - Two patients with pretreatment cirrhosis developed HCC and one died; - All the other patients with pretreatment cirrhosis or advanced fibrosis had improved fibrosis scores on long-term follow-up biopsy |
| D'Ambrosio *et al*[33], 2012 | INF-based | 38 | 61 mo | METAVIR scoring system, and the area of fibrosis was measured using morphometry | - Reversal of cirrhosis in 23.6% of the patients, regression of cirrhosis in 61%; regression of fibrosis in 36% of CHC patients |
| Enomoto *et al* [34], 2018 | DAA | 20 paired biopsy specimen | 41 ± 20 wk | Knodell scoring system and the METAVIR scoring system | - The inflammation grade significantly regressed, but the fibrosis stage did not; - Histological improvement, defined as a ≥ 2-point decrease in the Knodell inflammatory score and no worsening of the fibrosis, was found 55% patients. |
| Pan *et al*[35], 2018 | INF-based/DAA | 15 | 3 yr | METAVIR and Batts - Ludwig grading systems | - 13 of patients had improved liver stiffness (to < 9.5 kPa) |
| Chu *et al*[36], 2019 | INF-based | 31 | 93 mo | METAVIR scoring system and HAI | - Fibrosis regression, stable, and progression were 19%, 45%, and 36%; - A total of 71% of patients achieved inflammation improvement, whereas 6% and 23% of patients had stable disease and disease-progression, respectively |
| Cheng *et al* [37], 2021 | DAA | 21 | 6 mo | METAVIR fibrosis score and HAI | - Fibrosis scores improved in 61.9% of the patients; - 24.8% stable course; - 14.3% progression of fibrosis |

DAA: Direct acting antiviral; INF: Interferon; HAI: Histological Activity Index; HCC: Hepatocellular carcinoma; CHC: Chronic hepatitis C.

factors of fibrosis regression, including single nucleotide polymorphisms, since so few patients have received liver biopsy following HCV SVR due to its limitations[11]. The popularity of non-invasive methods of liver fibrosis staging was related to the advancement of DAA treatments, which had abolished the role of liver biopsy. As a result, most current research looking for fibrosis regression rely on paired or bi-paired non-invasive methods[47,48].

Despite the broad validation of the diagnostic and prognostic value of non-invasive modalities of liver fibrosis assessment, including liver stiffness measurement (LSM) in patients with HCV. LSM is a widely used non-invasive tool for the diagnosis and assessment of degrees of liver fibrosis and has high accuracy[49]. The proper interpretation of LSM in patients after SVR remains unclear. Many studies have shown a substantial reduction in LSM following SVR in HCV patients treated with DAAs[50-52]. It's still unclear if the drop in LSM after HCV eradication is due to HCV's necro-inflammatory activity being suppressed and changes in hepatic inflammation, rather than the regression of liver fibrosis. However, a large Canadian cohort of HIV-HCV co-infected patients that prospectively evaluated long-term changes in LSM before and after SVR due to DAAs confirmed that LSM after SVR likely indicates a true fibrosis reversal[53].

## ANTIVIRAL TREATMENT FOR HCV

Introducing the INF-free DAA has changed the natural history of chronic HCV in the past decade and revolutionized HCV treatment and viral cure. Indeed, improved quality of life is now a reality in most of patients. DAA regimens are safe and highly effective, resulting in SVR higher than 90%[4]. The last therapeutic regimens approved by Food and Drug Administration and European Medicines Agency are pan-genotypic, once-daily, all-oral DAA combinations that have the potential to close the gaps in the

current DAA treatment portfolio. Eight-twelve wk of treatment is now the standard of care, and viral eradication can be achieved in > 95% across different patient populations[54]. As a result, major scientific recommendations have been modified to promote DAA medication for all people who have chronic hepatitis C[4]. Furthermore, a recent large cohort research found that DAA therapy is linked to a lower risk of death and hepatocellular carcinoma (HCC), confirming SVR's long-term impact[55]. However, the risk of liver-related events persists in patients with HCV who have cleared the virus, particularly in those who had advanced fibrosis and cirrhosis prior to treatment[11].

## EVALUATION OF FIBROSIS

In the INF era, liver biopsy was the most accurate approach for making treatment choices as a precise assessment of liver fibrosis[56]. Therefore, the variable protocols of DAA therapies for HCV treatment had adopted reliance on non-invasive modalities of assessment of liver fibrosis. However, the existing non-invasive clinical and laboratory scores for assessing liver fibrosis performed poorly and inaccurately, failing to separate the phases of liver fibrosis' dynamic progression. Additionally, sophisticated imaging methods such as transient elastography (TE), shear wave (SW), acoustic radiation force impulse elastography (ARFI), and magnetic resonance elastography are available to quantify liver stiffness (LS) utilising a fibroscan instrument (MRE)[57]. However, it is important to interpret LSM cautiously as many studies denoted that liver stiffness could be affected by the presence of hepatic steatosis; and that the presence of severe steatosis, detected by histology or by US, should always be taken into account in order to avoid overestimations of liver stiffness[58,59]. In fact, higher LSM values in the presence of liver steatosis have been reported in patients with chronic HCV[60]. It was also reported that high body mass index BMI values negatively affected the diagnostic reliability[61]. Other limitations include the presence of tissue abnormalities, such as edema or inflammation which can interfere with LSM, independently of fibrosis stressing that LSM should be cautiously interpreted in such cases[62].

Finally, there is no perfect single test solution, as serological markers are good at assessing the advanced fibrosis stages only, making them inaccurate in mild to moderate fibrosis cases. So, it was suggested to use two non-invasive methods for assessing liver fibrosis, one imaging and the other serum marker, to be more effective and reliable[2]. Despite the high costs, time-consuming matter, and refusal of some patients, MRI elastography is a promising and more accurate tool for assessing liver fibrosis[47].

A widely accessible, reliable, accurate, reproducible, simple, and dynamic assessment of liver fibrosis development and reversal is still needed. In hepatology research, this seems to be an unmet need. Because the quantity of deposited collagens in each stage are not multiples of the preceding stage, the difference between liver fibrosis stages is a qualitative rather than a quantitative linear measure[63]. Late stages of fibrosis need more collagenases than early stages. Similarly, the non-uniform deposition of collagen in connection to time intervals is obvious, and variations in LS measurement in later stages may be within the same stage of fibrosis for the large number of people included[64].

## HISTOPATHOLOGICAL FEATURES OF FIBROSIS REGRESSION

There is no agreement on the suggested histological staging system for chronic viral hepatitis after therapy. Histological examination is best done on paired liver samples, one taken before treatment begins and the other taken at least six mo after the end of treatment (EOT). Fibrosis regression was formerly defined as a drop of at least one point in either the METAVIR or the histological activity index score from baseline to post-treatment assessment. The four-stage METAVIR fibrosis grading system was used to determine the fibrosis stage[37]. Stage 4 cirrhosis is further split into three categories based on fibrous septa thickness and nodule size, which correctly associated with clinical stage and the probability of hepatocellular carcinoma recurrence following curative resection[65]. Another scoring method is the hepatic repair complex, which is based on important histology features that indicate cirrhosis regression. The delicate perforated septa, isolated thick collagen fibres, thin peri-portal fibrous spikes, hepatic vein remnants with prolapsed hepatocytes, split septa interrupted by clusters or cords of hepatocytes, and aberrant parenchymal vein are all histological observations that support this system [66]. The Beijing classification, P-I-R Score, is a novel tool for dynamic assessment of fibrosis advancement versus regression (predominantly regressive, indeterminate, and predominantly progressive). This system was proposed by Sun *et al*[66] to evaluate chronic HBV before and after treatment[67].

More rigorous efforts are still required to respect the heterogeneous nature of the cirrhosis process to incorporate regression features and formulate a valid scoring system for better evaluation of fibrosis and activity regression in chronic liver diseases. In the assessment of fibrosis regression after HCV therapy, however, the improvement of digital pathology and the introduction of morphometry in determining collagen proportional area was noteworthy. Furthermore, second-harmonic generation/

two-photon excitation fluorescence (SHG/TPEF), a quantitative measure of liver fibrosis width, is thought to be the most accurate predictor of fibrosis regression[33].

Multiple studies addressed fibrosis regression following HCV treatment with INF and DAAs using invasive and non-invasive tools as shown in table 1 and 2. Conversely, reversal of liver inflammation and fibrosis was achieved in a significant number of patients treated with DAAs using histological assessment by liver biopsy[13-37].

## HEPATOCELLULAR CARCINOMA (HCC) POST-DAAS AND RELATION TO FIBROSIS REGRESSION

A 76% reduction in the risk of developing HCC in patients achieving SVR following IFN therapy has been documented by a meta-analysis[68]. However, some studies have pointed out that DAAs could instead augment the development of HCC. Despite the conflicting data regarding this issue in many studies, it was settled that HCV eradication has a protective effect against HCC development, regardless antiviral therapy. Response to treatment (SVR or non-SVR) was the sole independent predictor of HCC recurrence following curative treatment, rather than the type of antiviral treatment (IFN or DAA)[69].

Following the achievement of SVR, fibrosis regression reached its plateau for about one year. In addition, the fibrosis regression does not prevent the development of HCC years after treatment as liver, although deprived of the pro-inflammatory viral trigger, still has a potentially carcinogenic persistence [70,71]. Therefore, because of the insufficient data regarding the decrease in HCC risk after SVR with DAAs, patients, especially those with severe fibrosis, should be committed to frequent HCC screening.

## EFFECT OF FIBROSIS REGRESSION ON CLINICAL OUTCOMES

There is strong accumulating evidence that HCV eradication in all patients, besides patients with baseline cirrhosis, leads to improved clinical outcomes. A wide range of effects of HCV elimination exist. These include an overall reduction in mortality and the risk of HCC in patients with advanced fibrosis, and a reduction in extrahepatic manifestations, *e.g.*, HCV-related non-Hodgkin's lymphoma, other lymphoproliferative disorders, and cryoglobulinemic vasculitis[72,73]. Additionally, DAA-induced HCV clearance has been shown to decrease the risk of cardiovascular events in addition to the incidence of type 2 DM incidence probably by restoring the disordered glucose homeostasis[74,75]. Improvement in fibrosis, which seems to be a main driver of cirrhosis sequelae, will almost certainly lead to clinical improvement, even in patients with portal hypertension[11]. The long-term effects of fibrosis regression, however, are yet unknown. Furthermore, it has to be determined if the improved clinical outcome is due to successful causative therapy or fibrosis regression. Wu *et al*[76] looked at patients with compensated cirrhosis precipitated by HBV and found that changes in LS throughout the first 26 wk might predict decompensations and HCC with antiviral therapy. This might indicate that fibrosis regression has clinical implications. Cirrhosis regression was linked to lower morbidity and increased mortality in another HCV retrospective study[77]. However, there is currently a dearth of direct and convincing evidence that biopsy-proven fibrosis regression improves clinical outcomes. The more serious the underlying liver disease (particularly in individuals with advanced fibrosis and portal hypertension), the less likely the patient is to evade problems. Some individuals with severe liver disease and sequelae, on the other hand, may improve[11].

## CONCLUSION

Despite the amazing progress in HCV treatment using DAAs, information about its role in fibrosis regression is still inadequate. If the non-invasive methods for assessing liver fibrosis are suitable for assessing regression, it needs much research. Regression of liver fibrosis in cirrhotic patients and those with advanced fibrosis will remain a hope that both physicians and patients seek.

## FOOTNOTES

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**Country/Territory of origin:** Egypt

**ORCID number:** Aisha Mahmoud Elsharkawy 0000-0002-7150-173X; Reham Samir 0000-0001-7469-6483; Mohamed El-Kassas 0000-0002-3396-6894.

**Corresponding Author's Membership in Professional Societies:** Egyptian Association for Research and Training in Hepatogastroenterology, No. 001.

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ORIGINAL ARTICLE

Basic Study

# COVID-19 liver and gastroenterology findings: An *in silico* analysis of SARS-CoV-2 interactions with liver molecules

Gabrielle Caroline Peiter, Cristiano de Bem Torquato de Souza, Lucca Miketen de Oliveira, Luis Gustavo Pagliarin, Valentina Nunes Fontoura dos Anjos, Filipe Antônio França da Silva, Fabrício Freire de Melo, Kádima Nayara Teixeira

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**Gabrielle Caroline Peiter, Cristiano de Bem Torquato de Souza, Lucca Miketen de Oliveira, Luis Gustavo Pagliarin, Valentina Nunes Fontoura dos Anjos, Kádima Nayara Teixeira,** Universidade Federal do Paraná, Campus Toledo, Toledo 85919-899, Paraná, Brazil

**Filipe Antônio França da Silva, Fabrício Freire de Melo,** Universidade Federal da Bahia, Campus Anísio Teixeira, Vitória da Conquista 45029-094, Bahia, Brazil

**Corresponding author:** Kádima Nayara Teixeira, PhD, Professor, Universidade Federal do Paraná, Campus Toledo, Road 182, Km 320/321, Toledo 85919-899, Paraná, Brazil.
kadimateixeira@ufpr.br

## Abstract

### *BACKGROUND*

Coronavirus disease 19 (COVID-19) has not only been shown to affect the respiratory system, but has also demonstrated variable clinical presentations including gastrointestinal tract disorders. In addition, abnormalities in liver enzymes have been reported indicating hepatic injury. It is known that severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) might infect cells *via* the viral receptor angiotensin-converting enzyme 2 (ACE2) which is expressed in several organs including the liver. The viral Spike glycoprotein binds to ACE2 and must be cleaved by Furin and Type 2 Serine Protease to enter the cells. After that, the Akt/mTOR signaling pathway is activated and several COVID-19 changes are triggered.

### *AIM*

To analyze liver and gastrointestinal symptoms and cell signaling pathways triggered by SARS-CoV-2 infection due to virus-liver interactions *in silico*.

### *METHODS*

In this *in silico* study, the three-dimensional structures of the Akt, mTORC1 and Furin (receptors) were selected from the Protein Data Bank (PDB) and the structures of inhibitors (ligands) MK-2206, CC-223 and Naphthofluorescein were selected from PubChem and ZINC databases. Ligand files were downloaded as 2D structures and converted to optimized 3D structures using ViewerLite 4.2 software. Marvin Sketch® software was used to calculate prediction of the

protonated form of inhibitors in a physiological environment (pH 7.4). AutoDock Tools (ADT) software was used to calculate and delimit the Grid box used in the molecular docking of each structure selected in the PDB. In addition, protonated ligands were prepared for molecular docking using ADT software. Molecular docking was performed using ADT software tools connected to Vina software. Analysis of the amino acid residues involved in ligand interactions, as well as ligand twists, the atoms involved in interactions, bond type and strength of interactions were performed using PyMol® and Discovery Studio® (BIOVIA) software.

***RESULTS***

Molecular docking analysis showed that the mTORC1/CC-223 complex had affinity energy between the receptor and ligand of -7.7 kcal/moL with interactions ranging from 2.7 to 4.99 Å. There were four significant chemical bonds which involved two of five polypeptide chains that formed the FKBP12–Rapamycin-Binding (FRB) domain. The strongest was a hydrogen bond, the only polar interaction, and Van der Waals interactions shown to be present in 12 residues of mTORC1's FRB domain. With regard to the Akt/MK-2206 complex there were three Van der Waals interactions and 12 chemical bonds in which seven residues of Akt were involved with all five rings of the MK-2206 structure. In this way, both ASP 388 and GLN 391 bind to the same MK-2206 ring, the smaller one. However, LYS 386 had four chemical bonds with the inhibitor, one with each structure ring, while LYS 387 binds two distinct rings. One of the MK-2206 inhibitor's rings which binds to LYS 387 also binds simultaneously to ILE 367 and LEU 385 residues, and the fifth ring of the structure was involved in a bond with the ALA 382 residue. The hydrogen bonds were the shortest bonds in the complex (2.61 and 3.08 Å) and all interactions had an affinity energy of -8.8 kcal/moL. The affinity energy in the Furin/Naphhofluorescein complex was -9.8 kcal/moL and involved six interactions ranging from 2.57 to 4.98 Å. Among them, two were polar and the others were non-polar, in addition to twelve more Van der Waals interactions. Two distinct hydrogen bonds were formed between Furin and its inhibitor involving GLN 388 and ALA 532 residues. ALA 532 also binds to two distinct rings of Naphthofluorescein, while TRP 531 residue has two simultaneous bonds with the inhibitor.

***CONCLUSION***

Liver infection and signaling pathways altered by SARS-CoV-2 can be modulated by inhibitors that demonstrate significant interaction affinity with human proteins, which could prevent the development of infection and symptoms.

**Key Words:** Bioinformatics; Cell signaling pathway; COVID-19; Liver injury; SARS-CoV-2

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**Core Tip:** The classification of coronavirus disease 19 (COVID-19) as a respiratory disease caused the focus of studies to be directed in this direction. Therefore, mild clinical symptoms, such as gastrointestinal symptoms, have been little studied. Following the knowledge that COVID-19 is a systemic disease, studies on liver damage have become important. This study analyzed liver molecules targeted by severe acute respiratory syndrome coronavirus-2 infection using bioinformatics. Although these molecules are present in various organs due to the liver's central role in systemic metabolism, trying to understand metabolic changes in this organ will help understand systemic changes induced by the virus.

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## INTRODUCTION

At the end of 2019, in Wuhan (China), the emergence of a new coronavirus [severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)] was reported, which is classified as belonging to the *Betacoronavirus* genus and possessing as genetic material a single strip positive RNA, being capable of resulting in disease [coronavirus disease 19 (COVID-19)] that can evolve to severe acute respiratory syndrome. Although it is known that this disease mainly affects the respiratory system, it was identified

that it can manifest clinic signs and symptoms related to other organs, such as nausea, vomiting, abdominal pain and diarrhea. With regard to the liver, alterations in lesion markers including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total serum bilirubin were reported, indicating hepatic injury[1,2]. Between 14%-53% of patients with COVID-19 present with abnormalities in transaminase levels[3,4] which is associated with severity of the disease, and abnormal transaminase levels can indicate a higher chance of poor prognosis and Intensive Care Unit requirement[5-7]. The associated causes of liver injury, beyond direct viral impact, include the use of drugs during treatment, hypoxia due to pulmonary symptoms, previous hepatic lesions and co-morbidities[4]. Some of the drugs used can cause hepatotoxicity, liver injury and dysregulation, as shown for hydroxychloroquine, Azithromycin and Remdesivir, respectively[8,9]. One of the SARS-CoV-2 presentations on this system can be acute non-icteric hepatitis before the most common symptoms (fever and respiratory symptoms) [10] and those already related to the liver, nausea, diarrhea and abdominal pain. The viral cellular entrance and its dissemination is on a vast spectrum of the organism's systems, for example the liver-related, is a consequence of the expression of a common cellular receptor [angiotensin-converting enzyme 2 (ACE2)]. However, for effective cellular intrusion the participation of other human proteins such as Type 2 Serine Protease (TMPRSS2) and Furin (Convertase Proprotein of the Subtilisin Type)[1,2, 11-14]. Therefore, concerning the entrance mechanisms, it has been identified that the viral Spike glycoprotein possesses affinity and binds to the ACE2 (responsible for the adhesion stage), as it is structurally divided into two subunits: S1 (N-terminus, that connects to the receptor) and S2 (C-terminus, that takes part in the penetration process)[2,12,13]. Between both units, S1 and S2, there is a cleavage site for Furin, that triggers the activation and conformational change of viral Spike glycoprotein after it completes this action[2,13,14]. Following the initial activation process, further cleavage between the S1/S2 and S2' sites is essential for viral entrance. The TMPRSS2 protein performs this activity on the Spike glycoprotein of SARS-CoV-2, which allows fusion between the viral and cellular membranes, entry of the viral genetic material and the development of infection[1,11,12]. Following viral entrance, during the course of infection, modulation of the signaling pathway Akt/mTOR, that regulates apoptosis, cell survival, transcription and translation, occurs which also occurs during infection by other viruses[15-17]. This signaling possibly increases factors of viral translation while blocking mechanisms of cellular death, generating greater pathogenicity. Based on this, recent studies have indicated the possibility of using already existing drugs that interfere with this pathway for the treatment of COVID-19, including MK-2206, an Akt inhibitor[18].

Despite these facts, studies directly investigating the interaction between SARS-CoV-2 and liver cells, specifically the entrance mechanisms, the biochemical cascades and methods for possible infection inhibition still require further investigation. Therefore, the present study aims to associate the hepatic alterations triggered by SARS-CoV-2 with the activation and/or inhibition of transduction pathways of cellular signals in the viral infection process. In addition, possible intervention in the signaling pathways with inhibitors was analyzed to suggest potential treatments for SARS-CoV-2 infection.

## MATERIALS AND METHODS

### *Receptors and ligands preparation*

Akt, mTORC1 and Furin enzymes are inhibited by MK-2206, CC-223 and Naphthofluorescein, respectively. The three-dimensional structures of proteins/enzymes (receptors) Akt, mTORC1 and Furin were selected from the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) and their files were obtained in the extension.pdb (input file). The structures of the inhibitors (ligands) MK-2206, CC-223 and Naphthofluorescein were selected from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and ZINC databases (https://zinc12.docking.org/). The ligand files were downloaded as 2D structures in the extension.sdf and converted into.pdb (input file) by optimizing the 3D structure using ViewerLite 4.2 software (Accelrys Inc.). The selected receptor's three-dimensional structure was prepared for molecular docking simulations using AutoDock Tools (ADT) software (MGL, The Scripps Research Institute); possible ligands (ions, peptides) were eliminated, water molecules were deleted and hydrogen atoms were added. The location and dimensions of the Grid box (virtual box that delimits the region where the ligand will perform possible interactions with the receptor) were then determined using ADT software. The Grid box data and coordinates were used in molecular docking. The ligands interact with their receptors in a physiological environment at pH 7.4; thus, to reliably simulate their interaction, calculations for the prediction of their protonation state were carried out using Marvin Sketch® software (ChemAxon®). Protonated ligands were prepared for molecular docking using ADT software. This software detects the torsion points of the inhibitors and calculates their angle of torsion.

### *Molecular docking*

Molecular docking procedures for a rigid receptor and a flexible ligand were used. A grid of points in x, y, and z directions was built with a grid spacing of 1.0 Å using the AutoGrid component of the software. Molecular docking simulations were performed using ADT software connected to Vina software[19].

The software used associates two components: A search algorithm and a score function. The algorithm is responsible for the search of possible combinations in the bonds, exploring the rotational, translational and conformational degrees of freedom of the ligand, as well as of the proteins. The score function is then used to choose the best binding modes. These functions are obtained according to the force fields of molecular mechanics and empirical parameters from free energy calculations, thus an affinity energy is calculated. The cut-off for stable interactions is considered an affinity energy < −6.0 kcal/moL[20]. The results are based on the first docking conformer of the ligands with reference Root-Mean-Square Deviation of atomic positions (RMSD) of 0[21]. The analyses of amino acid residues of receptors involved in the interactions with ligands, as well as the twists of the ligand, atoms involved in the interactions, type, strength and length of the interactions were performed using PyMol® software (pymol.org/2) and Discovery Studio® software from BIOVIA.

## RESULTS

### *Receptors and ligands structures*

The receptors' 3D structures selected were the 5WBH-FKBP12–Rapamycin-Binding (FRB) domain of mTOR; 1GZN-PKB kinase domain and 5JXI-Furin convertase. All structures are human and present less than 2.5 Å of resolution. The structure of inhibitor MK-2206 was selected from ZINC databank-ZINC36382821 and the structures of inhibitors CC-223 (CID58298316), and Naphthofluorescin (CID3124834) were selected from the PubChem databank.

### *Interactions of protein-inhibitor complexes*

**mTORC1/CC-223 complex**: Molecular docking was used to analyze if there was an interaction between mTORC1 enzyme and CC-223 in its protonated form. In this way, the most stable mTORC1/CC-223 complex showed an affinity energy of -7.7 kcal/moL (Figure 1). Four significant chemical bonds were observed between the receptor and the ligand involving four distinct residues and bond size ranging from 2.7 to 4.99 Å (Table 1). Of these bonds only one was polar (hydrogen bond) and according to their size, this was the strongest (2.7 Å); the other bonds had a non-polar character. In addition, 12 other residues were involved in Van der Waals interactions (Figure 2). The four significant bonds in the mTORC1/CC-223 complex involved two (C and D) of five polypeptide chains (A-E) that formed the FRB domain.

**Akt/MK-2206 complex**: Analysis of the interaction between the Akt enzyme and its protonated inhibitor-MK-2206, showed that the complex was maintained by 12 chemical bonds, in addition to three Van der Waals interactions (Figure 3 and Table 1). All five rings of the MK-2206 structure were involved in bonds with amino acid residues of Akt enzyme. Seven residues of Akt were involved in chemical bonds; ASP 388 and GLN 381 residues were bound to an oxygen atom and a nitrogen atom of MK-2206 by a hydrogen bond, respectively. Hydrogen bonds were the shortest of the Akt/MK-2206 complex (2.61 and 3.08 Å). ASP 388 was bound to MK-2206 by a Pi-Alkyl bond (hydrophobic character). Both ASP 388 and GLN 391 were bound to the same MK-2206 ring, the smaller one. LYS 386 had four chemical bonds with the inhibitor (3.08-5.25 Å), one with each ring structure; three of them were Pi-Cation and the other was Pi-Alkyl. LYS 387 was bound to two distinct rings of the MK-2206 inhibitor by Pi-Alkyl bonds (5.19 and 5.22). One of the MK-2206 inhibitor's rings that bound LYS 387 also bound simultaneously to two other residues-ILE 367 (Pi-Alkyl) and LEU 385 (Amide-Pi). These bonds involved four of five rings of MK-2206. The fifth ring of the structure was involved in a single Pi-Alkyl bond with the ALA 382 residue. All bonds and their distribution conferred an affinity energy of -8.8 kcal/moL (Figure 1).

**Furin/naphthofluorescein complex**: Molecular docking was used to analyze whether there was an interaction between Naphthofluorescein and human Furin. In this way, the simulation helped to better understand the interaction dynamics between the protein and the inhibitor. After analyzing the protonation state of Naphthofluorescein, the interaction affinity was calculated by AutoDock Vina, and was -9.8 kcal/moL for the complex formed. It was possible to observe that Naphthofluorescein interacted with human Furin through six interactions with a length ranging from 2.57 to 4.98 Å. Two distinct hydrogen bonds were formed between Furin and its inhibitor involving GLN 388 and ALA 532 residues. Among them, two were polar and the others were non-polar, in addition to twelve more Van der Waals interactions. ALA 532 was also bound to two distinct rings of Naphthofluorescein by a Pi-Sigma bond and Pi-Alkyl bond. TRP 531 residue showed two simultaneous bonds with the inhibitor (Figure 4 and Table 1).

## DISCUSSION

Liver is the main organ involved in drug metabolism and xenobiotic detoxification; therefore, its proper

**Table 1 Type of interaction and bond size between amino acid residues of proteins and inhibitors**

| Protein | Residues | Type | Bond size (Å) |
|---|---|---|---|
| mTORC1 | HIS 2024 | Pi-Alkyl | 4.99 |
| | GLU 2080 | Carbon hydrogen bond | 3.45 |
| | GLN 2099 | Hydrogen bond | 2.70 |
| | ARG 2110 | Carbon hydrogen bond | 3.44 |
| | 12 residues | Van der Waals | - |
| Akt | ILE 367 | Pi-Alkyl | 5.04 |
| | ALA 382 | Pi-Alkyl | 5.02 |
| | LEU 385 | Amide-Pi | 3.90 |
| | LYS 386 | Pi-Alkyl | 5.25 |
| | LYS 386 | Pi-Cation | 3.08 |
| | LYS 386 | Pi-Cation | 4.19 |
| | LYS 386 | Pi-Cation | 4.94 |
| | LYS 387 | Pi-Alkyl | 5.22 |
| | LYS 387 | Pi-Alkyl | 5.19 |
| | ASP 388 | Hydrogen bond | 3.08 |
| | ASP 388 | Pi-Sigma | 3.67 |
| | GLN 391 | Hydrogen bond | 2.61 |
| | 3 residues | Van der Waals | - |
| Furin | TRP 531 | Pi-Pi T-shaped | 4.97 |
| | TRP 531 | Pi-Pi T-shaped | 4.95 |
| | ALA 532 | Hydrogen bond | 2.57 |
| | ALA 532 | Pi-Alkyl | 4.98 |
| | ALA 532 | Pi-Sigma | 3.94 |
| | GLN 488 | Hydrogen bond | 3.12 |
| | 12 residues | Van der Waals | - |

functioning is essential for the effectiveness of pharmacological treatments. As altered liver function is reported in up to half of patients with COVID-19, it is important to clearly understand the possible mechanisms involved in liver injury in order to optimize the treatment outcome of this disease[22].

Moderate microvesicular steatosis and mild inflammation in the lobular and portal area are pathological findings in liver tissue in patients with COVID-19[23]; thus, this may contribute to the incidence of elevated levels of hepatic transaminases reported in this disease[24]. In the early stage of COVID-19, infected individuals have positive SARS-CoV-2 RNA in fecal and blood samples, and present gastrointestinal symptoms such as diarrhea, abdominal pain, nausea, and vomiting[25] suggesting that SARS-CoV-2 could infect liver cells[26].

In SARS-CoV-2 infection, it is known that the viral Spike glycoprotein interacts with the ACE2 present in humans, leading to entry of the virus. However, for its entry to occur, there is a need for activation of the glycoprotein by host cell proteases which occurs between the S1/S2 subunits of Spike generating a conformational change in the S2 subunit and allowing the interaction of SARS-CoV-2 with ACE2, completing virus entry[12].

As SARS-CoV-2 interacts with the ACE2 receptor of host cells to invade them[27]; thus, cells that have this receptor are susceptible to infection. The level of ACE2 expression is low in hepatocytes (2.6%), but in bile duct cells (cholangiocytes) this expression is high (59.7%)[28]. Therefore, SARS-CoV-2 does not necessarily directly infect liver cells, but causes bile duct dysfunction that plays an important role in liver regeneration and immune response[26].

In fact, according to Xu *et al*[23] (2020), no direct cytopathic effect of SARS-CoV-2 on the liver was found in pathological autopsy findings. On the other hand, the findings of Pirola and Sookoian[29] support the possibility that SARS-CoV-2 may cause direct liver injury by the viral cytopathic effect. These authors showed that the three host cell proteins-ACE2, Furin and TMPRSS2, responsible for viral

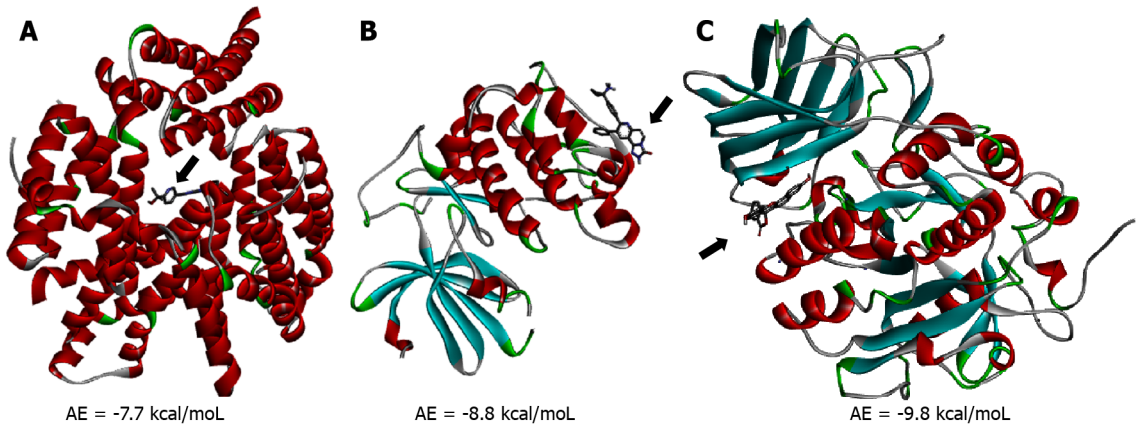


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**Figure 1 Panoramic view of the enzyme/inhibitor complexes.** A: mTORC1/CC-223 complex; B: Akt/MK-2206 complex; C: Furin/Naphthofluorescein complex. Arrow points to the inhibitor. Alpha helices are shown in red, Beta sheets are shown in blue, Random coils are shown in green and Turns are shown in grey. AE: Affinity energy.

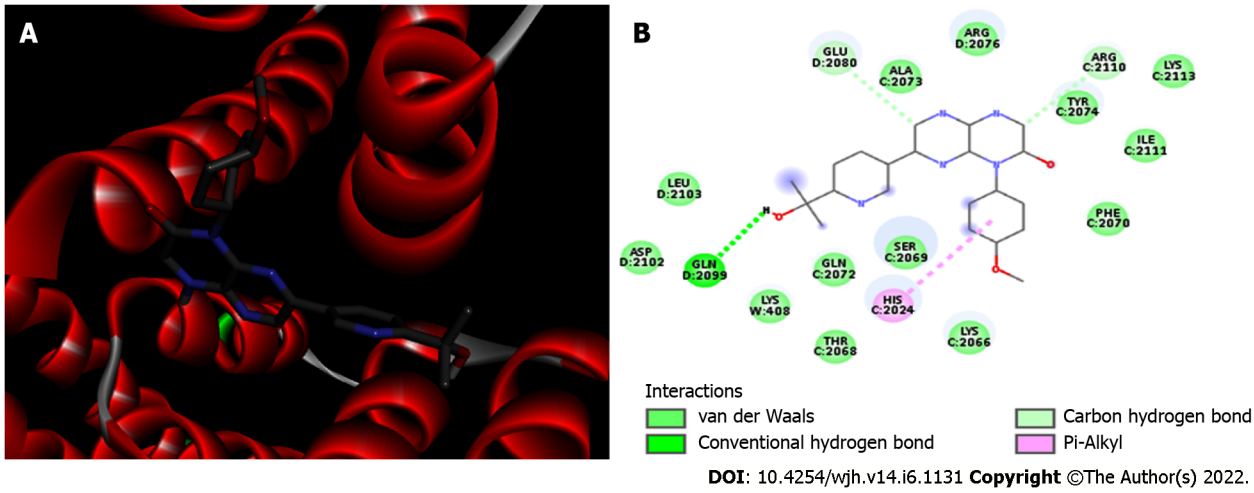


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**Figure 2 mTORC1/CC-223 complex.** A: 3D view of the CC-223 inhibitor (sticks) bound to the FKBP12–Rapamycin-Binding domain of mTORC1 (Protein Data Bank 5WBH). mTORC1 is shown in red alpha-helices; B: 2D diagram showing the mTORC1 residues that bind to CC-223. The internal legend indicates the bond type by color. Amino acid residues are shown in 3-letter code; the number indicates the position within the chain, and the capital letters (A, C and D) indicate which chain the residue belongs to.

infection are expressed in liver tissue. Although ACE2 has low expression in hepatocytes compared with cholangiocytes, TMPRSS2[1] and Furin[30] are expressed more in hepatocytes.

The Spike glycoprotein of SARS-CoV-2 facilitates viral entry into host cells; the surface unit S1 binds to the cellular receptor-ACE2, while the transmembrane unit S2 facilitates fusion of both viral and cellular membrane. Membrane fusion depends on S protein cleavage by host cell proteases at the S1/S2 and the S2′ sites[31], and among these proteases, TMPRSS2 and Furin play major roles in proteolytic activation of a broad range of viruses[32] including SARS-CoV-2. After infection, the signaling pathways of the host cell are affected to promote viral replication. Activation of the Akt/mTOR signaling pathway and through a cascade of events, mTORC1 and Akt activate host transcription and translation of specific genes. The activation of Akt/mTOR signaling during SARS-CoV-2 infection could be to sustain protein synthesis by increased access to translation components[33].

An analysis of the summary of the SARS-CoV-2 infection process indicates that host cell proteases and signaling cascade proteins could be a potential target for therapeutic interventions for COVID-19 symptoms, including gastrointestinal symptoms due to liver damage.

This study analyzed the interactions of SARS-CoV-2 with molecules which are necessary for the success of infection and are expressed in the liver using bioinformatics tools and *in silico* enzymatic inhibition tests to try to associate such interactions with the gastrointestinal findings and liver injuries in COVID-19. It was shown that the interaction affinity of some proteins present in the human body with their respective inhibitors that can act in their pathways and prevent the development of infection. The

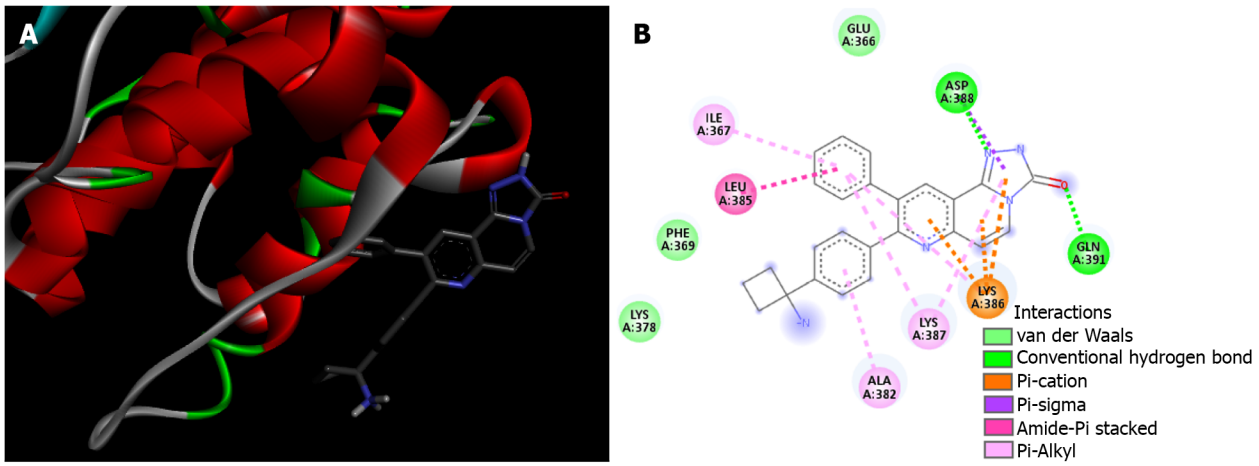


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**Figure 3 Akt/MK-2206 complex.** A: 3D view of MK-2206 inhibitor (sticks) bound to Akt kinase domain (Protein Data Bank 1GZN). Akt is shown in red alpha-helices, green random coils and grey turns; B: 2D diagram showing the Akt residues that bind to MK-2206. The internal legend indicates the bond type by color. Amino acid residues are shown in 3-letter code; the number indicates the position within the chain, and the capital letters (A) indicate which chain the residue belongs to.

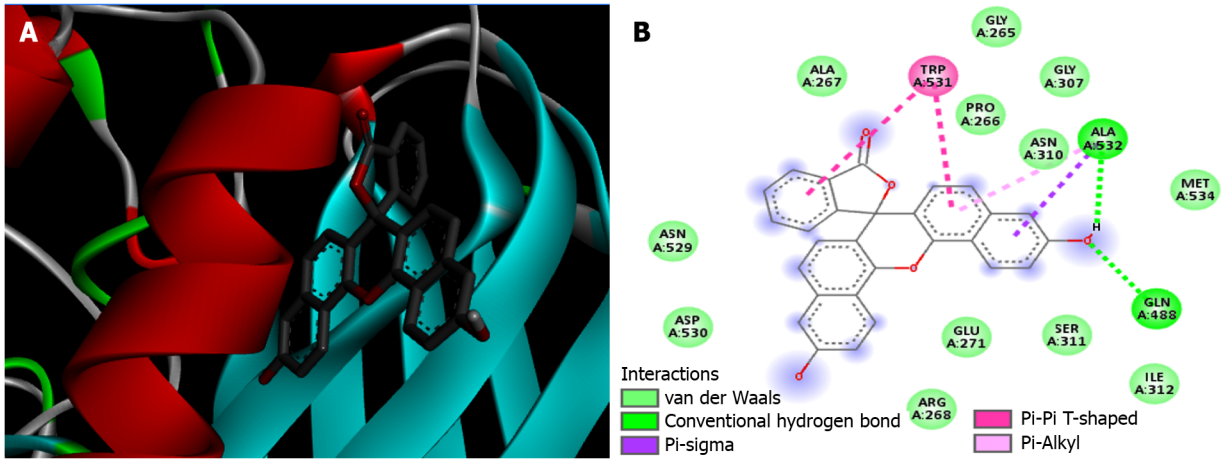


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**Figure 4 Furin/Naphthofluorescein complex.** A: 3D view of Naphthofluorescein inhibitor (sticks) bound to Furin (Protein Data Bank 5JXI). Furin is shown in blue beta sheets, red alpha-helices, grey random coils and green turns; B: 2D diagram showing the Furin residues that bind to Naphthofluorescein. The internal legend indicates the bond type by color. Amino acid residues are shown in 3-letter code; the number indicates the position within the chain, and the capital letters (A) indicate which chain the residue belongs to.

target proteins were the Furin enzyme, involved in cell invasion, and mTORC1 and Akt enzymes belonging to the signaling pathway.

To verify the interaction between the mTORC1/CC-223, Akt/MK-2206 and Furin/naphthofluorescein complexes at the molecular level, molecular docking was used. Thus, if there is an interaction between proteins and inhibitors, simulation helps us to understand the dynamics that occur *in silico*[34].

The interaction affinity calculated by AutoDock Vina for the mTORC1/CC-223 complex was -7.7 kcal/moL, for the Akt/MK-2206 complex it was -8.8, and for the complex formed by Furin/naphthofluorescein it was -9.8 kcal/moL. These values are considered significant since values lower than -6.0 kcal/moL already constitute stable interactions *in silico* analysis[35].

The complex with the highest interaction affinity was that formed by Furin and its inhibitor. This significantly low affinity energy value indicates a more stable complex, in other words, indicates that the inhibitor will have higher biological activity[36]. Unlike other coronaviruses, SARS-CoV-2 has a potentially critical insertion of a Furin cleavage site upstream of the S1 cleavage site in the Spike glycoprotein reducing its dependence on host cell proteases for infection. The high affinity between ACE2 and the Spike glycoprotein cleaved by Furin allows SARS-CoV-2 to maintain its efficient entry into cells while preventing the action of the immune system which can contribute to the widespread infection capacity of the virus[14,37]. As ACE2 is present in type 2 alveolar cells, the gastrointestinal

tract and the liver, these tissues would be more affected by COVID-19[38]; therefore, inhibiting the action of Furin would prevent infection of these tissues and consequently the associated symptoms.

Vankadari[39] in his study on Furin analyzed not only its structure but also how it would bind at S1/S2 subunits of the Spike glycoprotein. Thus, it was suggested that Furin binds to these subunits through the equatorial region present in the Spike glycoprotein which creates a 970 Å interface between the participants.

The Furin enzyme is required in various normal functions of the body[40]. Prolonged Furin blocking can therefore generate side effects or damage[41]. In this context, Furin's involvement in the viral invasion process could reduce the effectiveness of the action of this enzyme in normal physiological processes triggering pathological processes. In fact, studies suggest that Furin plays an important role in homeostasis and disease[42]; thus, it is possible that liver cell lesions would be reduced and AST and ALT levels would be normal. On the other hand, brief Furin inhibition can be well tolerated and has therapeutic benefit[41]; therefore, Naphthofluorescein has promising potential for treatment through this route given its high affinity for Furin *in silico.*

Another way of studying the symptoms resulting from liver injury triggered by SARS-CoV-2 infection is to analyze the signaling pathway affected by the virus. In fact, some studies point to the dysregulation in the Akt/mTOR signaling cascade, which could be a potential target in COVID-19 treatment[33].

It was observed in this study that the Akt/MK-2206 complex remains with 12 bonds, added to three Van der Waals interactions and all five rings of the MK-2206 inhibitor are bound to protein, suggesting stability of the complex, and the protein Akt would be unable to proceed with his cascade. Shi *et al*[43] in his findings on the inhibition of esophageal cancer growth through the PI3K/AKT/mTOR pathway, showed that MK-2206 would be a potential allosteric inhibitor of Akt, by decreasing cell proliferation, inducing cell cycle arrest and increasing apoptosis of cancer cells. Furthermore, Appelberg *et al*[33] observed that the Akt/mTOR/HIF-1 pathways participate in COVID-19 infection, in particular, Akt/mTOR are activated at the beginning of infection. It has also been shown that MK-2206 caused a decay in viral transcription in SARS-CoV-2 infected cells and supernatants by interacting with Akt. Based on these data and on the results obtained with molecular docking, in which significant affinity and stability of the bonds were observed, it is possible that the MK-2206 inhibitor may help to contain the development of COVID-19 by interacting with Akt, in a way that prevents continuity of the cascade triggered by this protein.

It is possible that in the mTORC1/CC-223 complex significant chemical bonds are established along the CC-223 structure involving three of four molecule rings. The same was observed for Van der Waals' interactions. The sum of all binding and interactions between mTORC1 and CC-223 contribute to the formation of a complex with significant affinity energy which contributes to its stability. Mortensen *et al*[44] showed that CC-223 as an inhibitor has high affinity for mTOR, and the pathway to which it belongs was unfeasible. It has been described that mTOR is relevant in cell growth, proliferation, survival and metabolism; in particular, mTORC-1 plays a role in protein synthesis and cell development. In view of this, CC-223 has been reported to cause inhibition of mTORC-1 *in vivo* upon administration of this compound in tumor-bearing mice, suppressing continuation of the cascade[44]. Added to this, in another study, Mortensen *et al*[45] also analyzed the interaction of compounds with the PI3K/AKT/mTOR pathway, thus, selectivity of the CC-223 inhibitor for the mTORC-1 protein was highlighted, as the former has high affinity for the latter and may inhibit it[45]. Furthermore, in line with the results obtained from molecular docking, it was found that binding of the mTORC-1 and CC-223 complex has a high affinity and stability, therefore, considering the potential of this inhibitor for blockade of the mTOR pathway, it may be used to prevent the spread of SARS-CoV-2 infection and, consequently, restrict disease spread. In this way, as the liver is the main regulatory organ of metabolism and the mTOR/Akt signaling pathway plays a key role in cellular metabolism, changes in this pathway are significantly reflected in the liver; thus, preventing changes in this pathway by the virus would help to slow down the symptoms.

## CONCLUSION

Current understanding suggests that the possible interaction between SARS-CoV-2 and liver cells occurs with enhancer factors that facilitate ACE2-mediated SARS-CoV-2 infection, such as Furin and TMPRSS2. In this study, we demonstrate, by means of molecular docking, significant affinity and stability of the bonds concerning some human hepatic proteins and their inhibitors. These work in signaling pathways due to interactions between mTORC1/CC-223, Akt/MK-2206 and Furin/naphthofluorescein complexes, and are capable of preventing infection development. Our *in silico* analysis shows the possible inhibitory mechanisms of cell viral receptors in the liver, which is consistent with other studies. Therefore, multi-target therapeutic drugs based on these pathways may be an option in COVID-19 patients, especially in severe/critical cases, preventing multiple organ dysfunction and perhaps leading to a more positive prognosis.

## ARTICLE HIGHLIGHTS

### *Research background*

Coronavirus disease 19 (COVID-19) has variable clinical manifestations, including gastrointestinal and hepatic disorders. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infects liver cells using angiotensin-converting enzyme 2, and the viral Spike glycoprotein must be cleaved by Furin or Type 2 Serine Protease. Following activation of the Akt/mTOR pathway several changes are triggered.

### *Research motivation*

Liver damage in COVID-19 is not well understood; therefore, molecular analysis of the infection process and cell signaling, *in silico*, can help in the discovery of targets for treatment of the disease.

### *Research objectives*

To analyze liver and gastrointestinal symptoms and cell signaling pathways triggered by SARS-CoV-2 infection due to virus-liver interactions *in silico*.

### *Research methods*

SARS-CoV-2/liver cell interactions, and signaling pathways activated by these interactions, were analyzed by inhibition studies using the molecular docking method.

### *Research results*

The mTORC1/CC-223 complex, Akt/MK-2206 complex and Furin/naphthofluorescein complex showed significant affinity energy, indicating stability and consequent effectiveness in inhibiting target molecules for COVID-19 therapy.

### *Research conclusions*

Liver disease and signaling pathways altered by SARS-CoV-2 can be modulated by inhibitors that demonstrate significant affinity for interactions with human proteins, which could prevent progression of the infection and its symptoms.

### *Research perspectives*

Evaluate the inhibition complexes studied using molecular dynamics and verify the possibility of structural changes of the drug to increase its efficiency and avoid possible adverse effects.

## FOOTNOTES

**Author contributions:** Peiter GC, de Souza CBT, Oliveira LM, dos Anjos VNF and Pagliarin LG performed the experiments, analyzed the results and wrote the manuscript; da Silva FAF analyzed the results and reviewed the manuscript; de Melo FF performed a critical analysis of the results and corrected the manuscript; Teixeira KN interpreted the data, performed a critical analysis of the results, corrected the manuscript and coordinated the study; all authors approved the final version of the manuscript.

**Institutional review board statement:** The study did not need approval by the Research Ethics Committee as it was an *in silico* study.

**Conflict-of-interest statement:** All the authors report no relevant conflicts of interest for this article.

**Data sharing statement:** No additional data are available.

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**Country/Territory of origin:** Brazil

**ORCID number:** Gabrielle Caroline Peiter 0000-0001-7965-4434; Cristiano de Bem Torquato de Souza 0000-0002-2036-5600; Lucca Miketen de Oliveira 0000-0003-4302-0012; Luis Gustavo Pagliarin 0000-0003-1447-4613; Valentina Nunes Fontoura dos Anjos 0000-0002-3346-662X; Filipe Antônio França da Silva 0000-0002-0550-1109; Fabrício Freire de Melo 0000-0002-5680-2753; Kádima Nayara Teixeira 0000-0002-2928-9181.

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Case Control Study

# Clinical outcomes of coronavirus disease 2019 in liver transplant recipients

Muhammad Shafiq, Cheryl Gibson

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**Muhammad Shafiq, Cheryl Gibson,** Department of General and Geriatric Medicine, University of Kansas Medical Center, Kansas City, KS 66160, United States

**Corresponding author:** Muhammad Shafiq, MD, Assistant Professor, Department of General and Geriatric Medicine, University of Kansas Medical Center, 4000 Cambridge Street, 6040 Delp & Mail Stop 1020, Kansas City, KS 66160, United States. mshafiq@kumc.edu

## Abstract

### *BACKGROUND*

Liver transplant patients are at higher risk of infection due to immunosuppression. Whether liver transplant recipients are also more susceptible to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and will have worse outcomes than the general population if they develop coronavirus disease 2019 (COVID-19) due to SARS-CoV-2 is a topic of ongoing studies, including ours.

### *AIM*

To assess the clinical outcomes of COVID-19 in liver transplant recipients.

### *METHODS*

This was a case-control study, with a database search performed (at the study site) from March 1, 2020 through February 28, 2021. Patients 18 years or older who tested positive for SARS-CoV-2 *via* polymerase chain reaction (PCR) were included in the study. Patients with infection other than pneumonia at the time of admission were excluded. After selection, patients who had been the recipient of liver transplant were considered cases and those without as controls. After being matched by age, sex, and obesity, two controls were randomly selected for each case. Death and hospitalization due to COVID-19 infection were the primary outcomes. Secondary outcomes were pertinent only to patients who were hospitalized, and they included duration of hospital stay, need for supplemental oxygen, presence of at least one type of end-organ damage, effects on liver enzymes, incidence of acute liver failure, effect on d-dimer levels, and incidence of venous thromboembolism (VTE). Chi-square or Fisher's exact test was used to compare all primary and secondary outcomes with the exception of duration of hospital stay and d-dimer levels, which were compared using the Wilcoxon signed-rank test. Alpha criterion was set at 0.05. Logistic regression was performed for each primary outcome (as the dependent variable). Statistical analyses were performed using R software.

***RESULTS***

Of the 470 Liver transplant recipients who were tested for COVID-19 *via* the PCR test, 39 patients tested positive (8.3%). There was no significant difference between cases and controls regarding death [odds ratio (OR): 2.04, 95% confidence interval (CI): 0.14–29.17; $P = 0.60$] and hospitalization rates (OR: 1.38, 95%CI: 0.59–3.24; $P = 0.46$). There also was no significant difference between cases and controls with respect to all secondary outcomes. Among all patients who had elevated liver enzymes, their levels were either normalized, improving, or remained stable at the time of discharge. No patient developed acute liver failure. Of the 31 hospitalized patients, 27 received a prophylactic anticoagulation dose and no patient developed VTE in either group. Among cases who were hospitalized, immunosuppression was decreased in 5 patients and there was no change in immunosuppression among the remaining 7 patients. One patient died in each of these two subgroups. Logistic regression analysis was done, but all of the models had poor model predictions as well as insignificant predictors (independent variables). Therefore, they could not be used for either prediction or inference.

***CONCLUSION***

Clinical outcomes of COVID-19 in liver transplant recipients are not different than those without transplantation. COVID-19 should not impact timely health care access and immunosuppression continuation among these patients.

**Key Words:** COVID-19; SARS-CoV-2; Liver transplant recipients; Clinical outcomes; Death; Hospitalization

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**Core Tip:** This was a case-control study that assessed the clinical outcomes of coronavirus disease 2019 (COVID-19) in liver transplant recipients. Our study did not show a significant difference in death or hospitalization rate due to COVID-19 between patients who had liver transplantation and those who did not. Our study also did not find any difference between these two groups in terms of duration of hospital stay, need for supplemental oxygen, presence of at least one type of end-organ damage, effect on liver enzymes, and d-dimer levels. Therefore, COVID-19 should not impact timely health care access and immunosuppression continuation among liver transplant recipients.

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## INTRODUCTION

According to the John Hopkins University Coronavirus Resource Center, more than 480 million people have contracted coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and more than 6.1 million people have died worldwide from it as of the writing of this manuscript[1]. In the United States alone, more than 79 million people have contracted COVID-19 and more than 900000 people have died from it[1]. This reflects the gravity of the situation.

COVID-19 primarily affects lungs, but evidence suggests the potential involvement and complications of other organ systems as well including cardiovascular, hematological, and neurological systems [2-5]. Liver injury, as demonstrated by elevated liver enzymes, has also been reported in many observational studies[6,7].

Patients with solid organ transplant are at higher risk of infection due to immunosuppression[8]. Whether SARS-CoV-2 causes more frequent infections, more hospitalizations, and is associated with worse outcomes among solid organ transplant patients have been the subject of many studies. In their systematic review of 215 studies, Raja *et al*[9] reported that the incidence of hospitalization was higher among patients with solid organ transplantation compared to patients with no transplant. Pooled incidence of all-cause mortality was 18.6% for all solid organ transplant recipients combined and 11.8% for liver transplant recipients in their study[9]; however, mortality comparison with non-transplant patients was not provided. Kulkarni *et al*[10] focused only on liver transplant recipients with COVID-19 in their systematic review of 18 studies and reported that the cumulative incidence of all-cause mortality was 17.4% among patients with liver transplant who had COVID-19. The authors also provided a

comparison and reported that there was no difference in mortality between non-transplant and liver transplant patients after being diagnosed with COVID-19, despite a change in immunosuppression in 55.9% of the liver transplant patients[10]. This fact, that all-cause mortality of COVID-19 is not different among liver transplant recipients compared to non-transplant patients, has been reported by Jayant *et al* [11] as well in their systematic review of 12 studies. Colmenero *et al*[12] also reported a higher risk of COVID-19 among liver transplant recipients, but surprisingly, mortality was lower for liver transplant recipients in their prospective study. Complete immunosuppression withdrawal also showed no benefit in their study[12].

The data on other outcomes of COVID-19 among liver transplant recipients besides hospitalization and mortality, such as duration of hospital stay and acute liver failure, are limited and heterogeneous. Since liver transplant recipients require specialized care and timely access to health care, we explored additional outcomes of COVID-19 among these patients besides mortality and hospitalization rates.

## MATERIALS AND METHODS

### Study design

This was a case-control study. After approval from the institutional review board, a list of patients was obtained with the help of the Clinical Informatics Department from the database of the study site from March 1, 2020 through February 28, 2021, using the selection criteria given below.

### Selection criteria

**Inclusion criteria:** Adult patients (age 18 years or above) who tested positive for SARS-CoV-2 *via* polymerase chain reaction (PCR).

**Exclusion criteria:** With the exception of bacterial pneumonia (which can be a direct complication of COVID-19 itself), patients with definitive evidence of any other infection, such as positive blood culture or positive urine analysis along with positive urine culture, were excluded (as the presence of another infection besides COVID-19 can also independently increase the risk of adverse outcomes and can be a confounding factor). Among patients who met the above inclusion and exclusion criteria, patients who had been the recipient of liver transplant were considered cases and those without liver transplant were considered controls.

### Outcomes

Death and hospitalization due to COVID-19 were the primary outcomes. Secondary outcomes were pertinent only to patients who were hospitalized and included duration of hospital stay (in days), need for supplemental oxygen, presence of at least one type of end-organ damage (*e.g.*, acute kidney injury or elevated troponins), effect on liver enzymes and incidence of acute liver failure due to COVID-19, effect on d-dimer levels and incidence of venous thromboembolism (VTE).

### Data analyses

Cases and controls were first matched by age, sex, and obesity. After controls were identified and matched with cases based on the aforementioned three variables, two controls were selected for each case (frequency matching) using random sampling. Cases and controls were compared with each other for primary and secondary outcomes.

Chi-square or Fisher's exact test was used to compare all primary and secondary outcomes with the exception of duration of hospital stay and d-dimer levels, which were compared between cases and controls using the Wilcoxon signed-rank test. Alpha criterion was set at 0.05. Logistic regression was performed for each primary outcome (as the dependent variable) as well. All statistical analyses were performed using R software.

## RESULTS

Of the 470 Liver transplant recipients who were tested for COVID-19 *via* PCR, 39 patients tested positive (8.3%), of whom 31 were symptomatic and 8 were asymptomatic. The general characteristics of cases and controls are given in Table 1. The characteristics of cases, pertinent to their liver transplantation, are given in Table 2.

No significant difference was found in death or rate of hospitalization due to COVID-19 between those who had liver transplantation (cases) and those who did not (controls), as detailed in Tables 3 and 4. A total of 31 patients were hospitalized (cases = 12, controls = 19). The mean duration of hospital stay for cases and controls was 8.25 ± 6.92 d and 9.84 ± 17.33 d, respectively. There was no significant difference in duration of hospital stay between the two groups ($P$ = 0.412).

**Table 1 General characteristics of cases and controls**

| Characteristics | Cases, *n* = 39 | Controls, *n* = 78 |
|---|---|---|
| Age, mean ± SD | 55.23 ± 13.51 | 55.32 ± 13.65 |
| Sex, males (%) | 26 (66.7) | 52 (66.7) |
| Race, *n* (%) | | |
| Caucasian | 31 (79.5) | 45 (57.7) |
| African American | 3 (7.7) | 11 (14.1) |
| Hispanic | 3 (7.7) | 14 (17.9) |
| Asian | 0 (0.0) | 2 (2.6) |
| Other/unknown | 2 (5.1) | 6 (7.7) |
| Presence of | | |
| Diabetes, *n* (%) | 17 (43.6) | 15 (19.2) |
| Hypertension, *n* (%) | 23 (59) | 30 (38.5) |
| Obesity, *n* (%) | 14 (35.9) | 28 (35.9) |
| Congestive heart failure, *n* (%) | 3 (7.7) | 6 (7.7) |
| Chronic kidney disease or hemodialysis, *n* (%) | 14 (35.9) | 8 (10.3) |

**Table 2 Characteristics of cases pertinent to liver transplantation**

| Characteristics | *n* | % |
|---|---|---|
| Additional renal transplantation | | |
| Yes | 6 | 15.4 |
| No | 33 | 84.6 |
| Duration of liver transplantation | | |
| < 1 yr | 3 | 7.7 |
| 1 to < 5 yr | 13 | 33.3 |
| 5 to 10 yr | 12 | 30.8 |
| > 10 yr | 11 | 28.2 |
| Etiology of end-stage liver disease prior to liver transplantation | | |
| Alcohol use | 7 | 17.9 |
| Chronic hepatitis C infection | 8 | 20.5 |
| Nonalcoholic fatty liver disease | 5 | 12.8 |
| Primary sclerosing cholangitis | 4 | 10.3 |
| Other | 15 | 38.5 |
| Current status of liver transplant | | |
| Working | 33 | 84.6 |
| Failed/cirrhotic | 6 | 15.4 |

In total, 8 of 12 cases (66.7%) and 14 of 19 controls (73.7 %) either had new supplemental oxygen requirement or an increase from baseline supplemental oxygen needs (if they were already on supplemental oxygen at baseline). There was no significant difference between cases and controls in terms of increase in oxygen requirements [odds ratio (OR): 0.722, 95% confidence interval (CI): 0.11-4.78; $P$ = 0.70]. Of the remaining 27 patients (4 patients died of COVID-19), only 5 had higher than baseline oxygen requirement at the time of discharge (cases = 2, controls = 3). Except for 3 patients (cases = 2 cases, controls = 1), all patients who had an increase in oxygen requirement from its baseline received both dexamethasone 6 mg daily and intravenous remdesivir. In all, 6 of 12 cases (50%) and 7 of 19 controls (36.8%) had at least one type of end-organ damage. There was no significant difference between

**Table 3 Death due to coronavirus disease 2019**

| Liver transplant recipient | Death due to COVID-19 | | Total | Odds ratio with 95% confidence interval | Two-sided *P* value |
|---|---|---|---|---|---|
| | Yes | No | | | |
| Yes | 2 | 37 | 39 | 2.04 (0.14–29.17) | 0.6 |
| No | 2 | 76 | 78 | | |
| Total | 4 | 113 | 117 | | |

COVID-19: Coronavirus disease 2019.

**Table 4 Hospitalization due to coronavirus disease 2019**

| Liver transplant recipient | Hospitalization due to COVID–19 | | Total | Odds ratio with 95% confidence interval | Two-sided *P* value |
|---|---|---|---|---|---|
| | Yes | No | | | |
| Yes | 12 | 27 | 39 | 1.38 (0.59–3.24) | 0.46 |
| No | 19 | 59 | 78 | | |
| Total | 31 | 86 | 117 | | |

COVID-19: Coronavirus disease 2019.

the two groups in terms of end-organ damage (OR: 1.71, 95%CI: 0.4-7.43; $P = 0.47$).

No liver enzyme data were available for 1 case and 4 controls. Analyses of the remaining 26 hospitalized patients (cases = 11, controls = 15) revealed that aspartate aminotransferase (AST) was elevated among 3 cases (27.27%) and 10 controls (66.66%), and this difference was not statistically significant (OR: 0.19, 95%CI: 0.03-1.03; $P = 0.05$). Alanine aminotransferase (ALT) enzyme elevation rate was also surprisingly low among cases, but there was no significant difference between the two groups (OR: 0.27, 95%CI: 0.021–2.0; $P = 0.22$). Only 1 patient had AST and ALT greater than 3 × the upper limit of normal (ULN). Among all patients who had elevated AST or ALT, their levels either normalized, were improving, or remained stable at the time of discharge. No patient developed acute liver failure.

No data were available on d-dimer level for 1 case and 1 control. For the remaining cases ($n = 11$) and controls ($n = 18$), the mean values of the first d-dimer level during hospitalization were 1800.7 ng/mL and 915 ng/mL, respectively. This difference in d-dimer levels was not statistically significant between the two groups ($P = 0.47$). One patient among cases did not receive anti-coagulation due to thrombocytopenia. One patient among controls was already on warfarin for history of atrial fibrillation. All other remaining patients (cases = 10, controls = 17) received a prophylactic dose of either subcutaneous unfractionated heparin ($n = 4$) or enoxaparin ($n = 23$). No patient developed VTE in either group. Among the cases who were hospitalized ($n = 12$), immunosuppression was decreased in 5 patients and did not change among the remaining 7 patients. One patient died in each of these two subgroups. Logistic regression analyses were conducted, but all of the models gave poor predictions as well as insignificant predictors (independent variables). Therefore, they could not be used for either prediction or inference.

## DISCUSSION

Our study did not show a significant difference in death or hospitalization rate due to COVID-19 between patients who had liver transplantation and those who did not. Our study also did not find a difference between these two groups in terms of duration of hospital stay, need for supplemental oxygen, presence of at least one type of end-organ damage, effect on liver enzymes, and effect on d-dimer levels. Although case count was low, reducing immunosuppression in a few patients did not have obvious effects on mortality and need for supplemental oxygen.

Co-morbidities such as obesity, diabetes, cardiovascular and chronic pulmonary diseases have been associated with worse outcomes among patients with COVID-19[13,14]. However, surprisingly, liver transplant status did not increase the risk of mortality for these patients in our study, and this finding is in line with the results of recently published systematic reviews[10,14]. Although higher hospitalization rate has been reported among solid organ transplant patients with COVID-19, our study did not show any such difference[9].

There is no reliable comparison available to date for duration of hospital stay for COVID-19 between patients who are or are not liver transplant recipients. On average, patients in each group spent more than 1 week in the hospital according to our study. Also, data on the need for supplemental oxygen among liver transplant recipients with COVID-19 are limited[15]. There is no comparison available to date for oxygen requirement among COVID-19 patients who have been liver transplant recipients' compared to those who are not. Most patients in both groups required supplemental oxygen in our study, but there was no statistically significant difference between the two groups in terms of oxygen requirements and only 5 of 27 discharged patients (cases = 2, controls = 3) required supplemental oxygen at the time of discharge.

Rabiee *et al*[16] reported moderate acute liver injury (ALT 2-5 × ULN) to be 22.2% ($n$ = 18) and severe acute liver injury (ALT > 5 × ULN) to be 12.3% ($n$ = 10) among liver transplant recipients who were diagnosed with COVID-19. Acute liver injury was lower among liver transplant recipients compared to patients with other chronic liver disease and COVID-19 according to their study[16]. Although we also noted overall lower values of ALT and AST among patients who had liver transplant compared to those who did not, this difference was not statistically significant in our study.

No reliable comparison exists to date for d-dimer levels and incidence of VTE in COVID-19 between patients who are and are not liver transplant recipients. D-dimer levels were higher in hospitalized liver transplant recipients compared to patients without any transplant; however, this difference was not statistically significant in our study. Most patients received prophylactic anti-coagulation against VTE, and no patient was diagnosed with VTE during their hospitalization.

In summary, our study shows that the clinical outcomes of COVID-19 between patients with and without liver transplant are not different. Important limitations of our study include the retrospective nature of the study, relatively small sample size, and the fact that it was a single-center study. Also, given the retrospective nature of the study, the severity of the comorbidities among cases and controls could not be estimated.

## CONCLUSION

Clinical outcomes of COVID-19 do not differ among patients with and without liver transplantation. Also, decreasing immunosuppression in limited liver transplant patients did not improve morbidity or mortality. Precautions, vaccination, and appropriate testing should be exercised but otherwise, the ongoing COVID-19 pandemic should not change how liver transplant patients are cared for, such as timely access to health care and continuation of immunosuppression.

## ARTICLE HIGHLIGHTS

### *Research background*

Liver transplant patients are at higher risk of infection due to immunosuppression. Whether liver transplant recipients are also more susceptible to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and will have worse outcomes than the general population if they develop coronavirus disease 2019 (COVID-19) due to SARS-CoV-2 is a topic of ongoing studies, including ours.

### *Research motivation*

Liver transplant recipients require specialized care and timely access to health care. However, the data on outcomes of COVID-19 among liver transplant recipients besides hospitalization and mortality is limited. This led to our interest to explore additional outcomes of COVID-19 among liver transplant recipients.

### *Research objectives*

The objective of the study was to assess clinical outcomes of COVID-19 in liver transplant recipients. Death and hospitalization due to COVID-19 were the primary outcomes. Secondary outcomes were pertinent only to patients who were hospitalized, and they included duration of hospital stay, need for supplemental oxygen, presence of at least one type of end-organ damage, effects on liver enzymes, incidence of acute liver failure, effect on d-dimer levels, and incidence of venous thromboembolism.

### *Research methods*

This was a case-control study. Patients 18 years or older who tested positive for SARS-CoV-2 *via* polymerase chain reaction were included in the study. Patients with infection other than pneumonia at the time of admission were excluded. Patients who had been the recipient of liver transplant were considered cases and those without as controls. Chi-square or Fisher's exact test was used to compare all primary and secondary outcomes with the exception of duration of hospital stay and d-dimer levels,

which were compared using the Wilcoxon signed-rank test. Alpha criterion was set at 0.05. Statistical analyses were performed using R software.

### *Research results*

There was no significant difference between cases and controls regarding death and hospitalization rates. There also was no significant difference between cases and controls with respect to all secondary outcomes.

### *Research conclusions*

Clinical outcomes of COVID-19 in liver transplant recipients are not different than those without transplantation. COVID-19 should not impact timely health care access and immunosuppression continuation among these patients.

### *Research perspectives*

Besides hospitalization and mortality, data on additional clinical outcomes of COVID-19 among liver transplant recipients is limited. Additional studies are needed to explore the full impact of COVID-19 among patients who have been the recipient of liver transplant.

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## FOOTNOTES

**Author contributions:** Shafiq M was involved in all aspects of this study, including but not limited to study design, data collection, data analyses, and writing of the abstract and manuscript; Gibson C assisted with the study design and data analyses.

**Institutional review board statement:** This study was reviewed and approved by the Institutional Review Board of University of Kansas Medical Center (Kansas City, KS, United States).

**Informed consent statement:** In accordance with the retrospective design of the study, based upon chart reviews, no informed consent was required.

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**Country/Territory of origin:** United States

**ORCID number:** Muhammad Shafiq 0000-0002-0717-1764; Cheryl Gibson 0000-0002-4025-8845.

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ORIGINAL ARTICLE

Retrospective Cohort Study

# Intensive care unit readmission in adult Egyptian patients undergoing living donor liver transplant: A single-centre retrospective cohort study

Manar Salah, Iman Fawzy Montasser, Hanaa A El Gendy, Alaa A Korraa, Gamal M Elewa, Hany Dabbous, Hossam R Mahfouz, Mostafa Abdelrahman, Mohammed Hisham Goda, Mohamed Mohamed Bahaa El-Din, Mahmoud El-Meteini, Heba A Labib

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**Manar Salah, Iman Fawzy Montasser, Hany Dabbous,** Department of Tropical Medicine, Ain Shams Center for Organ Transplantation, Ain Shams University, Cairo 11566, Egypt

**Hanaa A El Gendy, Alaa A Korraa, Gamal M Elewa, Hossam R Mahfouz, Heba A Labib,** Department of Anaesthesia and Critical Care, Ain Shams Centre for Organ Transplantation, Ain Shams University, Cairo 11566, Egypt

**Mostafa Abdelrahman, Mohammed Hisham Goda, Mohamed Mohamed Bahaa El-Din, Mahmoud El-Meteini,** Department of General Surgery and Liver Transplantation, Ain Shams Centre for Organ Transplantation, Ain Shams University, Cairo 11566, Egypt

**Corresponding author:** Iman Fawzy Montasser, MD, Professor, Department of Tropical Medicine, Ain Shams Centerfor Organ Transplantation, Ain Shams University, Abbassia Square, Khalifa El Mamoun Street, Cairo 11566, Egypt. imanfawzy2@gmail.com

## Abstract

### *BACKGROUND*

Patients who undergo living donor liver transplantation (LDLT) may suffer complications that require intensive care unit (ICU) readmission.

### *AIM*

To identify the incidence, causes, and outcomes of ICU readmission after LDLT.

### *METHODS*

A retrospective cohort study was conducted on patients who underwent LDLT. The collected data included patient demographics, preoperative characteristics, intraoperative details; postoperative stay, complications, causes of ICU readmission, and outcomes. Patients were divided into two groups according to ICU readmission after hospital discharge. Risk factors for ICU readmission were identified in univariate and multivariate analyses.

### *RESULTS*

The present study included 299 patients. Thirty-one (10.4%) patients were readmitted to the ICU after discharge. Patients who were readmitted to the ICU

were older in age (53.0 ± 5.1 *vs* 49.4 ± 8.8, $P = 0.001$) and had a significantly higher percentage of women (29% *vs* 13.4%, $P = 0.032$), diabetics (41.9% *vs* 24.6%, $P = 0.039$), hypertensives (22.6% *vs* 6.3%, $P = 0.006$), and renal (6.5% *vs* 0%, $P = 0.010$) patients as well as a significantly longer initial ICU stay (6 *vs* 4 d, respectively, $P < 0.001$). Logistic regression analysis revealed that significant independent risk factors for ICU readmission included recipient age (OR = 1.048, 95%CI = 1.005-1.094, $P = 0.030$) and length of initial hospital stay (OR = 0.836, 95%CI = 0.789-0.885, $P < 0.001$).

*CONCLUSION*

The identification of high-risk patients (older age and shorter initial hospital stay) before ICU discharge may help provide optimal care and tailor follow-up to reduce the rate of ICU readmission.

**Key Words:** Intensive care units; Liver transplantation; Patient readmission; Risk factors

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**Core Tip:** Patients undergoing living donor liver transplantation may suffer complications that require intensive care unit readmission. We retrospectively evaluated 299 patients who underwent living donor liver transplantation. We identified the incidence, causes, and outcomes of intensive care unit readmission after living donor liver transplantation. Older recipient age and longer length of initial hospital stay were recognized as significant independent risk factors for intensive care unit readmission. The identification of high-risk patients before discharge may help provide optimal care and tailor follow-up to reduce the rate of intensive care unit readmission.

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## INTRODUCTION

Liver transplantation is the only definitive treatment for end-stage liver disease[1]. As a major abdominal surgery, postoperative complications may occur and might require readmission, which may be serious and life threatening. In general, patients who require intensive care unit (ICU) readmission show higher morbidity, mortality, and prolonged hospital stays than those who do not require readmission[2-5].

Complications following living donor liver transplantation (LDLT) requiring ICU readmission may be serious and life threatening. Identification of the causes of ICU readmission is pivotal to establish effective strategies to reduce the rate of readmission, improve the quality of care and patient outcomes, and reduce health expenditures by medical institutions[6].

Most available reports are on hospital readmission after deceased donor liver transplantation (DDLT). To our knowledge, there are limited published data about hospital readmission of LDLT patients. We hypothesized that ICU readmission after LDLT is due to different reasons. Consequently, this study was conducted to identify the incidence, causes, and outcomes of ICU readmission after LDLT.

## MATERIALS AND METHODS

### Study design and settings

This retrospective cohort study was conducted by reviewing the hospital files of adult patients who underwent LDLT at Ain Shams University Hospital, Cairo, Egypt, during the period from January 1, 2008, to December 31, 2018.

### Ethical considerations

This study was approved by the Ethics Committee of the Faculty of Medicine, Ain Shams University,

Egypt (approval number: IRB/0006379). The confidentiality of the patients' data was maintained by assigning a code number to each patient.

### Inclusion criteria

We included adult Egyptian patients (18 years old or above) of either sex who underwent LDLT at our institution during the period from January 1, 2008, to December 31, 2018.

### Exclusion criteria

The following patients were excluded from the study: We excluded patients who were less than 18 years old, who died before discharge after LDLT, who underwent retransplantation before discharge from the ICU after the first liver transplant, and who were pregnant patients.

### Sampling method

The sample size was calculated using Epi Info™ software (Centers for Disease Control and Prevention, version 7.2.3.0), setting the type-1 error (α) at 0.05, an acceptable margin of error of 5%, and a 95% confidence interval. The results from a previous study[5] showed that the incidence of hospital readmission among cases undergoing liver transplantation was 17.1%. Calculation according to these values produced a minimal sample size of 218 cases.

### Study procedures

The hospital files of patients who met the eligibility criteria were thoroughly revised to extract relevant data. The collected data included patient demographics, donor characteristics, preoperative and intraoperative variables, postoperative stay, complications, causes for ICU readmission, and outcomes after ICU readmission.

The studied primary outcome was the incidence of ICU readmission. Readmission was defined as ICU readmission within ≤ 3 mo of initial ICU discharge. Patients were divided into two groups: those who were readmitted to the ICU (the readmission group) and those who were not (the control group). The secondary outcomes included the causes of first hospital readmission after discharge as well as the incidence of ICU readmission for more than one time and two-year survival.

Risk factors for ICU readmission were assessed by examining the contribution of collected patient and donor variables to the probability of ICU readmission.

### Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (IBM SPSS Statistics) for Windows, version 26 (IBM Corp., Armonk, NY, United States). For quantitative data, the Shapiro-Wilk test for normality was performed. For data that followed a normal distribution, values were expressed as the mean ± SD. Comparisons between two groups were carried out using an independent samples T test. For data that did not follow a normal distribution, the median and interquartile range (IQR; expressed as the 25$^{th}$-75$^{th}$ percentiles) were calculated, and the Mann–Whitney test was used to compare the two groups. For qualitative data, the variables were summarized as frequencies. Pearson's chi square tests for independence, Fisher's exact test or Fisher-Freeman-Halton exact test were used to examine the association between two categorical variables as appropriate. Binomial logistic regression was conducted to identify independent risk factors for ICU readmission, including all variables with a $P$ value < 0.1 in univariant analysis. Kaplan–Meier curves and log rank tests were performed to estimate two-year survival. A $P$ value < 0.05 was adopted to interpret the significance of statistical tests.

The statistical review of this study was performed by a biomedical statistician.

## RESULTS

The present study included 299 patients who underwent liver transplantation and were followed up for a median duration of 40 mo (ranging from less than one month to 136 mo) after surgery. Thirty-one (10.4%) patients were readmitted into the ICU within ≤ 3 mo of initial ICU discharge, among whom 7 (2.3% of total cases) had more than one ICU readmission. Hospital readmission was recorded in 10 (3.3%), among whom 5 (1.6% of total cases) were readmitted due to biliary complications and sepsis. Table 1 depicts the causes for ICU readmission.

The preoperative characteristics of the studied patients and donors are shown in Table 2. The mean age of the patients was 49.8 ± 8.6 years. Men outnumbered women (84.9% *vs* 15.1%, respectively). The mean BMI was 28.4 ± 4.3 kgm$^2$. The median MELD score was 16 (ranging from 6 to 29). Approximately one-third of the patients had one or more comorbidities; the most frequent were diabetes mellitus (26.4%), hypertension (8%), and IHD (1.3%). The most frequent liver disease was HCV (76.6%), followed by HCC (39.1%), PVT (12.4%), and cryptogenic (10.7%). Encephalopathy was diagnosed in 31.8% of patients. Regarding the donors, the mean age was 29.8 ± 6.3 years (ranging from 18 to 48), with a higher percentage of men than women (68.6% *vs* 31.4%, respectively); their mean BMI was 23.7 ± 2.6 kgm$^2$. The

**Table 1 Causes of intensive care unit readmission (total N = 31 out of 299 patients)**

| | N | % |
|---|---|---|
| Sepsis | 5 | 1.6 |
| Pulmonary complications | 3 | 0.9 |
| Cardiovascular complications | 3 | 0.9 |
| HA thrombosis | 3 | 0.9 |
| 7$^{Th}$ day syndrome | 1 | 0.3 |
| Acute cellular rejection | 1 | 0.3 |
| Acute Pancreatitis | 1 | 0.3 |
| Cerebrovascular stroke | 3 | 0.9 |
| Graft failure | 1 | 0.3 |
| hemorrhagic shock | 1 | 0.3 |
| Liver infarction | 1 | 0.3 |
| Metabolic disorders | 1 | 0.3 |
| Portal vein thrombosis | 1 | 0.3 |
| Prograf neurotoxicity | 2 | 0.6 |
| PV STENOSIS | 1 | 0.3 |
| Re -transplant[1] | 2 | 0.6 |
| Renal impairment | 1 | 0.3 |

[1]Re-transplantation for graft failure due to hepatic artery thrombosis in 1 case and small for size in the other case. HA: Hepatic artery; HV: Hepatic vein; LDLT: Living donor liver transplantation; N: Number; PV: Portal vein.

comparison between patients who were readmitted to the ICU and patients who were not readmitted showed that the former group was older in age (53.0 ± 5.1 *vs* 49.4 ± 8.8, $P = 0.001$) and had a significantly higher percentage of women (29% *vs* 13.4%, $P = 0.032$), diabetic patients (41.9% *vs* 24.6%, $P = 0.039$), hypertensive patients (22.6% *vs* 6.3%, $P = 0.006$), and renal patients (6.5% *vs* 0%, $P = 0.010$).

Table 3 summarizes the intraoperative and postoperative details of the studied patients. Synthetic grafts were used in 2.7% of patients. Vascular surgical complications were encountered in 15.7% of patients, mainly in the form of hepatic artery thrombosis (8%). No significant difference was detected between the two groups ($P > 0.05$).

Table 4 shows the follow-up details of the patients. The median LOS of initial ICU admission was significantly longer in the readmission group (6 *vs* 4 d, respectively, $P < 0.001$). Rejection occurred in 12% of all patients. The mortality rate was 29.8%, with a significantly higher percentage in the readmission group (64.5% *vs* 25.7%, $P < 0.001$).

Logistic regression analysis was conducted to identify risk factors for ICU readmission after discharge (Table 5). Significant independent risk factors included recipient age and length of initial hospital stay after discharge from the ICU. The increase in recipient age by one year was associated with an increased likelihood of ICU readmission by 4.8% (OR = 1.048, 95%CI = 1.005-1.094, $P = 0.030$). A negative relationship existed between the length of initial hospital stay and the probability of ICU readmission, as an increased length of stay resulted in a decreased risk of readmission (OR = 0.836, 95%CI = 0.789-0.885, $P < 0.001$).

Figures 1 and 2 illustrate the survival curve for all studied patients and according to ICU readmission, respectively. The OS rates for all patients at 1 and 2 years were 79.5% ± 2.3% and 75.2% ± 2.5%, respectively. The overall survival (OS) rates for non-ICU readmitted patients were 83.9% ± 2.3% and 79.2% ± 2.5% at 1 and 2 years, respectively. For ICU readmitted patients, the OS rate was 40.6% ± 9.1% at 1 year and persisted until 2 years. The log rank test showed a significant difference between the survival curves of the two groups ($P < 0.001$).

## DISCUSSION

Readmission after discharge from the hospital is considered among the important indicators of the quality of delivered health care services. Moreover, readmissions impose an additional considerable

**Table 2 Preoperative patients' and donors' characteristics (data were expressed as mean ± SD or number & percentage) (total N = 299)**

| | | Total (N = 299), % | | No ICU readmission (N = 268), % | | ICU readmission (N = 31), % | | Test statistic | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| Age (years); mean ± SD (Range) | | 49.8 ± 8.6; (19.0 - 67.0) | | 49.4 ± 8.8; (19.0 - 67.0) | | 53.0 ± 5.1; (42.0 - 64.0) | | 3.381[a] | 0.001[1] |
| Gender | Female | 45 | 15.1 | 36 | 13.4 | 9 | 29.0 | FE | 0.032[1] |
| | Male | 254 | 84.9 | 232 | 86.6 | 22 | 71.0 | | |
| BMI (Kg/m$^2$); mean ± SD (Range) | | 28.4 ± 4.3; (18.5 - 52.9) | | 28.2 ± 4.0; (18.5 - 42.0) | | 29.4 ± 6.5; (20.6 - 52.9) | | 0.936[a] | 0.356 |
| MELD score; Median [IQR] (Range) | | 16.0; [13.0 – 18.0] (6.0 - 29.0) | | 16.0; [13.0 -18.0] (6.0 - 29.0) | | 15.0; [12.0 – 20.0] (7.0 - 28.0) | | 0.538[b] | 0.590 |
| Positive medical history | | 103 | 34.4 | 86 | 32.1 | 17 | 54.8 | FE | 1.000 |
| | DM | 79 | 26.4 | 66 | 24.6 | 13 | 41.9 | 4.282[c] | 0.039[1] |
| | Hypertension | 24 | 8.0 | 17 | 6.3 | 7 | 22.6 | FE | 0.006[1] |
| | Bronchial asthma | 3 | 1.0 | 2 | 0.7 | 1 | 3.2 | FE | 0.281 |
| | IHD | 4 | 1.3 | 4 | 1.5 | 0 | 0.0 | FE | 1.000 |
| | Renal | 2 | 0.7 | 0 | 0.0 | 2 | 6.5 | FE | 0.010[1] |
| | Bilharziasis | 2 | 0.7 | 2 | 0.7 | 0 | 0.0 | FE | 1.000 |
| | Others | 9 | 3.0 | 8 | 3.0 | 1 | 3.2 | FE | 1.000 |
| Diagnosis | AIH | 10 | 3.3 | 10 | 3.7 | 0 | 0.0 | FE | 0.606 |
| | HCC | 117 | 39.1 | 107 | 39.9 | 10 | 32.3 | 0.686[c] | 0.408 |
| | PVT | 37 | 12.4 | 34 | 12.7 | 3 | 9.7 | FE | 0.780 |
| | Cryptogenic | 32 | 10.7 | 29 | 10.8 | 3 | 9.7 | FE | 1.000 |
| | ESLD | 10 | 3.3 | 8 | 3.0 | 2 | 6.5 | FE | 0.278 |
| | HCV | 229 | 76.6 | 204 | 76.1 | 25 | 80.6 | 0.317[c] | 0.573 |
| | HBV | 11 | 3.7 | 11 | 4.1 | 0 | 0.0 | FE | 0.612 |
| | BCS | 2 | 0.7 | 2 | 0.7 | 0 | 0.0 | FE | 1.000 |
| | PSC | 2 | 0.7 | 2 | 0.7 | 0 | 0.0 | FE | 1.000 |
| Encephalopathy | | 95 | 31.8 | 87 | 32.5 | 8 | 25.8 | 0.568[c] | 0.451 |
| Creatinine clearance (mL/min); mean ± SD (Range) | | 88.7 ± 26.5; (10.0 - 172.0) | | 88.5 ± 25.8; (10.0 - 170.0) | | 90.4 ± 32.7; (16.0 - 172.0) | | 0.384[a] | 0.702 |
| Serum creatinine (mg/dL); mean ± SD (Range) | | 0.97 ± 0.31; (0.30 - 2.40) | | 0.97 ± 0.32; (0.30 - 2.40) | | 0.96 ± 0.24; (0.50 - 1.30) | | 0.255[a] | 0.799 |
| Serum Albumin gm/dL; mean ± SD (Range) | | 3.0 ± 0.5; (1.8 - 4.8) | | 3.0 ± 0.5; (1.8 - 4.8) | | 2.8 ± 0.5; (1.8 - 3.8) | | 1.428[a] | 0.154 |
| Na mmol/L; mean ± SD (Range) | | 135.1 ± 5.2; (117.0 - 147.0) | | 135.1 ± 5.4; (117.0 - 147.0) | | 135.0 ± 3.6; (128.0 - 145.0) | | 0.137[a] | 0.891 |
| Total bilirubin mg/dL; Median [IQR] (Range) | | 1.3; [0.8 - 1.9] (0.2 - 27.0) | | 1.3; [0.7 - 1.9] (0.2 - 27.0) | | 1.4; [1.1 - 1.9] (0.6 - 5.6) | | 1.270[b] | 0.204 |
| Alkaline phosphatase IU/L; Median [IQR] (Range) | | 143.0; [97.0 - 221.0] (6.2 - 2369.0) | | 144.5; [97.0 - 231.5] (6.2 - 2369.0) | | 131.0; [98.0 - 167.0] (45.0 - 1410.0) | | 1.041[b] | 0.298 |
| Donor age (years); mean ± SD (Range) | | 29.8 ± 6.3; (16.0 - 48.0) | | 30.0 ± 6.3; (16.0 - 48.0) | | 28.0 ± 5.9; (18.0 - 39.0) | | 1.731[a] | 0.084 |
| Donor gender | Female | 94 | 31.4 | 83 | 31.0% | 11 | 35.5 | 0.263[c] | 0.608 |
| | Male | 205 | 68.6 | 185 | 69.0% | 20 | 64.5 | | |
| Donor BMI (Kg/m$^2$); mean ± SD (Range) | | 23.7 ± 2.6; (17.7 - 32.0) | | 23.8 ± 2.6; (17.7 - 32.0) | | 23.0 ± 2.3; (18.3 - 29.0) | | 1.545[a] | 0.124 |

[a]Independent samples T-test.
[b]Mann-Whitney test.
[c]Pearson's Chi square test for independence.

[1]Significant at $P < 0.05$. AIH: Autoimmune hepatitis; BMI: Body mass index; DM: Diabetes mellitus; FE: Fisher's exact test; IQR: Interquartile range; N: Number; SD: Standard deviation; ICU: Intensive care unit; IHD: Ischemic heart disease; HCC: Hepatocellular carcinoma; PVT: Portal vein thrombosis; ESLD: End-stage liver disease; HCV: Hepatitis C virus; HBV: Hepatitis B virus; BCS: Budd-Chiari syndrome; PSC: Primary sclerosing cholangitis.

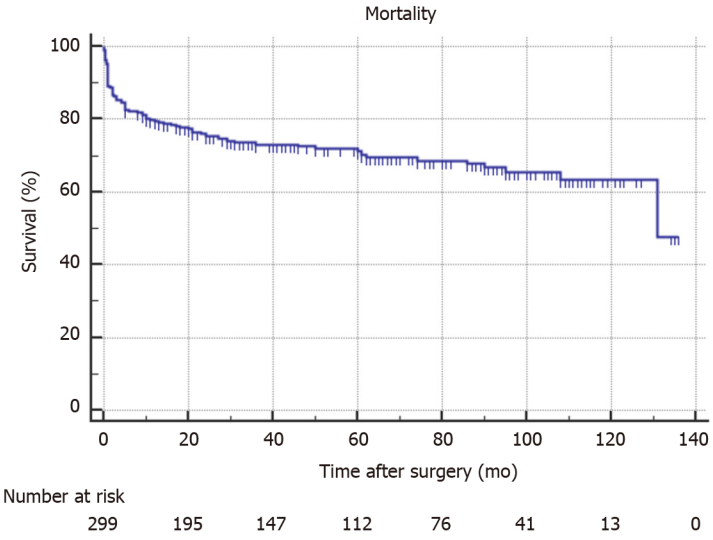


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**Figure 1 Kaplan-Meier curve showing survival after surgery in all patients.**

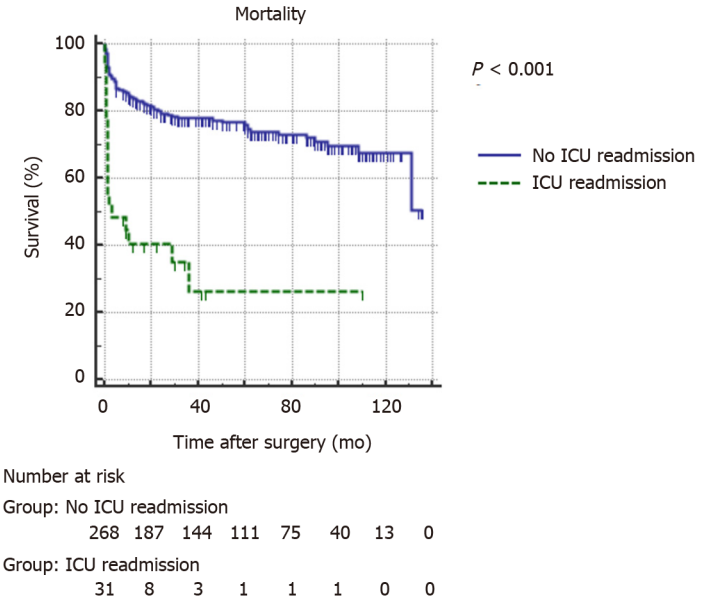


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**Figure 2 Kaplan-Meier curve showing survival after surgery in the studied groups.** Log rank test: $X^2 = 44.426$, $P < 0.001$. ICU: Intensive care unit.

burden on health care expenditure and on hospital resources[7]. Recipients of liver transplantation are susceptible to the administration of multiple drug regimens, and they endure metabolic changes in addition to the complications that may arise from surgery[5]. All of these factors increase the risk of hospital and ICU readmission in this group of patients[8,9]. The current study aimed to identify the incidence, causes, and outcomes of ICU readmission after LDLT. Our cohort consisted of 299 patients who underwent LDLT and were followed up for a median duration of 40 mo.

In the present study, the incidence of ICU readmission within the first 3 mo after discharge was 10.4% and that of multiple ICU readmissions was 2.3% of the total cases. The incidence of hospital readmission after discharge was 3.3%. In agreement with this low rate of readmission, Chen *et al*[5] reported a 3-mo hospital readmission rate of 9.4% in 791 patients who underwent either LDLT or DDLT. On the other

**Table 3 Intraoperative and postoperative data of the studied patients (total N = 299)**

| | | Total (N = 299), % | | No ICU readmission (N = 268), % | | ICU readmission (N = 31), % | | Test statistic | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| Waiting Time (days); Median [IQR] (Range) | | 86.0; [59.0 - 120.0] (20.0 - 546.0) | | 86.5; [56.5 - 120.0] (20.0 - 462.0) | | 74.0; [65.0 - 119.0] (29.0 - 546.0) | | 0.034[a] | 0.973 |
| Warm ischemia time (min); mean ± SD (Range) | | 48.6 ± 19.4; (20.0 - 145.0) | | 48.7 ± 19.0; (20.0 - 145.0) | | 47.9 ± 22.7; (20.0 - 145.0) | | 0.222[b] | 0.274 |
| Graft weight/GRWR; mean ± SD (Range) | | 1.06 ± 0.47; (0.01 - 6.30) | | 1.08 ± 0.49; (0.01 - 6.30) | | 0.98 ± 0.23; (0.01 - 1.30) | | 1.095[b] | 0.825 |
| Cold ischemia time (min); Median [IQR] (Range) | | 45.0; [30.0 - 60.0] (10.0 - 180.0) | | 45.0; [31.0 - 60.0] (10.0 - 180.0) | | 45.0; [30.0 - 50.0] (20.0 - 125.0) | | 0.864[a] | 0.387 |
| Packed red blood cells (units); Median [IQR] (Range) | | 4.0; [2.0 - 7.0] (1.0 - 28.0) | | 4.0; [2.0 - 7.0] (1.0 - 28.0) | | 3.0; [2.0 - 6.0] (1.0 - 17.0) | | 0.957[a] | 0.339 |
| PV Anastomosis | RPV/MPV | 255 | 85.3 | 230 | 85.8 | 25 | 80.6 | FE | 0.426 |
| | RPV/CPV | 34 | 11.4 | 29 | 10.8 | 5 | 16.1 | FE | 0.372 |
| | RPV/RPV | 4 | 1.3 | 4 | 1.5 | 0 | 0.0 | FE | 1.000 |
| | LPV/LPV | 1 | 0.3 | 1 | 0.4 | 0 | 0.0 | | |
| | LPV/MPV | 2 | 0.7 | 2 | 0.7 | 0 | 0.0 | | |
| | RPH/CHV | 2 | 0.7 | 2 | 0.7 | 0 | 0.0 | | |
| | RPV/CBV | 1 | 0.3 | 0 | 0.0 | 1 | 3.2 | | |
| | RPV/CHV | 1 | 0.3 | 1 | 0.4 | 0 | 0.0 | | |
| HA Anastomosis | RHA/RHA | 280 | 93.6 | 252 | 94.0 | 28 | 90.3 | FE | 0.708 |
| | RHA/LHA | 18 | 6.0 | 15 | 5.6 | 3 | 9.7 | FE | 0.606 |
| | RHA/SPA | 1 | 0.3 | 1 | 0.4 | 0 | 0.0 | | |
| HVs Anastomosis | RHV/RHV | 278 | 93.0 | 248 | 92.5 | 30 | 96.8 | FE | 0.708 |
| | RHV/IVC | 10 | 3.3 | 10 | 3.7 | 0 | 0.0 | FE | 0.606 |
| | LMHV/LMHV | 9 | 3.0 | 8 | 3.0 | 1 | 3.2 | FE | 1.000 |
| | RHV/MHV | 2 | 0.7 | 2 | 0.7 | 0 | 0.0 | | |
| Synthetic graft | | 8 | 2.7 | 7 | 2.6 | 1 | 3.2 | FE | 0.588 |
| Surgical vascular complications | | 47 | 15.7 | 39 | 14.6 | 8 | 25.8 | FE | 0.118 |
| | HA stenosis | 6 | 2.0 | 5 | 1.9 | 1 | 3.2 | | |
| | HA thrombosis | 24 | 8.0 | 19 | 7.1 | 5 | 16.1 | | |
| | HV stenosis | 3 | 1.0 | 3 | 1.1 | 0 | 0.0 | | |
| | HVT | 2 | 0.6 | 2 | 0.8 | 0 | 0.0 | | |
| | PV stenosis | 4 | 1.3 | 3 | 1.1 | 1 | 3.2 | | |
| | PV thrombosis | 7 | 2.3 | 6 | 2.2 | 1 | 3.2 | | |
| | Sub-diaphragmatic hematoma | 1 | 0.3 | 1 | 0.4 | 0 | 0.0 | | |

[a]Mann-Whitney test.

[b]Independent samples T-test. Significant at $P < 0.05$. FE: Fisher's exact test; IQR: interquartile range; N: Number; SD: Standard deviation; GRWR: Graft to recipient weight ratio; HA: Hepatic artery; HV: Hepatic vein; PV: Portal vein; RPV: Right portal vein; MPV: Main portal vein; CPV: Common portal vein; LPV: Left portal vein; CHV: Central hepatic vein; RHA: Right hepatic artery; LHA: Left hepatic artery; SPA: Splenic artery; RHV: Right hepatic vein; IVC: Inferior vena cava; LMHV: Left and middle hepatic veins; MHV: Middle hepatic vein; HVT: Hepatic vein thrombosis; ICU: Intensive care unit.

hand, much higher incidence rates of hospital readmission were reported by earlier studies[6,8-10].

Shankar *et al*[9] assessed risk factors for rehospitalization in 208 patients who underwent liver transplantation (among whom 8 patients only underwent LDLT) over a duration of 4 years. They reported a hospital readmission rate of 30.3% within 3 mo. Pereira *et al*[8] conducted an assessment of

**Table 4 Outcome of the studied patients (total N = 299)**

| | Total (N = 299) | | No ICU readmission (N = 268) | | ICU readmission (N = 31) | | Test statistic | *P* value |
|---|---|---|---|---|---|---|---|---|
| Initial Hospital length of stay (days); Median [IQR] (Range) | 21.0; [18.0 - 26.0](1.0 - 150.0) | | 22.0; [19.0 - 26.0] (5.0 - 120.0) | | 3.0; [2.0 - 9.0] (1.0 - 150.0) | | 8.003[a] | < 0.001[1] |
| Length of stay in initial ICU (days); Median [IQR] (Range) | 4.0; [3.0 - 5.0] (2.0 - 45.0) | | 4.0; [3.0 - 5.0] (2.0 - 45.0) | | 6.0; [5.0 - 7.0] (4.0 - 9.0) | | 6.676[a] | < 0.001[1] |
| Rejection (N, %) | 36 | 12.0 | 34 | 12.7 | 2 | 6.5 | FE | 0.557 |
| Mortality(N, %) | 89 | 29.8 | 69 | 25.7 | 20 | 64.5 | 19.978[b] | < 0.001[1] |

[a]Mann-Whitney test.

[b]Pearson's Chi square test for independence.

[1]Significant at $P < 0.05$. IQR: Interquartile range; N: Number; ICU: Intensive care unit.

**Table 5 Logistic regression analysis for risk factors of intensive care unit readmission**

| | Wald | *P* value | OR | 95%CI for OR | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| Patients' age (years) | 4.707 | 0.030[1] | 1.048 | 1.005 | 1.094 |
| Gender (male compared to female) | 2.722 | 0.099 | 0.399 | 0.134 | 1.188 |
| DM | 1.257 | 0.262 | 1.828 | 0.637 | 5.244 |
| Hypertension | 0.462 | 0.497 | 1.641 | 0.394 | 6.842 |
| Donors' age (years) | 1.700 | 0.192 | 0.954 | 0.888 | 1.024 |
| Length of initial ICU stay (days) | 1.255 | 0.263 | 1.055 | 0.960 | 1.159 |
| Length of initial hospital stay (days) | 37.306 | < 0.001[1] | 0.836 | 0.789 | 0.885 |

[1]Significant at $P < 0.05$. CI: Confidence interval; DM: Diabetes mellitus; OR: Odds ratio; ICU: Intensive care unit.

766 patients undergoing DDLT over an 8-year period. They found a 30-d readmission rate of 45%. Patel *et al*[10] evaluated 325 patients with DDLT over a 10-year period, with an overall 90-d readmission rate of 46%. Yataco *et al*[6] studied hospital readmission in 445 patients who underwent either DDLT or LDLT, with a 90-d hospital readmission rate of 42%. All of these studies included patients who underwent either DDLT only or a mixed sample of DDLT and LDLT. Nagaraja *et al*[11] assessed 140 LDLT patients and found the rate of readmission within 3 mo after discharge to be lower than reported in DDLT or a mixed sample (27.1%).

The wide variation in the reported readmission rates among studies may be explained by the difference in preoperative patient characteristics, as only 16.7% of studies included patients undergoing LDLT and had MELD scores above 19. In addition, institutional policies for patient selection before transplant and the criteria for readmission differ among the centres, potentially impacting the reported rates of readmission.

The most common causes of ICU readmission among our patients included sepsis (5/31 patients), followed by pulmonary and cardiac causes (3/31 each). Previous studies reported sepsis as the most common cause for hospital readmission, followed by biliary complications[6,11]. Meanwhile, sepsis due to biliary complications were reported among the causes for hospital readmission (1.6% of total cases) in our cohort.

We proceeded in the current study to identify potential risk factors that increase the likelihood of ICU readmission within 3 mo after discharge. Several variables were assessed in the literature as potential predictors of rehospitalization after liver transplantation.

The recipient's age was found on univariate and multivariate analyses in the current study to be significantly associated with an increased probability of ICU readmission. This association is supported by the results of Levy *et al*[12] and Patel *et al*[10]. However, several studies showed a lack of significant association with rehospitalization[5,6,8,9,11].

Regarding the recipient's sex, univariate analysis showed that women were significantly more likely to be readmitted to the ICU, but this association was not significant in multivariate analysis. Patel *et al*[10] reported a lower risk for men and an increased risk for women. Other previous studies reported the

lack of a significant effect of recipient sex on rehospitalization[5,6,8,9,11,12].

The presence of comorbidities was assessed in the present study. A higher percentage of ICU readmission was associated with diabetes, hypertension, and renal disease in univariate analysis, while multivariate analysis showed the lack of a significant effect on ICU readmission. The increased risk of rehospitalization with the presence of chronic illnesses was stated in the literature in patients undergoing surgery[13,14]. Our results are in line with previous studies assessing rehospitalization after liver transplantation, which did not show this significant association[5,8,11]. On the other hand, other preoperative morbid conditions, such as preoperative HCV infection[9] and PVT[8], were reported to increase the risk of hospital readmission, although such an association was not detected in our cohort.

While the current study results revealed a significantly longer initial ICU stay in ICU readmitted patients using univariate analysis, the association was not found to be significant on multivariate analysis. Similarly, Nagaraja *et al*[11] and Yataco *et al*[6] reported the lack of a significant difference between readmitted and non readmitted groups. In contrast, Levy *et al*[12] found that a higher percentage of non readmitted patients had an ICU stay less than 3 d than readmitted patients (67.8% *vs* 56.3%, $P = 0.0231$). Shankar *et al*[9] reported that a longer LOS in the ICU had a lower risk ratio.

Numerous other factors were identified by some researchers as predictors of rehospitalization but were nonsignificant in the current study, including the MELD score and postoperative complications.

We found that the initial hospital stay correlated negatively with the probability of ICU readmission (OR = 0.836, 95%CI = 0.789-0.885, $P < 0.001$), indicating an increased risk with shorter stays. The literature shows controversial reports concerning the relationship between the length of initial hospital stay and rehospitalization. A negative correlation was also observed by Kassin *et al*[13], Ladner *et al*[15], Pereira *et al*[8], and Chen *et al*[5]. Contradictory results were stated by Yataco *et al*[6], who found that an initial hospital stay longer than 7 d was significantly associated with hospital readmission. Prolonged hospital stay can potentially exert two contradictory effects on the probability of hospital readmission, which may depend largely on the range of stay. On the one hand, a longer stay can prevent discharge before full assessment, optimization of the patient, and adequate management of postoperative complications. Some postoperative complications, such as rejection, may not manifest within the first days after transplantation, and their detection after discharge leads to early hospital readmission. On the other hand, prolonged stay predisposes the patient to an increased risk of contracting nosocomial infection with a negative impact on the patient's health and outcomes.

A higher MELD score has been associated with higher health care costs and increased utilization of hospital resources[16,17]. Nevertheless, the MELD score was not found to be significantly associated with ICU readmission in this study, a finding shared by several previous studies assessing risk factors for hospital readmission[5,6,8-11]. The calculation of the MELD score is based on a limited set of laboratory measurements that are not able to capture all aspects of the patient's functional status.

We did not find a significant difference in the rate of postoperative complications between readmitted and nonreadmitted groups, a finding shared by Nagaraja *et al*[11]. However, Chen *et al*[5] found that the risk of readmission correlated positively with the number and severity of complications after liver transplantation. An increased risk in patients suffering postoperative complications was also observed by Pereira *et al*[8].

The mortality rate in our series was 29.8%. The OS rates for all patients at 1 and 2 years were 79.5% ± 2.3% and 75.2% ± 2.5%, respectively. Patients with ICU readmission had a significantly higher mortality rate than those without readmission (64.5% *vs* 25.7%, $P < 0.001$). The OS rates for ICU readmitted patients were significantly reduced compared to the non readmission group at one year (40.6% ± 9.1% *vs* 83.9% ± 2.3%) and two years (40.6% ± 9.1% *vs* 79.2% ± 2.5%) post transplantation. This association between readmission and mortality could be explained by the worsened health status of readmitted patients, which requires readmission and at the same time increases the risk of mortality. Moreover, ICU readmission may expose the patient to nosocomial infections, and the use of multiple medications may negatively affect renal function and result in further deterioration of the patient's health status.

In accordance with these findings, Pereira *et al*[8] found decreased OS at one year after transplantation in readmitted patients compared to nonreadmitted patients (88.2% *vs* 95.6%, $P < 0.05$). Nagaraja *et al*[11] reported that readmitted patients had a significantly higher mortality rate than nonreadmitted patients (8% *vs* 0%; $P = 0.01$). Chen *et al*[5] reported reduced OS in readmitted patients at 1 year (81.2% *vs* 94.1%) and 2 years (68.1% *vs* 88.2%). Patel *et al*[10] stated that readmitted patients had a significantly lower 5-year survival (75% *vs* 88%, $P = 0.008$). Nevertheless, Yataco *et al*[6] reported the lack of a significant difference in the 1-year survival rate between readmitted and nonreadmitted recipients.

The present study differs from previous studies by investigating ICU readmission and not all rehospitalizations, which may explain differences in results from those studies. We believe that ICU readmission imposes more negative effects on both patients and the resources of health care systems than rehospitalization into other hospital wards or units. Considering that the resources of the ICU are limited and the cost of care is higher than that encountered with hospital ward admission, the identification of specific causes and risk factors for ICU readmission is crucial. However, the present study bears some points of limitation. The retrospective nature of the study predisposes the collected data to inaccuracies. Moreover, patients may have been readmitted to other health care facilities, and such data may not be recorded in our institution's files. Being a single-centre experience hinders the generalization

of our results.

## CONCLUSION

Older recipient age and shorter initial hospital stay were significantly associated with ICU readmission. The overall survival rate for ICU readmitted patients was significantly lower than that for non-ICU readmitted patients. The identification of high-risk patients with these factors before discharge may help provide optimal care and tailor follow-up to reduce the rate of ICU readmission.

## ARTICLE HIGHLIGHTS

### *Research background*

Intensive care unit (ICU) admission and readmission following liver transplantation is important field in liver transplantation operation. Readmission causes and effect on prognosis in terms of morbidity and mortality are still needed to be further investigated

### *Research motivation*

To identify causes and outcome in recipients post living donor liver transplantation (LDLT) who required ICU readmission after initial discharge from ICU and to compare them with patients who did not require readmission

### *Research objectives*

A retrospective cohort study carried on recipients who had LDLT in single Egyptian center in the period betwenn 2008 and 2018. Patients were divided into two groups according to ICU readmission after initial hospital discharge. Risk factors for ICU readmission were identified in univariate and multivariate analyses.

### *Research methods*

Retrospective cohort study was conducted by reviewing the hospital files and records of adult patients who underwent LDLT at Ain Shams University Hospital, Cairo, Egypt, during the period from January 1, 2008, to December 31, 2018. Causes and outcome of ICU readmission were compared between both groups (Readmission group and non readmission group). Risk factors for ICU readmission were also assessed including donor and recipient factors. Binomial logistic regression was conducted to identify independent risk factors for ICU readmission, including all variables with a $P$ value < 0.1 in univariant analysis.

### *Research results*

Thirty-one (10.4%) patients were readmitted into the ICU within ≤ 3 mo of initial ICU discharge, among whom 7 (2.3% of total cases) had more than one ICU readmission. Biliary complication and sepsis was the most common cause of ICU readmission. Significant independent risk factors included recipient age and length of initial hospital stay after discharge from the ICU.

### *Research conclusions*

The study concluded that older recipient age and duration of hospital stay (word stay) before ICU readmission were significant risk factors for ICU readmission. The overall survival rate for ICU readmitted patients was significantly lower than that for non-ICU readmitted patients.

### *Research perspectives*

Further study are warranted to identity how to improve management of the risky patients and hence improve their survival.

## FOOTNOTES

**Author contributions:** Korraa AA, Elewa GM, and El Gendy HA designed the research; Montasser IF, Salah M, and Labib HA performed the research, wrote the paper, contributed analytical tools and analysed the data; Abdelrahman M and Goda MH contributed in data collection and analysis; Dabbous H, Bahaa M, and El-Meteini M revised the manuscript; All authors have read and approved the final manuscript.

**Institutional review board statement:** This study was approved by the Ethics Committee of the Faculty of Medicine, Ain Shams University, Egypt (approval number: IRB/0006379). The confidentiality of the patients' data was

maintained by assigning a code number to each patient.

**Clinical trial registration statement:** The trial is registered in clinical trial.gov (NCT04067739).

**Conflict-of-interest statement:** All the authors declare that they have no conflict of interest.

**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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**Country/Territory of origin:** Egypt

**ORCID number:** Manar Salah 0000-0001-9909-4016; Iman Fawzy Montasser 0000-0002-1351-978X; Hanaa A El Gendy 0000-0001-5888-1830; Alaa A Korraa 0000-0002-2529-109X; Gamal M Elewa 0000-0002-3272-8114; Hany Dabbous 0000-0001-5648-7733; Hossam R Mahfouz 0000-0001-7866-0138; Mostafa Abdelrahman 0000-0002-3925-1320; Mohammed Hisham Goda 0000-0002-3637-5248; Mohamed Mohamed Bahaa El-Din 0000-0002-8605-4397; Mahmoud El-Meteini 0000-0002-1839-3549; Heba A Labib 0000-0003-4194-3438.

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ORIGINAL ARTICLE

**Retrospective Cohort Study**

# Impact of alcohol consumption on treatment outcome of hepatocellular carcinoma patients with viral hepatitis who underwent transarterial chemoembolization

Attapon Rattanasupar, Arunchai Chang, Tanaporn Prateepchaiboon, Nuttanit Pungpipattrakul, Keerati Akarapatima, Apiradee Songjamrat, Songklod Pakdeejit, Varayu Prachayakul, Teerha Piratvisuth

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**Attapon Rattanasupar, Arunchai Chang, Keerati Akarapatima,** Division of Gastroenterology, Department of Internal Medicine, Hatyai Hospital, Hatyai 90110, Songkhla, Thailand

**Tanaporn Prateepchaiboon, Nuttanit Pungpipattrakul,** Department of Internal Medicine, Hatyai Hospital, Hatyai 90110, Songkhla, Thailand

**Apiradee Songjamrat, Songklod Pakdeejit,** Division of Intervention Radiology, Department of Radiology, Hatyai Hospital, Hatyai 90110, Songkhla, Thailand

**Varayu Prachayakul,** Siriraj Gastrointestinal Endoscopy Center, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkoknoi 10700, Bangkok, Thailand

**Teerha Piratvisuth,** NKC Institute of Gastroenterology and Hepatology, Songklanagarind Hospital, Faculty of Medicine, Prince of Songkla University, Hatyai 90110, Songkhla, Thailand

**Corresponding author:** Arunchai Chang, MD, Assistant Professor, Doctor, Division of Gastroenterology, Department of Internal Medicine, Hatyai Hospital, 182 Ruthakal road, Hatyai, Hatyai 90110, Songkhla, Thailand. busmdcu58@gmail.com

## Abstract

### *BACKGROUND*

Alcohol consumption increases the risk of hepatocellular carcinoma (HCC) in patients with pre-existing liver disease, including viral hepatitis. However, studies on the impact of alcohol consumption on the outcomes of HCC are limited. We hypothesized that alcohol had an additional effect with chronic viral hepatitis infection on treatment outcomes after transarterial chemoembolization (TACE) in patients with intermediate-stage HCC (Barcelona Clinical Liver Cancer [BCLC] -B).

### *AIM*

To evaluate the additional effect of alcohol on treatment outcomes of TACE among HCC patients with viral hepatitis.

### *METHODS*

This study, conducted at Hatyai Hospital in Thailand, included HCC patients over 18 years of age with chronic viral hepatitis. Records of HCC patients with viral hepatitis classified as BCLC-B who underwent TACE as the first treatment modality between 2014 and 2019 were retrospectively reviewed. Patients with chronic viral hepatitis only were categorized under group A, and those with chronic viral hepatitis and concurrent alcohol consumption were categorized under group B. Both groups were compared, and the Cox proportional-hazards model was used to identify the survival-influencing variables.

***RESULTS***

Of the 69 patients, 53 were categorized in group A and 16 in group B. There were no statistically significant differences in tumor characteristics between the two patient groups. However, Group A had a statistically significantly higher proportion of complete response (24.5% *vs* 0%, $P = 0.030$) and a higher median survival rate (26.2 mo *vs* 8.4 mo; log-rank $P = 0.012$) compared to group B. Factors associated with decreased survival in the proportional-hazards model included alcohol consumption (hazards ratio [HR], 2.377; 95% confidence interval [CI], 1.109-5.095; $P = 0.026$), presence of portal hypertension (HR, 2.578; 95%CI, 1.320–5.037; $P = 0.006$), largest tumor size > 5 cm (HR, 3.558; 95%CI, 1.824-6.939; $P < 0.001$), and serum alpha-fetoprotein level > 100 ng/mL (HR, 2.536; 95%CI, 1.377-4.670; $P = 0.003$).

***CONCLUSION***

In HCC BCLC B patients with chronic viral hepatitis, alcohol consumption is an independent risk factor for increased mortality and decreases the rate of complete response and survival after TACE.

**Key Words:** Alcohol misuse; Chronic viral hepatitis; Hepatocellular carcinoma; Risk factor; Survival; Transarterial chemoembolization

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**Core Tip:** Regular alcohol consumption is associated with increased hepatocellular carcinoma (HCC) risk, particularly in patients with pre-existing chronic liver diseases, including viral hepatitis B and C infection. However, data on the impact of alcohol consumption on HCC outcomes after treatment with transarterial chemoembolization (TACE) remain limited. This study is the first to address the additional effect of alcohol on treatment outcomes of transarterial chemoembolization TACE among HCC patients with viral hepatitis.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide[1]. As the incidence of HCC is almost the same as the number of annual deaths caused by this malignancy, it is also the third leading cause of cancer-related mortality worldwide[2]. Most HCC patients are diagnosed late, subsequently leading to poor clinical outcomes and often making palliative treatment their only option [2,3]. For patients with intermediate-stage HCC (Barcelona Clinical Liver Cancer [BCLC] B), transarterial chemoembolization (TACE) with preserved liver function has been shown to improve survival[2,3].

Chronic viral hepatitis infection, in particular with hepatitis B virus (HBV) and hepatitis C virus (HCV), are important risk factors for HCC. In fact, HBV and HCV are estimated to be responsible for 50%-90% of HCC cases worldwide[4]. Alcohol use disorder is associated with intravenous injections and bloodborne infections; heavy alcohol consumption has been reported to be much higher among individuals screened for chronic viral hepatitis than the general population[5]. Due to the strong association of alcohol misuse with alcohol-associated liver diseases, liver cirrhosis, and cancer[6,7], alcohol has been categorized as a human carcinogen[8]. Alcohol consumption enhances or accelerates hepatocarcinogenesis in patients with other pre-existing chronic liver diseases, especially chronic viral

hepatitis infection[9,10]. However, studies on the impact of alcohol consumption on HCC outcomes after treatment are limited.

The study's objective was to verify the additional effect of alcohol on treatment outcomes of TACE among intermediate-stage HCC (BCLC B) patients with viral hepatitis.

## MATERIALS AND METHODS

### Study design and patient population

This retrospective cohort study was conducted at Hatyai Hospital (a regional referral tertiary center in southern Thailand). The study protocol was approved by the Institutional Review Board of Hatyai Hospital (protocol number HYH EC 105-64-01) and conducted in accordance with the Declaration of Helsinki. The need for informed consent was waived because patient information was de-identified before analysis.

The inclusion criterion was HCC patients > 18 years of age with chronic viral hepatitis classified as BCLC B who underwent TACE as the first treatment modality between January 2014 and December 2019. The exclusion criteria were as follows: (1) Received any curative treatment for HCC; (2) infiltrative tumor or extrahepatic metastasis; (3) renal, cerebral, or cardiopulmonary dysfunction; (4) presence of other concurrent malignancy; and (5) insufficient data for analysis.

### Data collection

A retrospective review of the medical records of each patient was performed manually by two independent investigators (with at least five years of experience in the field of hepatology), and a third investigator (senior consultant who had an experience of more than ten years) was consulted to resolve disagreements or discrepancies. For each patient, data were extracted from the demographic and clinical variables (including age, sex, body mass index, comorbidities, clinical presentation, and laboratory results), tumor characteristics (including the number of tumors and the size and stage of the tumors) at the time of diagnosis. Data on clinical outcomes included the number of total sessions of TACE and responses after all treatments were completed.

### Treatment and evaluation of response

After discussion by a multidisciplinary team, TACE treatment was offered to patients and conducted after a consensus was reached between doctors and patients. Written informed consent was obtained from all the patients before the procedure. Contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) was performed to evaluate tumor status prior to TACE. Conventional TACE was performed by experienced interventional radiologists. A single intravenous dose of antibiotic prophylaxis with third-generation cephalosporin was routinely administered, except in patients who were prescribed antibiotics for other indications. After assessment of feeding vessels to the segment where the tumor was located, a mixture of a cytotoxic drug (such as doxorubicin or mitomycin C) and iodized oil (Lipiodol; Guerbet, Milan, Italy) was injected, followed by embolization using gelatin sponge particles under fluoroscopic monitoring. We routinely assessed the treatment response at 4-6 wk after the procedure using dynamic contrast-enhanced CT or MRI.

### Definitions and outcomes

HCC was diagnosed based on the American Association for the Study of Liver Disease (AASLD) criteria as previously described, and the BCLC system was used for tumor staging[2]. We stratified patients into two groups, namely "group A" consisting of patients with chronic viral hepatitis only and "group B" consisting of patients with concurrent chronic viral hepatitis and alcohol consumption. Viral hepatitis was defined as infection with either HBV or HCV as confirmed by a history of positive serological results (hepatitis B virus surface antigen and hepatitis C virus antibody) accompanied by the presence of HBV DNA or HCV RNA. Alcohol consumption was defined as daily alcohol consumption of at least 40 g[11]. The diagnosis of cirrhosis was based on clinical features, imaging, and histology. The presence of portal hypertension was confirmed if the patients had any of the following: (1) Ascites; (2) esophageal or gastric varices; and (3) splenomegaly accompanied by a platelet count < 100000/mm$^3$[2]. Hepatic function was assessed using the Child–Turcotte–Pugh score[12] and the model of end-stage liver disease [13]. The patient's performance status was classified according to the Eastern Cooperative Oncology Group Performance Status scale[14].

Complete response (CR) after treatment was defined as the disappearance of any intra-tumor enhancement in all target lesions, as demonstrated by dynamic enhanced cross-sectional imaging based on the modified Response Evaluation Criteria in Solid Tumors[15]. Overall survival (OS) was calculated from the date of diagnosis of HCC until either death (using data from the Thailand civil registrations) or the last follow-up date. The censored survival time was January 1, 2021.

### Statistical analysis

Categorical variables were expressed using descriptive statistics and assessed for statistically significant differences using Pearson's chi-square or Fisher's exact test. For continuous variables, data were presented as mean ± standard deviation (SD) or median and interquartile range (IQR) and tested for statistically significant differences using the Student's t-test and Wilcoxon rank-sum test. Survival analysis was performed using the Kaplan–Meier method, and the log-rank test was used to analyze statistical differences between the two groups. The Cox proportional hazards model was used to identify variables influencing survival. After univariate analysis, sex, age, and other variables with probabilities ($P$ values) < 0.2 were included in the multivariate analyses. All data analyses were performed using the statistical program Stata Version 15.1 (StataCorp LLC, College Station, TX, United States). Statistical significance was set at $P < 0.05$.

## RESULTS

### Baseline characteristics

A total of 69 patients met the inclusion criteria and were enrolled in the study. The average age was 55.5 ± 9.9 years, and 51 (73.9%) were men. Of these patients, 53 were classified into group A (chronic viral hepatitis only) and 16 into group B (concurrent chronic viral hepatitis and alcohol consumption). Comparisons of demographic data are shown in Table 1. The proportion of female patients in group A was higher than that in group B (34.0% *vs* 0%, $P = 0.007$). When compared between the two groups, serum albumin level in group A was significantly higher (mean ± SD = 3.6 ± 0.7 g/dL *vs* 3.2 ± 0.4 g/dL, $P = 0.017$), while serum aspartate aminotransferase (AST) level in group B was significantly higher (median [IQR] = 63.0 [42.0 to 116.0] mg/dL *vs* 96.5 [73.5 to 155.0] mg/dL). The proportion of patients with chronic hepatitis B tended to be higher in group A compared to group B (64.2% *vs* 37.5%, $P = 0.058$), while the proportion of patients with chronic hepatitis C tended to be higher in group B compared to group A (37.7% *vs* 62.5%, $P = 0.080$).

### Tumor characteristics and response

There were no significant differences in tumor characteristics between these two patient groups (Table 2). The median number of TACE sessions was not significantly different between the two groups; the proportion of patients who achieved CR after treatment was statistically significantly higher in group A than in group B (24.5% *vs* 0%, $P = 0.030$).

### Impact of alcohol consumption on OS

Based on the Kaplan–Meier method, the survival rate of patients in group A was significantly higher than in group B (median OS was 26.2 mo in group A and 8.4 mo in group B; log-rank $P = 0.012$) (Figure 1).

To identify the factors of OS after TACE in HCC patients with viral hepatitis, the Cox proportional-hazards model was used. In the multivariate analysis, factors associated with a decreased OS included alcohol consumption (hazards ratio [HR], 2.377; 95% confidence interval [CI], 1.109-5.095; $P = 0.026$), presence of portal hypertension (HR, 2.578; 95%CI, 1.320-5.037; $P = 0.006$), largest tumor size > 5 cm (HR, 3.558; 95%CI, 1.824-6.939; $P < 0.001$), and serum alpha-fetoprotein level > 100 ng/mL (HR, 2.536; 95%CI, 1.377-4.670; $P = 0.003$) (Table 3).

## DISCUSSION

This retrospective cohort study was based on a series of patients with intermediate-stage HCC (BCLC B) who underwent TACE and reflects "real-life" outcome data from a Government Hospital in a middle-income country. The principal findings of this study were as follows: First, HCC BCLC B patients with chronic viral hepatitis concurrent with alcohol consumption showed a decreased rate of CR and survival after TACE than those who had chronic viral hepatitis alone; and second, after adjusting for confounding factors, alcohol consumption was observed as an independent risk factor of increased mortality after TACE in individuals with chronic viral hepatitis.

It has been well documented that regular alcohol consumption is associated with increased HCC risk, with a significant dose-dependent response relationship between the amount of alcohol intake and the risk of HCC[16,17]. Recent meta-analysis demonstrated that consumption of even a small amount of alcohol is related to cancer risk[18]. The risk of HCC in alcohol consumption may differ depending on the severity of baseline liver status[19]. For patients with pre-existing chronic liver diseases, including HBV and HCV, alcohol consumption has a synergistic effect on the development of HCC, although the risk threshold remains uncertain[1,20,21]. However, the data on the impact of alcohol consumption on HCC outcomes after treatment remains limited.

**Table 1 Baseline demographic data of patients with viral hepatitis only (group A) and those with viral hepatitis concurrent with alcohol consumption (group B)**

| Variables | Group A (*n* = 53), % | Group (*n* = 16), % | *P* value |
|---|---|---|---|
| Female sex | 18 (34.0) | 0 (0) | 0.007 |
| Age (yr): mean ± SD | 56.1 ± 10.5 | 53.6 ± 7.5 | 0.365 |
| Body mass index (kg/m$^2$): mean ± SD | 23.2 ± 4.3 | 22.0 ± 3.1 | 0.298 |
| Underlying disease | | | |
| Diabetic mellitus | 10 (18.9) | 2 (12.5) | 0.718 |
| Hypertension | 9 (17.0) | 2 (12.5) | 1.000 |
| Dyslipidemia | 2 (3.8) | 0 (0) | 1.000 |
| Hepatitis B virus infection | 34 (64.2) | 6 (37.5) | 0.058 |
| Hepatitis C virus infection | 20 (37.7) | 10 (62.5) | 0.080 |
| Hepatitis B and C virus coinfection | 1 (1.9) | 0 (0) | 1.000 |
| Cirrhosis | 53 (100) | 16 (100) | N/A |
| Child–Turcotte–Pugh classification | | | 0.109 |
| A | 35 (66.0) | 7 (43.8) | |
| B | 18 (34.0) | 9 (56.2) | |
| Presence of portal hypertension | 36 (67.9) | 10 (62.5) | 0.687 |
| Laboratory data | | | |
| Hemoglobin (g/dL): mean ± SD | 12.3 ± 1.9 | 12.2 ± 1.9 | 0.883 |
| Platelet median (×10$^3$/mL): Median (IQR) | 119 (78 to 208) | 116 (64 to 175) | 0.803 |
| Serum creatinine (mg/dL): Median (IQR) | 0.9 (0.7 to 1.0) | 0.8 (0.7 to 0.9) | 0.257 |
| Serum Albumin (g/dL): mean ± SD | 3.6 ± 0.7 | 3.2 ± 0.4 | 0.017 |
| Total bilirubin (mg/dL): Median (IQR) | 1.0 (0.6 to 2.0) | 1.7 (0.9 to 2.1) | 0.155 |
| Aspartate aminotransferase (mg/dL), median (IQR) | 63.0 (42.0 to 116.0) | 96.5(73.5 to 155.0) | 0.013 |
| Alanine aminotransferase (mg/dL), median (IQR) | 41.0 (23.0 to 76.0) | 52.5 (45.0 to 85.0) | 0.151 |
| International normalized ratio: mean ± SD | 1.2 ± 0.2 | 1.2 ± 0.4 | 0.654 |
| Hepatitis B viral load (IU/mL): Median (IQR) | 1450 (Undetectable to 165000) | 32650 (13700 to 966000) | 0.706 |
| Alpha-fetoprotein (ng/mL): Median (IQR) | 20.5 (9.3 to 499.8) | 176.45 (13.3 to 992.2) | 0.207 |
| MELD: mean ± SD | 9 (7 to 12) | 11 (8 to 12) | 0.307 |
| ECOG score | | | 1.000 |
| 0 | 42 (79.2) | 13 (81.2) | |
| 1 | 11 (20.8) | 3 (18.8) | |

SD: Standard deviation; IQR: Interquartile range; IU: International unit; MELD: Model for end-stage liver disease; ECOG: Eastern Cooperative Oncology Group; N/A: Not applicable.

To the best of our knowledge, this was the first study evaluating the impact of alcohol consumption on treatment outcomes among patients with intermediate-stage HCC after TACE in individuals with chronic viral hepatitis. According to the tumor characteristics, there were no significant differences in the number and size of tumors between the two groups. Patients with chronic viral hepatitis concurrent with alcohol consumption developed a lower rate of CR and had decreased survival rate after TACE than those who had chronic viral hepatitis alone. These results underscore that alcohol consumption provides worse outcomes after TACE when concomitant with chronic viral hepatitis. There are many possible reasons to explain this finding.

First, patients with alcohol-related HCC are linked to poor general conditions, including performance status and hepatic reserve[22]. This is consistent with the results of our study demonstrating liver status in patients with viral hepatitis infection and alcohol consumption which was poorer in both synthesis

**Table 2 Comparison of tumor characteristics and response between patients with viral hepatitis only (group A) and those with viral hepatitis concurrent with alcohol consumption (group B)**

| Variables | Group A (*n* = 53), % | Group (*n* = 16), % | *P* value |
|---|---|---|---|
| Multinodular (> 1 lesion) | 44 (83.0) | 14 (87.5) | 1.000 |
| Largest tumor size (cm): Median (IQR) | 5.3 (3.7 to 9.0) | 4.3 (2.6 to 9.0) | 0.399 |
| Largest tumor sized > 5 cm | 27 (50.9) | 9 (56.2) | 0.710 |
| Number of TACE sessions: median (IQR) | 2 (1 to 3) | 2 (1 to 3) | 0.301 |
| Achieved complete respond | 13 (24.5) | 0 (0) | 0.030 |

IQR: Interquartile range; TACE: Transarterial chemoembolization.

**Table 3 Univariate and multivariate Cox proportional-hazards model of predictive factors of overall survival after transarterial chemoembolization in individuals with chronic viral hepatitis**

| Factor | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95%CI | *P* value | HR | 95%CI | *P* value |
| Female sex | 0.722 | 0.384-1.358 | 0.312 | 1.103 | 0.516-2.359 | 0.800 |
| Age, every 1-year increase | 0.977 | 0.947-1.008 | 0.148 | 1.000 | 0.968-1.034 | 0.979 |
| Body mass index < 18.5 | 0.937 | 0.439-2.002 | 0.867 | | | |
| Hepatitis B infection | 0.841 | 0.487-1.453 | 0.535 | | | |
| Hepatitis C infection | 1.270 | 0.737-2.191 | 0.389 | | | |
| Alcohol consumption | 2.185 | 1.172-4.074 | 0.014 | 2.377 | 1.109-5.095 | 0.026 |
| Serum albumin > 3.5 g/dL | 0.717 | 0.414-1.240 | 0.234 | | | |
| Alpha-fetoprotein > 100 ng/mL | 2.174 | 1.249-3.783 | 0.006 | 2.536 | 1.377-4.670 | 0.003 |
| Child–Turcotte–Pugh classification | | | 0.115 | | | 0.793 |
| A | 1 | (reference) | | 1 | (reference) | |
| B | 1.558 | 0.898-2.704 | | 1.114 | 0.498-2.492 | |
| MELD score > 10 | 1.133 | 0.652-1.968 | 0.657 | | | |
| Presence of portal hypertension | 1.743 | 0.952-3.191 | 0.072 | 2.578 | 1.320-5.037 | 0.006 |
| ECOG | | | 0.270 | | | |
| 0 | 1 | (reference) | | | | |
| 1 | 1.436 | 0.755-2.731 | | | | |
| Multinodular (> 1 lesion) | 1.141 | 0.512-2.543 | 0.747 | | | |
| Largest tumor sized > 5 cm | 2.203 | 1.242-3.906 | 0.007 | 3.558 | 1.824-6.939 | < 0.001 |

MELD: Model for End-Stage Liver Disease; ECOG: Eastern Cooperative Oncology Group.

(lower albumin level) and evidence of inflammation (higher AST level) than that in patients with viral hepatitis only. The impaired clinical status could be caused by the direct effect of ethanol on the liver, alcohol-associated malnutrition, or brain cognitive dysfunction occurring in chronic alcohol abuse[23,24]. Poorer general conditions at the time of HCC detection were associated with a higher rate of non-HCC-related complications than viral-related-HCC, which resulted in shorter survival[22,25-28]. In addition, continuing alcohol abuse precludes providing treatment options for best supportive care as a result of worsening survival[29]. In Thailand, most patients with HCC who abused alcohol still had concurrent alcohol consumption, leading to ongoing liver function deceleration and limited treatment options[22,28,29]. This could explain why patients with chronic viral hepatitis and alcohol abuse had a lower rate of CR and shorter OS than patients with chronic viral hepatitis alone in this study.

Second, alcohol can accelerate the progression of liver disease in patients with chronic viral hepatitis (B or C) by several mechanisms. Alcohol increases intestinal permeability to various substances,

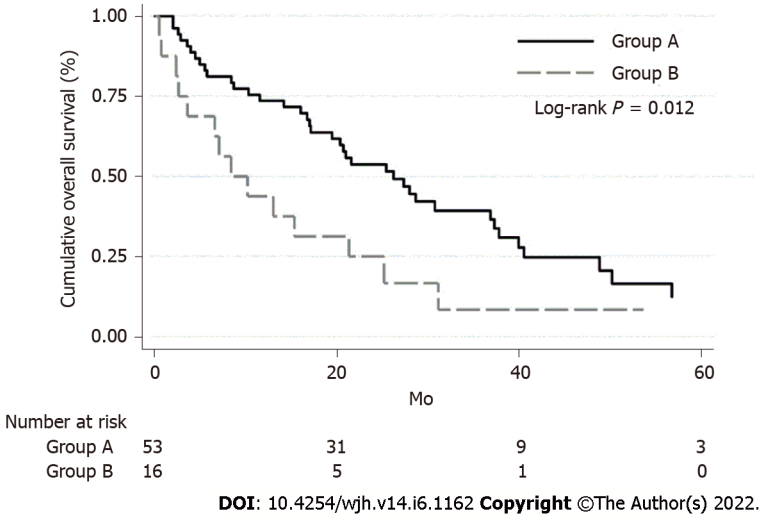


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**Figure 1 Kaplan-Meier curves of cumulative overall survival rates after transarterial chemoembolization in patients with hepatocellular carcinoma Barcelona Clinical Liver Cancer Stage B with viral hepatitis only (group A) compared with those with viral hepatitis concurrent with alcohol consumption (group B).**

especially bacteria-derived liposaccharides from the gut to the liver, stimulating Kupffer cell activity and promoting inflammatory cascade resulting in progression of fibrosis[30,31]. Acetaldehyde, which is derived from the metabolism of ethanol, is a carcinogen and a highly toxic substance that plays a major role in the necroinflammation of hepatocytes[1]. Besides the direct biological impact of alcohol, the association between alcohol consumption and chronic viral hepatitis infection has been identified. Chronic alcohol consumption led to increased replication of viral hepatitis virus (both HBV and HCV) [32,33] and altered immune response, which is associated with promoting hepatocyte injury resulting in hepatic deterioration[34,35]. Heavy alcohol drinking was associated with rapid progression of fibrosis and development of cirrhosis in patients with HBV infection[36]. Among HCV patients, excessive alcohol consumption was strongly associated with decompensated cirrhosis[37]. In addition, HBV infection compromises the host function of antioxidant defense, which promotes alcoholic liver injury [38]. For these reasons, alcohol consumption and chronic viral hepatitis can synergize the lifetime risk of liver disease progression and ultimately increase the risk of death, as seen in our study[37,39].

Third, alcohol may alter the biological pattern of HCC in patients with viral hepatitis. Kubo *et al*[40] demonstrated that the proportion of well-differentiated HCC was lower among those with massive alcohol consumption than those without alcohol use. Undifferentiated HCC is more aggressive and metastatic[41]. Okada *et al*[42] also reported that patients with excessive alcohol consumption had a short tumor-free and overall survival after treatment.

Fourth, alcohol consumption is linked with different types of liver disease. Alcohol abuse, especially heavy alcohol consumption, cause changes in lipid metabolism resulting in aggravation of non-alcoholic liver disease (NASH), which affects treatment outcomes. NASH-related HCC is associated with poorer OS than HCC in patients with cirrhosis from other etiologies[43].

In addition to the impact of alcohol on treatment outcome, our study revealed the other factors that affect the risk of mortality, including the presence of portal hypertension, serum AFP > 100 ng/mL and larger tumor size. Consistent with the findings of a previous study, Scheiner *et al*[44] demonstrated that portal hypertension was a significant poor prognostic factor in HCC patients undergoing TACE. After TACE, transient hepatic hypoxia enhanced the upregulation of vascular endothelial growth factor, which plays a significant role in cirrhosis progression and dysfunction[44,45]. Tumor burden is another factor that affects the prognosis of HCC. A larger tumor size provides higher tumor volume resulting in a worse prognosis, which is consistent with the results of our study. A previous study reported that the elevation of serum AFP levels correlated with the tumor size in HCC[46]. In our study, patients with serum AFP levels of more than 100 ng/mL showed an increase in the risk of death with an odds ratio of 2.5. This supports that AFP is not only a diagnostic tool but also a prognostic tool of HCC.

Our study has several limitations. First, this was a single center study conducted in a tertiary care center in a developing country in Southeast Asia. According to a previous study by our group, the rate of adherence to the international guidelines of HCC treatment in developing Asian countries was decreased because of the regional culture in which the aggressive treatment options were not preferred extensively in patients with non-curative malignancies[47]. Second, this study was retrospective in nature. All variables were obtained from a review of medical records, which may have caused misclassification bias and missing data. Minimization of these errors was attempted using two independent reviewers, and a third reviewer made the final decision in case discrepancies were found. Third, some information that might affect survival (*e.g.*, data on non-alcoholic fatty liver disease, viral status, and

alcohol abstinence) was unavailable. Finally, the study population size was relatively small, and the number of patients among the two groups was disproportionate (53 in group A and 16 in group B). A future prospective study with a larger sample size and appropriately balanced heterogenous participants to eliminate bias and confirm the findings of this study is needed.

## CONCLUSION

In HCC BCLC B, patients with chronic viral hepatitis concurrent with alcohol consumption had a decreased CR rate and survival post-TACE than those who had viral hepatitis infection only. Alcohol consumption was observed as an independent risk factor of increased mortality after TACE in individuals with viral hepatitis. The burden of alcohol is high globally and is avoidable, although difficult to prevent. The results of this study remind us that alcohol consumption will continue to be important, and strategies modified for these factors to limit their impact at the individual and population levels need to be continued.

## ARTICLE HIGHLIGHTS

### *Research background*

Alcohol consumption increases the risk of hepatocellular carcinoma (HCC) in patients with pre-existing liver disease, including viral hepatitis. However, the impact of alcohol consumption on the outcomes of HCC remained questionable.

### *Research motivation*

We hypothesized that alcohol had an additional effect with chronic viral hepatitis infection on treatment outcomes after transarterial chemoembolization (TACE) in patients with intermediate-stage HCC (Barcelona Clinical Liver Cancer [BCLC] -B).

### *Research objectives*

We aims to evaluate the additional effect of alcohol on treatment outcomes of TACE among HCC patients with viral hepatitis.

### *Research methods*

We conducted a retrospective review the records of 69 HCC patients with viral hepatitis classified as BCLC B who underwent TACE as the first-line treatment between 2014 and 2019 at Hatyai Hospital. Patients with chronic viral hepatitis only were categorized under group A and those with chronic viral hepatitis and concurrent alcohol consumption were categorized under group B. Both groups were compared, and the Cox proportional hazards model was used to identify variables influencing survival.

### *Research results*

We find that patients who had chronic viral hepatitis alone had a statistically significantly higher proportion of complete response (24.5% *vs* 0%, $P = 0.030$) and a higher median survival rate (26.2 mo *vs* 8.4 mo; log-rank $P = 0.012$) than those with chronic viral hepatitis concurrent with alcohol consumption. Alcohol consumption was an independent factor associated with decreased survival in the proportional hazards model included (hazards ratio [HR], 2.377; 95% confidence interval [CI], 1.109-5.095; $P = 0.026$).

### *Research conclusions*

In HCC BCLC B patients with chronic viral hepatitis, alcohol consumption is an independent risk factor for increased mortality and decreases the rate of complete response and survival after TACE.

### *Research perspectives*

This research underscore that alcohol consumption leads to worse outcomes after TACE in intermediate stage HCC patients with chronic viral hepatitis.

## FOOTNOTES

**Author contributions:** Rattanasupar A designed and conceptualized the study, acquired the data and drafted the manuscript; Chang A designed and conceptualized the study, acquired, analyzed, and interpreted the data, and drafted the manuscript; Prateepchaiboon T designed and conceptualized the study, acquired, analyzed, and interpreted the data; Pungpipattrakul N, Akarapatima K, Songjamrat A, and Pakdeejit S acquired, analyzed, and interpreted the data; Prachayakul V and Piratvisuth T critically revised the manuscript for important intellectual

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**Country/Territory of origin:** Thailand

**ORCID number:** Attapon Rattanasupar 0000-0002-9595-5808; Arunchai Chang 0000-0002-0158-2685; Tanaporn Prateepchaiboon 0000-0003-1000-7794; Nuttanit Pungpipattrakul 0000-0002-5983-7273; Keerati Akarapatima 0000-0001-5907-0001; Apiradee Songjamrat 0000-0002-8008-2450; Songklod Pakdeejit 0000-0002-0144-3378; Varayu Prachayakul 0000-0001-6652-9044; Teerha Piratvisuth 0000-0002-5249-9208.

**Corresponding Author's Membership in Professional Societies:** Gastroenterological Association of Thailand.

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ORIGINAL ARTICLE

Retrospective Study

# Relationship between phase angle, steatosis, and liver fibrosis in patients coinfected with human immunodeficiency virus/hepatitis C virus

Sabrina Alves Fernandes, Cristiane Valle Tovo, André Luiz Machado da Silva, Letícia Pereira Pinto, Randhall B Carteri, Angelo A Mattos

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**Sabrina Alves Fernandes, Cristiane Valle Tovo, Letícia Pereira Pinto, Angelo A Mattos,** Postgraduate Program in Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre 90050-170, Brazil

**André Luiz Machado da Silva,** Department of Infectology Service, Hospital Nossa Senhora da Conceição, Porto Alegre 91350-200, Brazil

**Randhall B Carteri,** Department of Nutrition, Centro Universitário Metodista-IPA, Porto Alegre 90420-060, Brazil

**Randhall B Carteri,** Department of Nutrition, Centro Universitário CESUCA, Cachoeirinha 94935-630, Brazil

**Corresponding author:** Cristiane Valle Tovo, PhD, Research Assistant Professor, Research Associate, Research Scientist, Postgraduate Program in Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite 245, Porto Alegre 90050-170, Brazil. cristianev@ufcspa.edu.br

## Abstract

*BACKGROUND*

Malnutrition, lipodystrophy, and dyslipidemia are prevalent characteristics in patients with human immunodeficiency virus (HIV) infection with or without previous treatment. Such a clinical condition can lead to the hypothesis of the presence of hepatic steatosis with possible progression to fibrosis and the risk of hepatocellular carcinoma. Notably, a low phase angle (PA), evaluated by bioelectrical impedance analysis (BIA), is an independent prognostic marker of clinical progression and survival in HIV-infected patients.

*AIM*

To evaluate the relationship between PA and body composition with steatosis and hepatic fibrosis in HIV/hepatitis C virus (HCV)-coinfected patients.

*METHODS*

A retrospective observational study by convenience sampling of coinfected HIV/HCV patients, in which all patients underwent transient elastography

(Fibroscan) and BIA evaluation. Student's *t* test was used for group comparisons, and Spearman's or Pearson's correlation test was used when appropriate. The significance level was set at 5%, and analyses were performed using SPSS version 21.0.

***RESULTS***

Forty-three patients who received antiretroviral therapy met the inclusion criteria, and 23 (53.5%) were under treatment with protease inhibitors (PIs). There was no difference in PA between those who used PIs and those who did not ($P$ = 0.635). There was no correlation between fibrosis grade and PA ($P$ = 0.355) or lean mass ($P$ = 0.378). There was a significant inverse correlation between the controlled attenuation parameter (CAP) and lean mass ($P$ = 0.378), positive correlation between PA and lean mass ($P$ = 0.378), and negative correlation between PA and fatty mass ($P$ = 0.378), although the CAP and PA were not correlated. When evaluated by sex, no significant correlations were found.

***CONCLUSION***

PA determines the muscle function of HIV/HCV-coinfected patients, and the CAP values reinforce the association with lean mass, suggesting that patients require early nutritional interventions.

**Key Words:** Phase angle; Bioelectrical impedance; Coinfection; Human immunodeficiency virus; Hepatitis C virus; Nutrition

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**Core tip:** Patients living with human immunodeficiency virus (HIV) are often affected by malnutrition, which may be related to the progression of liver disease. A low phase angle (PA), assessed by bioelectrical impedance analysis, is a prognostic marker of clinical progression and survival in HIV-infected patients. This study aimed to assess the relationship between PA, steatosis, and liver fibrosis in HIV/hepatitis C virus (HCV)-coinfected patients. Forty-three HIV/HCV-coinfected patients were included in this study. PA determines the muscle functionality of patients coinfected with HIV/HCV, and the controlled attenuation parameter values reinforce the association with lean mass, suggesting that patients require early nutritional interventions.

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## INTRODUCTION

Patients living with human immunodeficiency virus (HIV) are frequently affected by malnutrition, which may contribute to the emergence of infections[1,2]. Individuals at all stages of HIV are at risk of nutritional deficiency, and nutritional status is a strong predictor of disease progression, survival, and functional status during the course of the disease, showing a direct relationship with cell integrity and function[3,4].

Furthermore, in patients coinfected with HIV and hepatitis C virus (HCV), physicians must consider not only the natural history of the disease, but also the patient's clinical treatment and previous clinical conditions, which significantly compromise physiological homeostasis[3]. One of the physiological mechanisms of lean mass increase is the role of anabolic hormones, mainly testosterone and insulin-like growth factor-1[3]. The associated mechanisms are responsible for catalyzing protein synthesis and enhancing the replication and differentiation of muscle cells. In HIV patients, testosterone levels decrease, which negatively affects the healthy body composition of this population. This modification of the physiological architecture causes these patients to present with lipodystrophy and malabsorption of nutrients, compromising their nutritional status[3]. Owing to the strong association between muscle mass loss and liver disease, regardless of obesity or metabolic syndrome, identifying a method that indicates these physiological impairments is of paramount importance.

Strikingly, through bioelectrical impedance analysis (BIA), it is possible to measure the phase angle (PA); defined as the relationship between two vectors of resistance and reactance, and a parameter

widely used and established as a prognostic factor in several diseases. Currently, low PA is an independent prognostic marker of clinical progression and survival in HIV-infected patients receiving antiretroviral therapy (ART)[5,6].

Nevertheless, nutritional assessment in patients with chronic liver disease has limitations due to body asymmetry (for example, ascites and edema) that these patients may present as a result of complications from liver cirrhosis, in addition to the lack of a gold standard method[7]. Therefore, PA is the nutritional assessment method with the best performance because it reflects muscle volume and functionality without the influence of confounding factors[8]. In this context, in a study that evaluated 129 patients with cirrhosis using different nutritional assessment methods, including body mass index (BMI), skin folds, subjective global assessment, handgrip strength, and PA, the authors concluded that PA was the only method associated with the condition. Another fact that draws attention to this study is the discrepancy in the percentage of malnourished patients measured by the methods, corroborating the statement that diagnosing the nutritional status of patients with chronic liver disease remains challenging. Identifying the nutritional status of patients is important to intervene early and consequently improve their clinical prognosis[8].

Moreover, coinfected HIV/HCV patients may also have a poor prognosis, as evidence suggests that HIV infection negatively impacts the progression of liver diseases, particularly increasing risks for fibrosis and hepatocellular carcinoma development[9,10], although this may be controversial[11].

Hence, to the best of our knowledge, no studies have assessed the role of PA and its body composition associated with hepatic steatosis and fibrosis in HIV/HCV-coinfected patients. The findings of this study can guide future interventions with a positive impact on the prognosis and quality of life of this population.

## MATERIALS AND METHODS

This was a retrospective study by convenience sampling conducted between January and July/2019 at the outpatient Gastroenterology and Hepatology Clinic of Santa Casa Hospital and the Infectiology Clinic of Hospital Nossa Senhora da Conceição, both tertiary reference centers in Porto Alegre, RS, Brazil.

### *Subject selection criteria*

The study included coinfected HIV/HCV patients. Patients with hepatitis B virus infection, significant alcohol consumption (> 14 drinks *per* week for women and > 21 for men), and hepatocellular carcinoma were excluded.

### *Diagnosis of HIV/HCV*

Serological tests were used to determine chronic HCV and HIV infections. A positive serological test for HCV by ELISA with a positive reverse transcription polymerase chain reaction for HCV RNA confirmed viremia. An ELISA with a confirmatory western blot test confirmed HIV infection.

### *Anthropometric measurements*

Body weight and height were measured on a mechanical scale using a Filizola stadiometer with a weight scale of 100 g and a height scale of 1 cm, which was previously calibrated. The patients were evaluated while wearing light clothing and barefoot. Height was determined using a fixed stadiometer on the wall, with the patient standing upright and barefoot. BMI was calculated as weight (kg) divided by height (in meters) squared[12].

### *BIA*

BIA was performed in all patients without previous specific preparation for fasting. The patients were evaluated in a comfortable dorsal decubitus position and relaxed without shoes, socks, or metallic fittings. The legs were spread apart, hands opened, and supported on a stretcher. The electrodes were positioned as follows: One was placed at the base of the middle toe on the right foot, and another slightly above the line of the ankle joint between the medial and lateral malleoli. Another pair of electrodes was distributed at the base of the middle finger of the right hand, slightly above the line of the right wrist joint, coinciding with the styloid process. The device used was Biodynamics® model 450 (multifrequential–800 A and 50 KhZ, and tetrapolar).

To assess cellular functionality and integrity, PA was measured, which was automatically provided by the equipment based on the values of R and Xc[13]. PA was classified according to the cut-off point of 5.4°, based on the reference parameters of the study by Fernandes *et al*[8], in which values below this point are considered predictive of poor prognosis, and the values above are predictors of good prognosis. Using BIA, the lean mass and fat mass of the coinfected patients were also measured.

### *Staging of liver fibrosis*

Liver fibrosis was evaluated by transient elastography (TE) (Fibroscan), performed by a specialized physician experienced in the procedure (at least 500 examinations performed). The physician was blinded to the patient's data. A FibroScan device (Echosens, Paris, France) was used, and the results were expressed in kPa. The examination was performed after a 4-h fast. Procedures were considered reliable and included in the analysis only when they presented at least 10 valid shots, a success rate of at least 60%, and an interquartile range of liver stiffness value ≤ 30%. The cutoff points for TE were established according to the Brazilian Society of Hepatology and Brazilian College of Radiology practice guidance for the use of elastography in liver diseases for HCV patients (7.1 kPa for F2, 9.5 kPa for F3, and 12.5 kPa for F4)[14]. The controlled attenuation parameter (CAP) was evaluated in all TE in a complementary manner to identify steatosis. The study population was stratified into two groups based on the stage of fibrosis at TE: With (≥ F3) and without (< F3) advanced fibrosis.

### *Statistical analysis*

The normality of the data was assessed using the Kolmogorov–Smirnov test. Parametric variables are described as means and standard deviations. Categorical variables were described by frequencies and percentages. Differences in PA, lean mass, and fatty mass percentages between groups considering the staging of fibrosis were analyzed using Student's *t*-test. Pearson's $\chi^2$ test was used to assess the association between the CAP and PA, and Spearman's correlation coefficient was used to assess the association between fibrosis and PA. The significance level adopted was 5%, and analyses were performed using SPSS version 21.0.

## RESULTS

Initially, of a total of 47 patients, four were excluded because they did not agree to perform TE or BIA; the remaining 43 patients were included in the analysis. Anthropometric and clinical characteristics are shown in Table 1. Male sex was more frequent (22; 51.2%), mean age was 46.2 ± 8.5 years, the HCV genotype 1 was the most frequent ($n$ = 30; 69.7%), and 27 (62.8%) presented advanced fibrosis (F3/F4). The mean BMI was 25.9 ± 4.9 kg/m², and participants showed a mean percentage of lean mass of 75.5 ± 9.2 and a mean percentage of fatty mass of 24.5 ± 9.2 (Table 1). In addition, using the PA cutoff points, only two patients (4.7%) were classified as malnourished.

All patients were taking ART, with 23 (53.5%) using schemes containing protease inhibitors (PIs), and 20 (46.5%) did not. There was no difference in PA between these groups using PIs [7.20 ± 0.70° *vs* 7.06 ± 1.09°; $t$ (37) = 0.479, $P$ = 0.635].

The groups were compared according to the fibrosis stage. The value of PA for patients with fibrosis (TE < F3; $n$ = 16) was 7.3 ± 1.0°, and for advanced fibrosis (TE ≥ F3; $n$ = 27) was 7.0 ± 0.7° [$t$ (41) = 0.936; $P$ = 0.355]. No differences were found between the percentages of lean mass for patients with fibrosis and those with advanced fibrosis [73.9% ± 9.7 *vs* 76.5 ± 8.9; $t$ (41) = -0.89; $P$ = 0.378]. The values of the percentage of fatty mass between patients with fibrosis and those with advanced fibrosis were also similar [26.1% ± 9.7 *vs* 23.5% ± 8.9; $t$ (41) = 0.886; $P$ = 0.381]. There was no correlation between fibrosis grade, PA, and anthropometric parameters (Tables 2 and 3).

The mean CAP was 241.1 ± 55.7 (Table 1). As shown in Table 3, there was a significant inverse correlation between the CAP and the percentage of lean mass (Pearson's $r^2$ = -0.493, $P$ = 0.01). Although no significant correlations between the CAP and PA were found, there was a positive correlation between PA and lean mass (Pearson's $r^2$ = 0.373, $P$ = 0.014) and a negative correlation between PA and fatty mass (Pearson's $r^2$ = -0.373, $P$ = 0.014). Additionally, when evaluated by sex, no significant correlations were found (Table 4).

## DISCUSSION

The present study evaluated the role of PA in HIV/HCV-coinfected patients. Notably, there was no correlation between the fibrosis grade and PA values. However, there was an inverse correlation between the CAP and fatty mass, a positive correlation between PA and lean mass, and a negative correlation between PA and fatty mass, although this correlation was not significant when evaluated according to sex and age. Although it is not possible to stratify patients by age because of the limited number of patients allocated to the study, the patients' mean represents the age group that presents the physiological degradation of skeletal muscle mass[15].

Importantly, the CAP quantifies liver steatosis; however, several covariates may hamper the analysis, including nonalcoholic fatty liver disease, diabetes, and BMI. However, most studies using the CAP evaluated small sample sizes and heterogeneous populations with variable BMI and diabetes prevalence, which may explain the differences in the proposed cutoffs[16]. The present study observed a mean value that was not considered high (241.1 ± 55.7, Table 1), and thus we cannot confirm that the

**Table 1 Anthropometric and clinical characteristics of the evaluated patients (*n* = 43) (mean ± SD)**

| Characteristic | |
|---|---|
| Male sex, *n* (%) | 22 (51.2) |
| Age, yr | 46.2 ± 8.5 |
| Body mass index | 25.9 ± 4.9 |
| Phase angle | 7.1 ± 0.8 |
| Lean mass, % | 75.5 ± 9.2 |
| Fatty mass, % | 24.5 ± 9.2 |
| **Genotype HCV, *n* (%)** | |
| 1 | 30 (69.7) |
| 2 | 02 (4.7) |
| 3 | 09 (20.9) |
| Missing data | 02 (4.7) |
| **Fibrosis, *n* (%)** | |
| F0 | 05 (11.6) |
| F1 | 10 (23.3) |
| F2 | 01 (2.3) |
| F3 | 10 (23.3) |
| F4 | 17 (39.5) |
| CAP | 241.1 ± 55.7 |

CAP: Controlled attenuation parameter; HCV: Hepatitis C virus.

**Table 2 Relationship between hepatic fibrosis and controlled attenuation parameter *vs* phase angle and lean mass (*n* = 43)**

| | Fibrosis[1] | | CAP[2] | |
|---|---|---|---|---|
| | rho | *P* value | $r^2$ | *P* value |
| Phase angle | 0.075 | 0.634 | 0.016 | 0.918 |
| Lean mass | 0.095 | 0.543 | 0.493[3] | 0.001 |

[1]Spearman's rho test.
[2]Pearson test.
[3]Correlation is significant at the 0.05 level (2-tailed).
CAP: Controlled attenuation parameter.

CAP has demonstrated significant steatosis in this case. Meanwhile, we demonstrated a significant inverse correlation between the CAP and lean mass. To justify these findings, we could consider the role of lipodystrophy, a common issue in patients with HIV, as well as the greater chance of steatosis in these patients related to ART, or even the greater occurrence of steatosis in some patients with HCV. Nonetheless, we could not prove the individual role of each parameter. Accordingly, moderate-to-severe steatosis in people living with HIV without viral hepatitis or excessive alcohol intake is associated with cumulative exposure to stavudine, elvitegravir, and raltegravir[17]. In the present study, none of the patients used schemes containing ART.

Concomitantly, of the nucleoside analog reverse transcriptase inhibitors currently available in Brazil for the treatment of people living with HIV (PLHIV), zidovudine (AZT) is the main drug related to adverse events, with para effects due to mitochondrial damage, such as myopathy, lipoatrophy, peripheral neuropathy, hepatic steatosis, and lactic acidosis[17]. Similarly, lamivudine and abacavir, other representatives of this class prescribed in Brazil, can cause damage due to mitochondrial dysfunction, but to a lesser extent than AZT[17]. Additionally, lipohypertrophy is a common feature in PLHIV patients treated with first-generation PIs such as indinavir, but it is not possible to prove a direct relationship between this adverse event and this class of drugs[18]. Those with greater total body fat

**Table 3 Comparison between groups in accordance with fibrosis staging**

| | Fibrosis (TE < F3; *n* = 16) | Advanced fibrosis (TE ≥ F3; *n* = 27) | *t*-test | *P* value |
|---|---|---|---|---|
| Phase angle° | 7.3° ± 1.0 | 7.0° ± 0.7 | *t* (41) = 0.936 | 0.355 |
| Lean mass (%) | 73.9% ± 9.7 | 76.5 ± 8.9 | *t* (41) = -0.89 | 0.378 |
| Fatty mass (%) | 26.1% ± 9.7 | 23.5% ± 8.9 | *t* (41) = 0.886 | 0.381 |

Values are represented as mean ± SD. TE: Transient elastography.

**Table 4 Relationship between phase angle and lean mass, fatty mass and controlled attenuation parameter**

| | Total (*n* = 43) | | Males (*n* = 22) | | Females (*n* = 21) | |
|---|---|---|---|---|---|---|
| | $r^2$ | *P* value | $r^2$ | *P* value | $r^2$ | *P* value |
| Lean mass (%) | 0.373[1] | 0.014 | 0.208 | 0.353 | 0.189 | 0.412 |
| Fatty mass (%) | -0.373[1] | 0.014 | -0.208 | 0.353 | -0.189 | 0.411 |
| CAP | 0.016 | 0.918 | 0.035 | 0.878 | 0.102 | 0.659 |

[1]Spearman's rho test. Correlation is significant at the 0.05 level (2-tailed).
CAP: Controlled attenuation parameter.

before ART and a positive energy balance may have an additional increase in trunk fat, including visceral, breast, and dorsocervical adiposity[19].

Accordingly, the findings of the present study corroborate those observed by Ruiz-Margáin *et al*[20], which evaluated patients with chronic liver disease. In this study, PA was directly proportional to skeletal muscle mass. Therefore, it is possible to observe that the skeletal muscle mass significantly guarantees the improvement of the physiological performance of patients.

Likewise, Osuna-Padilla *et al*[21], when evaluating PA in patients with HIV, also showed that this parameter could be a predictor of malnutrition. In addition, they identified PA cut-off points for men and women of 5.45° and 4.95°, respectively, with specificity and sensitivity > 70%, similar to the present study when evaluating PA, body composition, and the degree of hepatic fibrosis in patients coinfected HIV/HCV.

Recently, PA has gained importance as a nutritional status marker, with low values associated with malnutrition and nutritional risk at the time of hospital admission[22]. The main advantage of using PA is the possibility of its application even under unstable tissue hydration conditions, such as edema and ascites[23]. This fact deserves recognition since patients with HIV may have reduced muscle mass and increased fat mass.

Furthermore, a previous study of 539 adults with HIV demonstrated that lower BMI, lower PA, and loss of fatty mass were associated with more advanced HIV infection ($CD4^+$ lymphocyte count < 200 cells/$mm^3$)[24]. Hence, BIA is a good tool for detecting body cell mass loss in HIV and compares favorably with gold-standard methods. Nevertheless, one of the main clinical complications of advanced liver disease is protein–calorie malnutrition, which has a prevalence ranging from 10% to 100%, regardless of the stage and etiology of the disease. Thus, it is evident that the general prognosis of the disease worsens in the presence of malnutrition, contributing negatively to patients' quality of life [25].

A possible limitation of the present study was the small number of patients. As for strengths, we highlight the originality and importance of the data for early interventions aimed towards the clinical/nutritional treatment of patients coinfected with HIV/HCV, offering improved quality of life and prognosis. In addition, this study implemented an important tool, namely electrical bioimpedance, which does not depend on the operator. Another important and extremely relevant point is the evaluation of liver fibrosis by elastography, a noninvasive and promising method for the diagnosis of these patients.

## CONCLUSION

PA determines the muscle function of HIV/HCV-coinfected patients, and the CAP values reinforce the association with lean mass (both show a relationship with muscle mass, PA, and the CAP), suggesting that patients require early nutritional interventions.

## ARTICLE HIGHLIGHTS

### *Research background*

Human immunodeficiency virus/hepatitis C virus (HIV/HCV)-coinfected patients may have a poor prognosis, as evidence suggests that HIV infection negatively impacts the progression of liver disease, particularly increasing the risks of developing fibrosis and hepatocellular carcinoma, although this can be controversial. Both HIV and HCV negatively affect the nutritional status of patients, regardless of the stage of the disease. In addition, nutritional assessment in patients with chronic liver disease has limitations due to the body asymmetry (*e.g.*, ascites and edema) that these patients may experience as a result of complications from liver cirrhosis, in addition to the lack of a standard method.

### *Research motivation*

There is a strong association between muscle mass loss and liver diseases, regardless of obesity or metabolic syndrome, and identifying a method that indicates these physiological impairments is of paramount importance.

### *Research objectives*

To the best of our knowledge, no studies have assessed the role of phase angle (PA) and its body composition associated with hepatic steatosis and fibrosis in HIV/HCV-coinfected patients.

### *Research methods*

A retrospective observational study by convenience sampling with coinfected HIV/HCV patients, where all patients underwent transient elastography (Fibroscan) and bioelectrical impedance analysis evaluation. Student's *t*-test was used for group comparisons and Spearman's or Pearson's correlation tests were used when appropriate. The significance level adopted was 5% and the analyses were performed using the SPSS version 21.0.

### *Research results*

Of 43 patients who were analyzed, male sex was more frequent (22; 51.2%), mean age was 46.2 ± 8.5 years, HCV genotype 1 was the most frequent ($n$ = 30; 69.7%), and 27 (62.8%) presented with advanced fibrosis (F3/F4). There was no correlation between the fibrosis grade and the PA ($P$ = 0.355). Also, there was no correlation between the fibrosis grade and the lean mass ($P$ = 0.378). The mean controlled attenuation parameter (CAP) was 241.1 ± 55.7, and there was a significant inverse correlation between CAP and percentual of lean mass ($P$ = 0.01). Although no significant correlations between CAP and PA were found, there was a positive correlation between PA and lean mass ($P$ = 0.014), and a negative correlation between PA and fatty mass ($P$ = 0.014). Additionally, when evaluated by sex, there were no significant correlations.

### *Research conclusions*

The PA determines the muscle function of the HIV/HCV-coinfected patients, and the CAP values reinforce the association with lean mass (both show a relationship with muscle mass, the PA and the CAP), suggesting patients who need early nutritional intervention.

### *Research perspectives*

Identifying clinical factors that potentiate a poor prognosis of patients coinfected with HIV/HCV, such as malnutrition, is of relevance. With this information, it is possible to act early in the management of these patients and increase the effectiveness of the therapeutic response, with a consequent improvement in the prognosis and quality of life of this population.

## FOOTNOTES

**Author contributions:** Fernandes SA was responsible for the conception and design, data collection, statistical analysis, and manuscript writing; Tovo CV was responsible for the conception and design, statistical analysis, manuscript writing, and critical revision; da Silva ALM was responsible for data collection, manuscript writing, and critical revision; Pinto LP was responsible for data collection, statistical analysis, and critical revision; Carteri RB was responsible for the statistical analysis, manuscript writing, and critical revision; Mattos AA was responsible for conception and design, statistical analysis, manuscript writing, and critical revision.

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**Country/Territory of origin:** Brazil

**ORCID number:** Sabrina Alves Fernandes 0000-0001-8504-603X; Cristiane Valle Tovo 0000-0002-7932-5937; André Luiz Machado da Silva 0000-0002-1517-1377; Letícia Pereira Pinto 0000-0001-8543-1587; Randhall B Carteri 0000-0003-4124-9470; Angelo A Mattos 0000-0003-2417-9765.

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ORIGINAL ARTICLE

Retrospective Study

# DNA and RNA oxidative damage in hepatocellular carcinoma patients and mortality during the first year of liver transplantation

Leonardo Lorente, Sergio T Rodriguez, Pablo Sanz, Agustín F González-Rivero, Antonia Pérez-Cejas, Javier Padilla, Dácil Díaz, Antonio González, María M Martín, Alejandro Jiménez, Purificación Cerro, Julián Portero, Manuel A Barrera

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**Leonardo Lorente,** Department ofIntensive Care, Hospital Universitario de Canarias, La Laguna 38320, Tenerife, Spain

**Sergio T Rodriguez, María M Martín,** Intensive Care Unit, Hospital Universitario Nuestra Señora Candelaria, Santa Cruz de Tenerife 38010, Spain

**Pablo Sanz, Javier Padilla, Manuel A Barrera,** Department of Surgery, Hospital Universitario Nuestra Señora de Candelaria, Santa Cruz de Tenerife 38010, Spain

**Agustín F González-Rivero, Antonia Pérez-Cejas,** Department of Laboratory, Hospital Universitario de Canarias, La Laguna 38320, Spain

**Dácil Díaz, Antonio González,** Department of Digestive, Hospital Universitario Nuestra Señora de Candelaria, Santa Cruz de Tenerife 38010, Spain

**Alejandro Jiménez,** Research Unit, Hospital Universitario de Canarias, La Laguna 38320, Spain

**Purificación Cerro,** Transplant Unit, Hospital Universitario Nuestra Señora Candelaria, Santa Cruz de Tenerife 38010, Spain

**Julián Portero,** Department of Radiology, Hospital Universitario Nuestra Señora Candelaria, Santa Cruz de Tenerife 38010, Spain

**Corresponding author:** Leonardo Lorente, MD, PhD, Attending Doctor, Medical Assistant, Department of Intensive Care, Hospital Universitario de Canarias, Ofra, s/n., La Laguna 38320, Tenerife, Spain. lorentemartin@msn.com

## Abstract

*BACKGROUND*

Oxidative damage of DNA and RNA has been associated with mortality of patients with different diseases. However, there is no published data on the potential use of DNA and RNA oxidative damage to predict the prognosis of patients with hepatocellular carcinoma (HCC) undergoing liver transplantation (LT).

*AIM*

To determine whether patients with increased DNA and RNA oxidative damage prior to LT for HCC have a poor LT prognosis.

***METHODS***

Patients with HCC who underwent LT were included in this observational and retrospective study. Serum levels of all three oxidized guanine species (OGS) were measured prior to LT since guanine is the nucleobase that forms DNA and RNA most prone to oxidation. LT mortality at 1 year was the end-point study.

***RESULTS***

Surviving patients ($n$ = 101) showed lower serum OGS levels ($P$ = 0.01) and lower age of the liver donor ($P$ = 0.03) than non-surviving patients ($n$ = 13). An association between serum OGS levels prior to LT and 1-year LT (odds ratio = 2.079; 95% confidence interval = 1.356-3.189; $P$ = 0.001) was found in the logistic regression analysis.

***CONCLUSION***

The main new finding was that high serum OGS concentration prior to LT was associated with the mortality 1 year after LT in HCC patients.

**Key Words:** DNA oxidative damage; Hepatocellular carcinoma; Liver transplantation; Mortality; Oxidized guanine species

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**Core Tip:** The potential use of DNA and RNA oxidative damage to predict prognosis of patients with hepatocellular carcinoma who underwent liver transplantation is unknown. In this retrospective study serum levels of the three oxidized guanine species before liver transplantation in 114 patients were measured. One-year survivor patients showed lower serum oxidized guanine specie levels than non-survivor patients ($P$ = 0.01). These preliminary results could induce studies to clarify the potential role of oxidative damage in the prognosis of liver transplantation patients due to hepatocellular carcinoma and to explore the use of antioxidant agents to reduce oxidative stress in those patients.

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## INTRODUCTION

Liver transplantation (LT) could be the treatment of choice in some patients with hepatocellular carcinoma (HCC)[1-4], which is the most common malignant liver tumor and is responsible for many deaths. LT may be an appropriate choice because it treats liver failure and removes the liver tumor[5-8].

The possible contribution of the oxidative state in chronic liver disease progression and in hepatocarcinogenesis development has been suggested[9-12]. RNA, DNA, lipids and proteins could be damaged by reactive oxygen species during oxidative stress. The five types of nucleobases present in RNA and DNA are adenine, guanine, cytosine, uracil and thymine; but only four types of those nucleobases constitute RNA and DNA. In both, RNA and DNA, guanine, adenine and cytosine are present. In addition, uracil is present in RNA and thymine in DNA. Guanine is the nucleobase most prone to oxidation since it has the lowest redox potential[13-16]. The three species of oxidized guanine species (OGS) are 8-hydroxyguanine from DNA or RNA, 8-hydroxyguanosine from RNA, and 8-hydroxy-2′-deoxyguanosine from DNA.

An association between DNA and RNA oxidative damage and mortality has been found in patients with other diseases such as sepsis[17]. Greater DNA oxidative damage (assessed by concentrations of 8-hydroxy-2′-deoxyguanosine in liver biopsy samples) has been found in patients with chronic hepatic disease with HCC than without it[18,19]. However, there is no published data about the potential use of DNA and RNA oxidative damage to predict the prognosis of patients with HCC and who underwent LT. Therefore, the aim in our study was to analyze the potential association between increased oxidative DNA and RNA damage before LT for HCC and poorer LT prognosis.

## MATERIALS AND METHODS

### Design and patients

We included patients who underwent LT due to HCC between May 2001 to May 2017. LT were carried out in the Hospital Universitario Nuestra Señora de Candelaria (Santa Cruz de Tenerife, Spain). This observational and retrospective study was performed after the approval by the Institutional Review Board. Patients were included after the written informed consent was obtained by the LT recipient or a family member. All LT donors were brain dead. Serum samples were obtained before LT and frozen at -80 ºC, and serum concentrations of 8-hydroxy-2'-deoxyguanosine were determined in those samples.

### Variables

Sex, age, nodule size, degree of tumor differentiation, Child-Pugh score[20], infiltration, serum alpha-fetoprotein level, macrovascular invasion, multinodular tumor, portal hypertension (determined either by clinical data or by hepatic venous pressure gradient), microvascular invasion, model for end-stage liver disease score[21] by hepatic function, treatment before LT, LT technique and inside Milan criteria [22] before and after LT were registered. In addition, age of LT donor was registered. One-year LT survival was considered our end-point study.

### Serum samples and determination of OGS concentrations

Serum samples were taken about 2 h before LT. Afterwards samples were placed in a -80 ºC freezer. We had previously determined serum caspase-3 levels in some of these patients[23], and in this research we determined serum OGS levels. We used kits called DNA/RNA Oxidative Damage ELISA Kit® (by Cayman Chemical Corporation in Ann Arbor, United States) to determine serum OGS concentrations. The detection limit of these kits was 0.45 ng/mL. All determinations were carried out in the same Laboratory Department blinded to clinical data.

### Statistical methods

Categorical variables, presented as frequency (percentage), were compared using the $\chi^2$ test. Continuous variables, presented as median (percentiles 25 and 75), were compared using the test of Mann-Whitney. The ability of serum OGS concentrations prior to LT to predict 1-year LT mortality was analyzed using receiver operating characteristic curve. The Kaplan-Meier 1-year LT survival curves were constructed with a serum OGS concentration cut-off (3.3 ng/mL) selected on the basis of Youden's J-index. The association between serum OGS levels and 1-year LT controlling for serum caspase-3 levels and age of liver donor was analyzed using the logistic regression analysis. MedCal 15.2.1 (Ostend, Belgium) and SPSS 17.0 (by SPSS Inc. in Chicago, IL, United States) were used to perform the statistical analyses.

## RESULTS

We included 114 patients in the study, of which 101 remained alive 1 year after LT and 13 died during the first year after LT. Surviving LT patients in comparison to non-surviving patients showed lower serum OGS concentrations prior to LT ($P$ = 0.01) and lower liver donor age ($P$ = 0.03) (Table 1). No significant differences between surviving and non-surviving patients regarding sex, liver receptor age, nodule size, serum alpha-fetoprotein levels, degree of tumor differentiation, microvascular invasion, multinodular tumor, infiltration, macrovascular invasion, Child-Pugh score, model for end-stage liver disease score, portal hypertension, treatment prior to LT, LT technique and inside Milan criteria before and after LT were observed (Table 1). Significant differences were not found ($P$ = 0.20) in serum OGS concentrations in regard to the cause of death: 8 (61.5%) sepsis, 3 (23.1%) multiple organ failure, 1 (7.7%) recurrence of hepatitis C virus infection and 1 (7.7%) recurrence of HCC.

In logistic analysis, an association was found between serum OGS and 1-year LT mortality, controlling for serum caspase-3 and liver donor age [odds ratio = 2.079; 95% confidence interval (CI): 1.356-3.189; $P$ = 0.001] (Table 2). On the receiver operating characteristic analysis, the area under the curve of pre-LT serum OGS concentrations for predicting 1-year LT mortality was found to be 71% (95%CI: 55%-88%; $P$ = 0.009) (Figure 1).

Serum OGS levels with a cut-off point of 3.3 ng/mL showed a sensitivity of 69% (39%-91%), specificity of 66% (56%-74%), positive likelihood ratio of 2.1 (1.3-3.2), negative likelihood ratio of 0.5 (0.2-1.1), positive predictive value of 21% (14%-29%) and negative predictive value of 94% (88%-98%) for 1-year LT mortality prediction. The Kaplan-Meier survival analysis showed a higher 1-year LT mortality risk in patients with serum OGS levels prior to LT above 3.3 ng/mL (hazard ratio = 4.2; 95%CI: 1.36-13.11; $P$ = 0.01) (Figure 2).

**Table 1 Clinical and biochemical characteristics of 1-year liver transplantation survivor and non-survivor patients**

| | 1 yr survivor patients, *n* = 101 | 1 yr non-survivor patients, *n* = 13 | *P* value |
|---|---|---|---|
| Serum OGS (ng/mL)–median (p 25-75) | 2.80 (2.20-4.00) | 4.00 (2.70-10.25) | 0.01 |
| Age of liver recipient (yr)-median (p 25-75) | 58 (52-62) | 57 (55-63) | 0.61 |
| Serum alpha-fetoprotein (ng/dL)-median (p 25-75) | 7.4 (4.0-21.6) | 8.4 (4.3-130.5) | 0.62 |
| Protein (g/dL)-median (p 25-75) | 6.70 (6.10-7.10) | 6.70 (5.58-7.63) | 0.90 |
| Leukocytes count–median × $10^3/mm^3$ (p 25-75) | 4.57 (3.48-6.01) | 4.52 (3.27-7.77) | 0.89 |
| Albumin (g/dL)-median (p 25-75) | 3.29 (2.89-3.99) | 3.47 (3.14-3.93) | 0.45 |
| Creatinine (mg/dL)-median (p 25-75) | 0.90 (0.78-1.10) | 1.02 (0.75-1.10) | 0.27 |
| BMI ($kg/m^2$)-median (p 25-75) | 27.3 (24.3-29.7) | 28.7 (24.9-31.8) | 0.26 |
| Nodules size (cm)-median (p 25-75) | 2.9 (2.0-3.4) | 3.2 (1.8-4.9) | 0.40 |
| MELD score-median (p 25-75) | 15 (11-18) | 15 (13-17) | 0.77 |
| Age of liver donor (yr)-median (p 25-75) | 51 (35-62) | 62 (49-72) | 0.03 |
| Gender female, *n* (%) | 19 (18.8) | 0 | 0.12 |
| Child-Pugh score, *n* (%) | | | 0.06 |
| A | 46 (45.5) | 10 (76.9) | |
| B | 29 (28.7) | 3 (23.1) | |
| C | 26 (25.7) | 0 | |
| Infiltration, *n* (%) | 32 (31.7) | 3 (23.1) | 0.75 |
| Macrovascular invasion, *n* (%) | 4 (4.0) | 0 | 0.99 |
| Microvascular invasion, *n* (%) | 19 (18.8) | 2 (15.4) | 0.99 |
| Multinodular tumor, *n* (%) | 27 (26.7) | 4 (30.8) | 0.75 |
| Portal hypertension, *n* (%) | 64 (63.4) | 9 (69.2) | 0.77 |
| Treatment previously to LT, *n* (%) | 56 (55.4) | 8 (61.5) | 0.77 |
| PEI, *n* (%) | 26 (25.7) | 5 (38.5) | 0.33 |
| RFA, *n* (%) | 6 (5.9) | 0 | 0.99 |
| TACE, *n* (%) | 18 (17.8) | 3 (23.1) | 0.71 |
| Liver resection, *n* (%) | 3 (3.0) | 0 | 0.99 |
| Mixed treatment, *n* (%) | 3 (3.0) | 0 | 0.99 |
| Transplantation technique, *n* (%) | | | 0.99 |
| By-pass | 44 (43.6) | 6 (46.2) | |
| Piggy back | 57 (56.4) | 7 (53.8) | |
| Degree of tumor differentiation, *n* (%) | | | 0.11 |
| Well | 76 (75.2) | 11 (84.6) | |
| Moderate | 24 (23.8) | 1 (7.7) | |
| Poor | 1 (1.0) | 1 (7.7) | |
| Inside Milan criteria previously to LT, *n* (%) | 96 (95.0) | 12 (92.3) | 0.53 |
| Inside Milan criteria after LT, *n* (%) | 85 (84.2) | 10 (76.9) | 0.45 |

OGS: Oxidized guanine species; MELD: Model for end-stage liver disease; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation; LT: Liver transplantation; TACE: Transarterial chemoembolization; BMI: Body mass index.

**Table 2 Logistic regression analysis for the variables associated with 1-year liver transplantation mortality**

| | Odds ratio | 95%CI | *P* value |
|---|---|---|---|
| Age of liver donor (age) | 1.087 | 1.019-1.160 | 0.01 |
| Serum oxidized guanine species levels (ng/mL) | 2.079 | 1.356-3.189 | 0.001 |
| Serum caspase-3 levels (ng/mL) | 4.178 | 1.709-10.211 | 0.002 |

CI: Confidence interval.

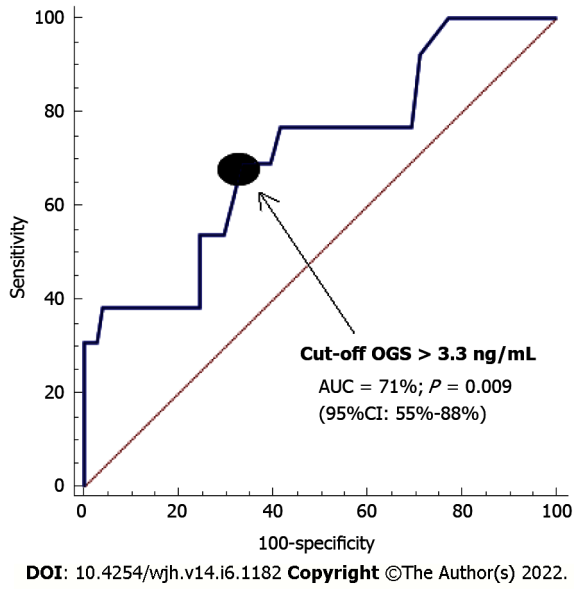


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**Figure 1 On the receiver operating characteristic analysis, the area under the curve of pre-liver transplantation serum oxidized guanine species concentrations for predicting 1-yr liver transplantation mortality was found to be 71% (95% confidence interval: 55%-88%; *P* = 0.009).** OGS: Oxidized guanine species; CI: Confidence interval; AUC: Area under the curve.

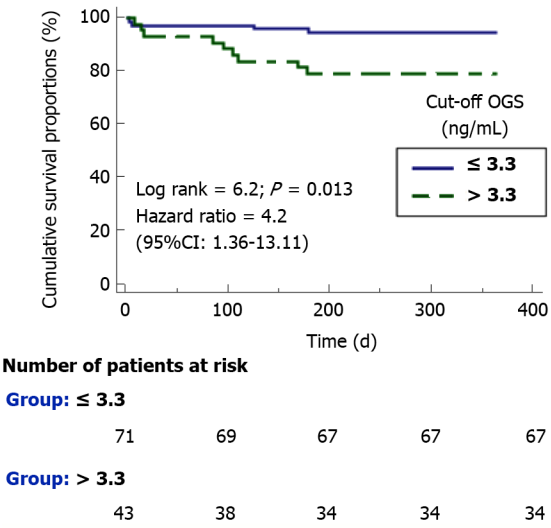


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**Figure 2 The Kaplan-Meier survival analysis showed a higher 1-yr liver transplantation mortality risk in patients with increased serum oxidized guanine species levels prior to liver transplantation (hazard ratio = 4.2; 95% confidence interval: 1.36-13.11; *P* = 0.01).** OGS: Oxidized guanine species; CI: Confidence interval.

## DISCUSSION

To our knowledge, our study is the first reporting data about the determination of DNA and RNA oxidative damage to predict prognosis of patients with HCC who underwent LT. The main finding was that high serum OGS prior to LT was associated with the mortality 1 year after LT. Greater oxidative DNA damage (assessed by 8-hydroxy-2′-deoxyguanosine concentration in liver biopsy specimens) has been found in patients with chronic liver disease with HCC compared to those without[18,19]. However, the association between serum OGS concentration and LT mortality is a new finding of our study.

These higher serum OGS levels found in non-surviving LT patients are in line with those found in patients with other diseases, such as sepsis[17], and could be in relation with a higher oxidative status that could favor multiple organ dysfunction and death of patients.

There were some limitations of our study. First, we have not determined serum 8-hydroxy-2′-deoxyguanosine change after LT to explore which is a better serum marker for prognosis (before or after LT). Second, we have not determined serum 8-hydroxy-2′-deoxyguanosine in healthy controls or chronic liver patients without HCC. However, the objective of our study was to determine whether patients with increased oxidative DNA and RNA damage before undergoing LT for HCC have poorer LT prognosis. Third, we have not determined other markers of oxidative stress for nucleic acids, such as abasic sites or 8-nitroguanosine 3′,5′-cyclic monophosphate. Fourth, we have not determined 8-hydroxy-2′-deoxyguanosine in the liver to explore its correlation with serum levels. Fifth, the regression analysis did not allow the introduction of more variables due to the low number of deceased patients. However, one strength of our study was that the association between mortality and serum OGS has been also previously found in patients with other diseases such as sepsis[17].

The possible contribution of an oxidative state in chronic liver disease progression and in hepatocarcinogenesis development has been suggested. In addition, the potential use of antioxidant agents in patients with chronic liver diseases has also been suggested[9-12]. Therefore, these preliminary results could induce studies to clarify the potential role of oxidative damage in the prognosis of LT patients due to HCC and to explore the use of antioxidant agents to reduce oxidative stress in those patients.

## CONCLUSION

The main new finding was that high serum OGS concentrations prior to LT were associated with mortality 1 year after LT in HCC patients.

## ARTICLE HIGHLIGHTS

### *Research background*

Oxidative damage of DNA and RNA has been associated with mortality of patients with various diseases.

### *Research motivation*

There is no published data on the potential use of DNA and RNA oxidative damage to predict the prognosis of patients with liver transplantation (LT) due to hepatocellular carcinoma (HCC).

### *Research objectives*

The aim in our study was to analyze the potential association between increased oxidative DNA and RNA damage before LT due to HCC and poorer LT prognosis.

### *Research methods*

In this observational, retrospective study, patients with HCC who underwent LT were included. Serum levels of all three oxidized guanine species (OGS) were measured prior to LT because guanine is the nucleobase with a higher risk of oxidation. LT mortality at 1 year was the end point of the study.

### *Research results*

Surviving patients ($n$ = 101) showed lower serum OGS levels ($P$ = 0.01) and lower age of liver donor ($P$ = 0.03) than non-surviving patients ($n$ = 13). An association between serum OGS prior to LT and 1-year LT (odds ratio = 2.079; 95% confidence interval: 1.356-3.189; $P$ = 0.001) was found in the logistic regression analysis.

### *Research conclusions*

The main new finding was that high serum OGS concentration prior to LT was associated with 1-year

LT mortality.

### *Research perspectives*

These preliminary results could induce studies to clarify the potential role of oxidative damage in the prognosis of LT patients due to HCC and to explore the use of antioxidant agents to reduce oxidative stress in those patients.

## FOOTNOTES

**Author contributions:** Lorente L was responsible for conception, design and coordination of the study, made substantial contributions to acquisition of data and analysis and interpretation of data and drafted the manuscript; Rodriguez ST, Sanz P, Portero J, Díaz D, González A, Martín MM, Cerro P, Portero J and Barrera MA made substantial contributions to acquisition of data and provided useful suggestions; González-Rivero AF and Pérez-Cejas A participated in blood determination levels; Jiménez A made substantial contributions to analysis and interpretation of data; All authors critically read and approved the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Institutional review board statement:** The Institutional Board of the Hospital Universitario Nuestra Señora de Candelaria (Santa Cruz de Tenerife, Spain) approved the study protocol.

**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 the authors report no relevant conflicts of interest for this article.

**Data sharing statement:** The datasets generated during the current study are available from the corresponding author on reasonable request.

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**Country/Territory of origin:** Spain

**ORCID number:** Leonardo Lorente 0000-0003-4902-4065; Sergio T Rodriguez 0000-0003-1635-3604; Pablo Sanz 0000-0003-4770-0111; Agustín F González-Rivero 0000-0002-9712-6990; Antonia Pérez-Cejas 0000-0002-8267-4556; Javier Padilla 0000-0001-8103-7267; Dácil Díaz 0000-0002-8836-9771; Antonio González 0000-0002-4180-0376; María M Martín 0000-0003-0239-873X; Alejandro Jiménez 0000-0001-8732-2616; Purificación Cerro 0000-0002-1270-5046; Julián Portero 0000-0001-9619-197X; Manuel A Barrera 0000-0001-8216-6212.

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ORIGINAL ARTICLE

Retrospective Study

# Direct-acting antivirals for hepatitis C virus-infected patients with hepatocellular carcinoma

Kazuto Tajiri, Hiroyuki Ito, Kengo Kawai, Yoshiro Kashii, Yuka Hayashi, Aiko Murayama, Masami Minemura, Terumi Takahara, Yukihiro Shimizu, Ichiro Yasuda

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**Kazuto Tajiri, Yuka Hayashi, Aiko Murayama, Masami Minemura, Terumi Takahara, Ichiro Yasuda,** Department of Gastroenterology, Toyama University Hospital, Toyama 930-0194, Japan

**Hiroyuki Ito,** Department of Gastroenterology, Takaoka Municipal Hospital, Takaoka 933-8550, Japan

**Kengo Kawai, Yukihiro Shimizu,** Gastroenterology Center, Nanto Municipal Hospital, Nanto 932-0211, Japan

**Yoshiro Kashii,** Department of Gastroenterology, Saiseikai Toyama Hospital, Toyama 931-8533, Japan

**Corresponding author:** Kazuto Tajiri, MD, PhD, Associate Professor, Department of Gastroenterology, Toyama University Hospital, 2630 Sugitani, Toyama 930-0194, Japan. tajikazu@med.u-toyama.ac.jp

## Abstract

***BACKGROUND***

Hepatocellular carcinoma (HCC) in hepatitis C virus (HCV)-infected patients has a high risk of recurrence. Although eradication of HCV is expected to reduce this risk, the risk in patients with a history of HCC may be high after treatment with direct-acting antivirals (DAAs).

***AIM***

To determine the risk factors for HCC recurrence in patients with HCV and a history of HCC.

***METHODS***

The risk of HCC recurrence in patients with a history of HCC and/or of HCC occurrence in patients without a history of HCC after DAA therapy was retrospectively analyzed in 311 HCV patients treated at our institution and several neighboring hospitals. The frequency and predictors of HCC recurrence/ occurrence after DAA treatment were included in these analyses. The clinical course of HCC before and after DAA treatment was also evaluated.

***RESULTS***

HCV patients with a history of HCC were older and had greater progression of

liver fibrosis and diabetes than patients without a history of HCC. Median recurrence-free survival (RFS) was 1092 d in patients with a history of HCC, and post-DAA HCC recurrence/occurrence was observed in 29 patients (53.7%) with and 5 (1.9%) without a history of HCC over 6 years ($P < 0.001$). RFS in patients with a history of HCC did not differ significantly before and after DAA treatment. The frequency of HCC recurrence/occurrence in patients with a history of HCC was lower after than before DAA treatment. Multivariate analysis showed that the incidence rate of HCC recurrence/occurrence before DAA treatment was the only independent predictor of HCC recurrence/occurrence after DAA treatment. Liver function was well preserved and clinical course was good in patients with HCC recurrence/occurrence after DAA therapy.

***CONCLUSION***

DAA therapy in patients infected with HCV is also effective in patients with a history of HCC. Curative treatment for HCC is desirable before DAA therapy. The frequency of HCC recurrence/occurrence before DAA therapy was associated with a significantly increased risk of HCC recurrence after DAA therapy. Careful observation after DAA therapy is required in patients with a history of HCC.

**Key Words:** Direct-acting antivirals; Hepatitis C virus; Hepatocellular carcinoma; Recurrence; Liver fibrosis; Curative treatment

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**Core Tip:** To estimate the therapeutic value of direct-acting antivirals (DAAs) in hepatitis C virus (HCV)-infected patients with a history of hepatocellular carcinoma (HCC), the clinical course of HCV patients with or without a history of HCC after DAA therapy was retrospectively analyzed. DAA treatment did not increase the incidence rate of HCC recurrence/occurrence or enhance malignant transformation of HCC in patients with a history of HCC. The risk of HCC recurrence after DAA therapy was significantly associated with the frequency of HCC recurrence/occurrence before DAA therapy.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies and a major cause of cancer-related deaths worldwide. Although HCC detected at an early stage can often be cured by surgical resection or local ablative therapy, HCC is often diagnosed at an advanced stage, precluding curative treatment and resulting in a high mortality rate[1]. Viral hepatitis is associated with the development of HCC, with hepatitis C virus (HCV) and hepatitis B virus (HBV) infections being major causes of HCC, along with nonviral etiologies such as alcoholic liver disease and nonalcoholic fatty liver disease[2]. HCV-related HCC often recurs after curative therapies for HCC, such as surgical resection or ablative therapies, with 5-year recurrence rates ranging from 60%-80%[3].

Interferon-based HCV eradication reduces the incidence rates of HCC[4]. The anti-HCV and anti-carcinogenic effects of interferon reduce liver inflammation, contributing to reductions in the rate of HCC recurrence/occurrence. It is unclear, however, whether HCV eradication with direct-acting antivirals (DAAs) increase the risk of HCC, as DAA treatment disrupts immune surveillance during rapid elimination of HCV[5]. Large-scale studies, however, have shown that DAA eradication of HCV increases the risk of HCC, whereas basal liver fibrosis is associated with the risk of HCC[6-8]. Because other studies have reported that DAA eradication results in malignant transformation, suggesting that DAA had adverse carcinogenic effects[5,9], these carcinogenic risks should be especially considered in patients with a history of HCC. The effects of DAA therapy have therefore been assessed in patients with a history of HCC. Studies have suggested that factors associated with pre-existing malignant potential, such as advanced liver fibrosis, high serum alpha-fetoprotein (AFP) concentration, and the presence of precancerous nodules, might lead to HCC recurrence in patients with a history of HCC[10-14].

This study retrospectively evaluated the risks of HCC recurrence/occurrence, defined as HCC recurrence in patients with a history of HCC and/or of HCC occurrence in those without a history of HCC, and the clinical course of HCC in HCV patients treated with DAA. The results of this study suggest that a history of HCC prior to DAA treatment is a major factor contributing to HCC recurrence/occurrence after DAA treatment.

## MATERIALS AND METHODS

### Patients

This study enrolled HCV patients treated with DAA at Toyama University Hospital, Takaoka Municipal Hospital, Nanto Municipal Hospital, and Saiseikai Toyama Hospital (all in Toyama, Japan) between November 2014 and July 2020. HCV infection was confirmed by HCV-RNA quantification and the genotype of HCV was determined in all patients. The fibrosis-4 (Fib-4) index, a useful noninvasive method of assessing liver fibrosis[15], was also evaluated in all patients. Liver cirrhosis was diagnosed by hepatologists, each with over 20 years' of experience, based on the results of imaging modalities such as ultrasonography (US), computed tomography (CT), and elastography, and the titers of fibrosis markers such as platelet count and Fib-4 index. HCC was diagnosed based on histological and/or imaging data such as contrast-enhanced CT or magnetic resonance imaging (MRI), according to the diagnostic criteria of the American Association for the Study of Liver Diseases[16]. Before DAA therapy, all patients were screened using US, CT, or MRI to rule out the presence of viable HCC. This multicenter study was performed in accordance with the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Toyama University (Approval No. R2019-131).

### Treatment with DAAs

Before the start of DAA therapy, patients with viable HCC were treated with surgery, radiofrequency ablation (RFA), or transarterial chemoembolization (TACE). Patients who did not show viable HCC lesions on contrast-enhanced CT or MRI performed 1 to 3 mo after HCC treatment were considered eligible for DAA therapy. Treatment regimens were determined by hepatologists according to HCV treatment guidelines[17,18]. Treatment regimens included daclatasvir plus asunaprevir (DCV + ASV) in patients with HCV genotype 1b from 2014 to 2016; sofosbuvir plus ledipasvir (SOF + LDV) for patients with HCV genotypes 1b and 2a/2b from 2015 to 2020; SOF plus ribavirin (SOF + Rib) for patients with HCV genotypes 2a/2b from 2015 to 2017; and glecaprevir and pibrentasvir (GLE + PIB) for patients with any HCV genotype from 2017 to 2020. Other regimens considered included ombitasvir, paritaprevir, and ritonavir from 2016 to 2017; elbasvir plus grazoprevir in 2017; and SOF plus velpatasvir from 2019 to 2020 depending on the patient's condition and the timing of treatment. Patients were monitored every 4 wk during DAA treatment, and every 12 wk thereafter, with HCC evaluated by imaging modalities. A sustained viral response (SVR) was defined as complete clearance of HCV-RNA clearance 12 wk after the end of DAA treatment. The flow chart of this study is shown in Supplementary Figure 1. Patients were monitored for a median 1311 d (range: 28 d to 2231 d) after the end of DAA therapy.

### HCC Treatment

HCC treatment in each patient was determined by discussions among surgeons, hepatologists, and radiologists at each institution and was based on Japanese practice guidelines for HCC[19]. Treatments of patients with early-stage HCC included surgical resection or RFA. Treatments of patients with multiple HCCs included TACE or systemic chemotherapy such as sorafenib, according to liver function and tumor progression and following treatment guidelines.

### Statistical analyses

Variable distributions were reported as mean ± SD. Categorical variables were compared by the Fisher's exact test. Continuous variables were compared by the Student's *t*-test or the Mann-Whitney U test. Survival was evaluated using the Kaplan-Meier method, with differences in survival curve compared by log-rank tests. The incidence rates of HCC recurrence/occurrence were reported as person-years. All statistical analyses were performed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, United States), with $P < 0.05$ considered statistically significant.

## RESULTS

### Patients and recurrence/occurrence of HCC

A total of 311 patients, 143 (46.0%) men and 168 (54.0%) women, were included in this study (Table 1). Of these 311 patients, 87 (28.0%) had cirrhosis, 229 (73.6%) were infected with HCV genotype 1b, and 53 (17.0%) had a previous history of HCC. Their mean Fib-4 index was 3.87 ± 3.24 and their mean AFP concentration was 12.0 ± 35.2 ng/mL. The 53 patients with a history of HCC were significantly older

**Table 1 Characteristics of patients**

| | Overall | With HCC | Without HCC | *P* value[1] |
|---|---|---|---|---|
| Case | 311 | 53 | 258 | |
| Age in yr | 68.1 ± 13.5 | 75.8 ± 6.7 | 66.5 ± 14.1 | < 0.01 |
| Male/Female | 143/168 | 27/26 | 116/142 | 0.45 |
| Diabetes, yes/no | 47/264 | 19/34 | 28/230 | < 0.01 |
| Habitual alcohol use[2], yes/no | 56/255 | 12/41 | 44/214 | 0.33 |
| Liver cirrhosis, yes/no | 224/87 | 18/35 | 52/206 | < 0.01 |
| Genotype, 1b/2a, 2b/others | 229/73/10 | 45/7/1 | 183/66/9 | 0.04 |
| Alb in g/dL | 3.9 ± 0.4 | 3.5 ± 0.4 | 4.0 ± 0.4 | < 0.01 |
| ALT in U/L | 44.3 ± 45.0 | 40.3 ± 22.0 | 45.1 ± 48.3 | 0.48 |
| Plt as × $10^4$/μL | 16.0 ± 5.9 | 12.9 ± 5.6 | 16.7 ± 5.8 | < 0.01 |
| Fib-4 index | 3.87 ± 3.24 | 6.27 ± 4.64 | 3.37 ± 2.60 | < 0.01 |
| AFP in ng/mL | 12.0 ± 35.2 | 23.7 ± 52.4 | 9.4 ± 29.6 | 0.047 |

[1]Statistical significance set up as $P < 0.05$ as compared between with HCC *vs* without HCC.
[2]Habitual alcohol use is defined as daily alcohol consumption of > 20 g a woman or > 30 g for a man.
Alb: Albumin; AFP: Alpha fetoprotein; ALT: Alanine aminotransferase; Fib-4: Fibrosis-4; HCC: Hepatocellular carcinoma; Plt: Platelet.

(75.6 years *vs* 66.5 years; $P < 0.01$) than the 258 patients with no history of HCC. The rates of diabetes, a risk factor for HCC after DAA treatment[20] (35.8% *vs* 3.1%; $P < 0.01$) and liver cirrhosis (34.0% *vs* 20.2%; $P < 0.01$) were significantly higher, whereas the rates of HCV genotype 2 (13.2% *vs* 25.6%; $P = 0.04$) were significantly lower, in patients with than without a history of HCC. In addition, serum albumin concentrations (3.5 g/dL *vs* 4.0 g/dL; $P < 0.01$) and platelet counts (12.9 × $10^4$/mL *vs* 16.7 × $10^4$/mL; $P < 0.01$) were significantly lower, whereas Fib-4 index (6.27 *vs* 3.37; $P < 0.01$) and AFP concentrations (23.7 ng/mL *vs* 9.4 ng/mL; $P = 0.047$) were significantly higher in patients who had a previous history of HCC. Of the 311 patients, 56 (21.9%) had a history of habitual alcohol use, but these rates did not differ significantly in patients with and without a history of HCC. Thus patients with a history of HCC were older and had more advanced liver fibrosis progression and diabetes than patients without a history of HCC.

### Treatment with DAA

Patients infected with HCV genotype 1b were administered DCV + ASV, SOF + LDV, GLE + PIB, or other regimens in accordance with contemporary guidelines. Similarly patients infected with HCV genotypes 2a/2b were administered SOF + Rib, SOF + LDV, GLE + PIB, or other regimens; and patients with other genotypes such as genotypes 3a/3b/4s were administered GLE + PIB. SVR was achieved by 52 (98.1%) of the 53 patients with and by 250 (96.9%) of the 258 patients without a history of HCC ($P = 1.00$). Several patients who did not initially achieve SVR were switched to another DAA regimen, with SVR achieved in all treated patients. Post-DAA treatment AFP levels were higher in patients with, than without, a history of HCC history, both at end of treatment and SVR, but these concentrations were lower than those before DAA therapy (Table 2).

### HCC after DAA-therapy

Following DAA therapy, HCC recurrence/occurrence was found in 29 patients (53.7%) with and 5 (1.9%) without a history of HCC, with 3-year incidence rates of 50.9% (27/53) and 1.2% (3/258), respectively. Median recurrence-free survival (RFS) in patients with a history of HCC was 1092 d, whereas none of those without a history of HCC died during the 6-year study period ($P < 0.001$; Figure 1A).

### HCC before and after DAA treatment

HCC recurrence and other parameters before and after DAA therapy were compared in patients with a history of HCC. Median RFS did not differ significantly in patients with HCC recurrence before and after DAA therapy [1293 d (range 554-2032 d) *vs* 1053 d (range 741-1443 d); $P = 0.884$) (Figure 2A), with incidence rates of HCC recurrence of 1/1.25 and 1/2.99 person-years, respectively (Figure 2B). HCV clearance induced by DAA treatment did not increase HCC recurrence rate. Univariate analysis showed that AFP concentration at SVR and frequency of HCC recurrence before DAA treatment were risk factors for HCC recurrence after DAA treatment, whereas multivariate analysis showed that only the

**Table 2 Treatment with direct-acting antivirals**

| Regimens | With HCC | Without HCC |
|---|---|---|
| DCV + ASV/SOF + LDV/SOF + Rib/GLE + PIB/others | 13/24/6/6/4 | 45/103/35/58/20 |
| SVR at 12 wk post-treatment, yes/no | 52/1 | 250/8 |
| AFP at EOT in ng/mL | 7.64 ± 6.81[a] | 3.93 ± 3.99 |
| AFP at SVR in ng/mL | 6.60 ± 6.27[a] | 3.68 ± 2.64 |

[a]$P < 0.05$ as compared between with HCC *vs* without HCC.

AFP: Alpha fetoprotein; ASV: Asunaprevir; EOT: End of treatment; DCV: Daclatasvir; GLE: Glecaprevir; HCC: Hepatocellular carcinoma; LDV: Ledipasvir; PIB: Pibrentasvir; Rib: Ribavirin; SOF: Sofosbuvir; SVR: Sustained viral response.

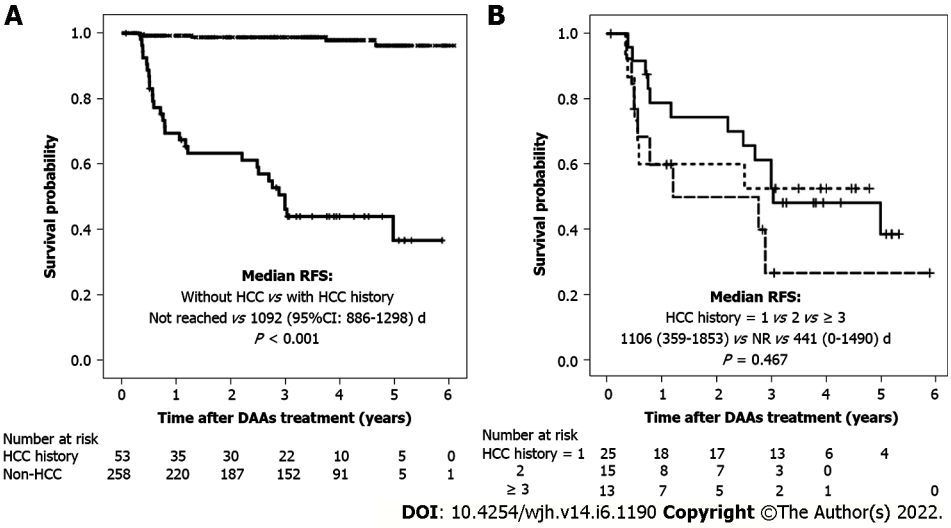


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**Figure 1 Kaplan-Meier analysis.** A: Hepatocellular carcinoma (HCC) recurrence/occurrence after direct-acting antiviral (DAA) treatment of patients with (solid line) and without (dotted line) a history of HCC; B: Kaplan-Meier analysis of HCC events after DAA treatment in patients with 1 (solid line), 2 (dotted line), and ≥ 3 (dashed line) HCC events before DAA treatment. Numbers in parenthesis = 95% confidence interval. NR: Not reached; RFS: Recurrence-free survival; CI: Confidence interval; HCC: Hepatocellular carcinoma.

frequency of HCC recurrence before DAA treatment was an independent predictor of HCC recurrence after DAA treatment (Table 3). Only a history of HCC before DAA treatment contributed to the risk of HCC recurrence after DAA treatment, whereas HCV clearance by DAA alone did not. The 1-year rates of HCC recurrence after DAA treatment in patients with 1, 2, and ≥ 3 HCC events before DAA treatment were 28%, 40% and 38.5%, respectively (Figure 1B).

### *Clinical course after HCC recurrence*

All 29 patients with a history of HCC who experienced HCC recurrence after DAA therapy had been treated according to HCC treatment guidelines[19]. Six and seventeen of these patients underwent surgical resection and RFA, respectively. Multiple recurrences were observed in 6 patients, including one with portal invasion. These 6 patients were subsequently treated with TACE, hepatic artery infusion chemotherapy, or sorafenib. Two died due to advanced HCC, with survival times following DAA therapy completion of 49.7 and 52.6 mo, respectively.

## DISCUSSION

This study found that DAA-induced eradication of HCV did not increase the risk of HCC recurrence, with multivariate analysis showing that a prior history of HCC was the only independent factor predicting the risk of HCC recurrence after DAA therapy. DAA treatment, however, did not worsen the clinical course of subsequent HCC events. Rather, liver reserve function was preserved following DAA treatment, allowing curative and continuous treatment of HCC. Although malignant transformation after DAA treatment has been reported[5,9], this study found that DAA therapy itself was not the causal agent.

**Table 3 Univariate and multivariate analyses for hepatocellular carcinoma recurrence after direct-acting antiviral treatment**

| Factors | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* value | HR | 95% CI | *P* value |
| Age | 0.98 | 0.92-1.04 | 0.52 | | | |
| CH or LC | 0.50 | 0.21-1.21 | 0.12 | | | |
| Diabetes | 1.12 | 0.79-1.59 | 0.53 | | | |
| Habitual alcohol use | 1.08 | 0.68-1.51 | 0.51 | | | |
| Fib-4 | 1.01 | 0.93-1.09 | 0.86 | | | |
| AFP at baseline | 1.01 | 1.00-1.01 | 0.22 | | | |
| AFP at EOT | 1.09 | 1.00-1.19 | 0.05 | 1.10 | 1.00-1.01 | 0.05 |
| AFP at SVR | 1.01 | 1.00-1.01 | 0.04 | 1.01 | 1.00-1.01 | 0.08 |
| Duration between first HCC and DAAs treatment | 1.00 | 1.00-1.00 | 0.18 | | | |
| Number of HCC occurrence | 1.32 | 1.06-1.64 | 0.02 | 1.61 | 1.18-2.19 | < 0.01 |

AFP: Alpha fetoprotein; CH: Chronic hepatitis; CI: Confidence interval; DAAs: Direct-acting antivirals; EOT: End of treatment; HCC: Hepatocellular carcinoma; HR: Hazard ratio; LC: Liver cirrhosis; SVR: Sustained viral response.

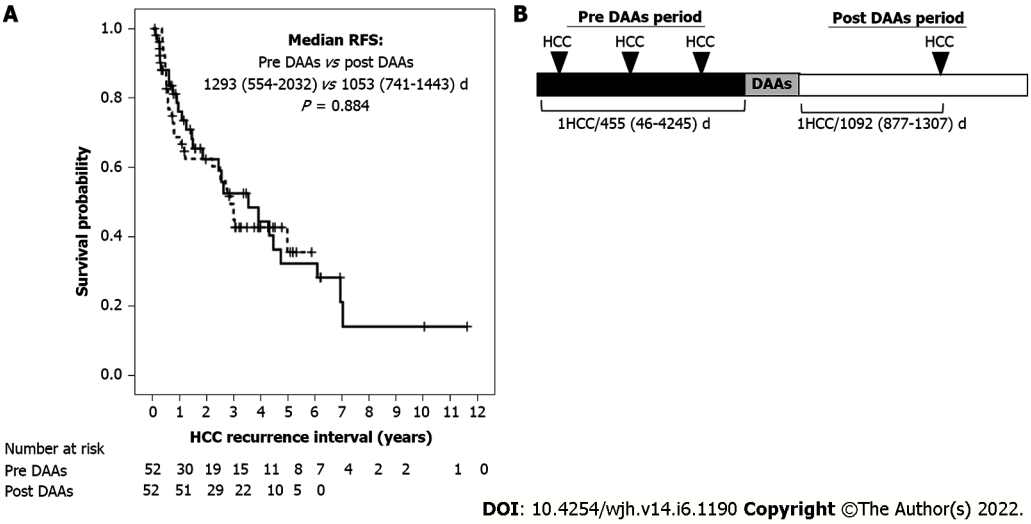


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**Figure 2 Median recurrence-free survival in patients with hepatocellular carcinoma recurrence before and after direct-acting antivirals treatment.** A: Kaplan-Meier analysis of hepatocellular carcinoma (HCC) recurrence before (solid line) and after (dotted line) direct-acting antiviral (DAA) treatment in patients with a history of HCC; B: Schema of HCC events. Solid triangle = one event. Black bar = period in days. Numbers in parenthesis = 95% confidence interval. RFS: Recurrence-free survival; HCC: Hepatocellular carcinoma; DAA: Direct-acting antiviral.

In this study, SVR rates in DAA-treated patients were similar in those with (98.1%) and without (96.9%), a previous history of HCC. Systematic reviews, however, have reported lower SVR rates in patients with a history of HCC[21]. This study found that treatment with DAAs was highly effective in eradicating HCV in patients with a history of HCC, despite their being older and more likely to have liver fibrosis and diabetes mellitus than patients without a history of HCC. DAAs are also effective in patients with advanced HCC[22-24]. HCV eradication by DAAs ameliorates liver inflammation and suppresses liver fibrosis progression, preserving or improving liver function. Since the introduction of DAAs as treatment for HCV, mortality rates in patients with HCV-associated HCC have improved compared with mortality rates in patients with HBV-related and nonviral HCC[25]. These findings suggest that HCV eradication might prolong overall survival in patients with HCV-related HCC.

Although HCV eradication by DAAs has been suggested to increase the subsequent risk of HCC, most studies have found that preexisting risk factors for HCC development were present at the time of DAA initiation. The progression of liver fibrosis and the presence of cirrhosis have been shown to be associated with HCC development[6-8]. Chronic HCV infection leads to the progression of liver fibrosis,

the factor that contributes most to HCC development through various epigenetic changes and the creation of a microenvironment favorable to carcinogenesis[26]. The risk of HCC recurrence/occurrence after DAA treatment was shown to be higher in patients with than without advanced liver fibrosis[27], suggesting that earlier achievement of SVR before the development of fibrosis may reduce the likelihood of HCC recurrence/occurrence.

Serum AFP concentration has also been found to predict HCC development[10,13]. Higher AFP concentration is a major biomarker for HCC occurrence after SVR[28,29], as well as being associated with liver inflammation, making AFP concentration at the end of treatment very important[30]. AFP concentrations before and after DAA treatment should therefore be measured to estimate the risk of HCC recurrence/occurrence. Another factor associated with HCC development is the presence of preexisting hepatic nodules[14]. Although all patients in the present study who were treated with DAAs were evaluated by imaging modalities, some did not undergo enhanced CT or MRI. Thus, the exact proportion of patients with dysplastic nodules was unclear. For example, a patient found to have a 1.5 cm dysplastic nodule in the liver on ethoxybenzyl-diethylenetriamine pentaacetic acid enhanced (EOB)-MRI developed HCC from the dysplastic nodule 3-years after DAA completion, akin to hypervascular transformation of 9 mm hypovascular nodules with a 3-year incidence rate of 30%[31]. Certain types of DAAs, such as SOF and DCV, were found to have greater oncogenic potential through off-target DAA effects[32]. In the present study, HCC recurrence/occurrence was not frequent in patients treated with SOF or DCV (data not shown).

Collectively, DAA treatment was effective in patients with a history of HCC, as shown by their high SVR rates. DAAs eliminated hepatic inflammation and suppressed the progression of hepatic fibrosis, leading to preserved liver function. Improvement or preservation of liver function provides benefits in the management of HCC. Further prospective studies are required to evaluate the risk of DAA-associated transformation of precancerous lesions to HCC and the effects of specific DAAs on the risks of HCC recurrence/occurrence.

Multivariate analysis of patients in the present study also found that liver fibrosis, diabetes mellitus, and serum AFP concentration before DAA treatment were unassociated with HCC recurrence/occurrence after DAA treatment. Rather, the only factor significantly associated with HCC recurrence/occurrence after DAA treatment was history of prior HCC events. DAA treatment has been reported effective in patients with multiple prior courses of HCC recurrence[33], suggesting the need for careful screening for HCC before DAA treatment of patients with a history of HCC, as well as diligent follow-up of these patients after DAA therapy. Estimating the risk of HCC after DAA treatment is important, with the degree of liver fibrosis predicting the risk HCC recurrence[34,35]. A previous history of HCC and stratification by the Fib-4 index can be used to construct a novel predictive model for HCC development after DAA treatment[36]. The need for careful screening and follow-up in patients with a history of HCC increases with the number of times patients have experienced HCC recurrence.

This study had several limitations. First, its retrospective design precluded accurate determination of the effects of DAA treatment on the risks of HCC recurrence/occurrence. Second, the number of patients included in the present study, especially of those with a history of multiple HCC events, was relatively small. Third, not all patients underwent EOB-MRI, preventing actual determination of their HCC or non-HCC status. Although all underwent enhanced CT or US performed by experienced hepatologists rather than EOB-MRI, further studies are required to evaluate precancerous lesions and HCC more precisely. In addition, other risk factors for HCC, including tobacco use, obesity, and metabolic diseases, were not analyzed.

## CONCLUSION

DAA treatment of HCV-infected patients can also preserve liver function in patients with HCC. Curative treatment of HCC is desirable before DAA therapy. A history of multiple courses of HCC events before DAA treatment significantly increases the risk of HCC recurrence. Careful HCC screening prior to DAA treatment and thorough follow-up observation after DAA treatment is recommended in such patients.

## ARTICLE HIGHLIGHTS

### *Research background*

Treatment with direct-acting antivirals (DAAs) has provided many benefits to hepatitis C virus (HCV)-infected patients. Hepatocellular carcinoma (HCC) development after treatment with DAAs remains a serious issue.

### *Research motivation*

The effect of DAA treatment on the risk of HCC development is an important clinical question.

### *Research objectives*
To clarify the risk of HCC development after DAA treatment in patients HCV-infected patients at high risk for HCC development.

### *Research methods*
HCC occurrence after DAA treatment was retrospectively evaluated in patients with and without a history of HCC.

### *Research results*
The frequency of HCC recurrence/occurrence was similar before and after treatment with DAAs. The number of HCC occurrences before DAA treatment was an independent risk factor for HCC recurrence/occurrence.

### *Research conclusions*
HCV-infected patients with a history of multiple HCCs should be monitored carefully for HCC recurrence.

### *Research perspectives*
An effective screening method should be established for patients at high risk of HCC recurrence/occurrence.

## FOOTNOTES

**Author contributions:** Tajiri K designed and performed the research and wrote the paper; Ito H, Kawai K, Murayama A, Hayashi Y, Minemura M, Takahara T, and Shimizu Y contributed to the management of patients; Shimizu Y and Yasuda I supervised the work.

**Institutional review board statement:** This study was reviewed and approved by the ETHICs Committee of Toyama University.

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient had agreed to treatment with confirmed written consent.

**Conflict-of-interest statement:** All the authors report no relevant conflicts of interest for this article.

**Data sharing statement:** No additional data are available.

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**Country/Territory of origin:** Japan

**ORCID number:** Kazuto Tajiri 0000-0002-2373-8601; Hiroyuki Ito 0000-0001-7356-8527; Kengo Kawai 0000-0003-3465-1293; Yoshiro Kashii 0000-0002-4813-9699; Yuka Hayashi 0000-0003-4992-1161; Aiko Murayama 0000-0003-1084-3012; Masami Minemura 0000-0001-7489-7955; Terumi Takahara 0000-0002-2363-671X; Yukihiro Shimizu 0000-0002-8216-9269; Ichiro Yasuda 0000-0002-6888-0310.

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ORIGINAL ARTICLE

Retrospective Study

# Use of doppler ultrasound to predict need for transjugular intrahepatic portosystemic shunt revision

Nikki Duong, Marcus Healey, Kunal Patel, Brian J Strife, Richard K Sterling

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**Nikki Duong, Richard K Sterling,** Division of Gastroenterology, Hepatology, and Nutrition, Virginia Commonwealth University Medical Center, Richmond, VA 23219, United States

**Marcus Healey,** Department of Internal Medicine, Virginia Commonwealth University Medical Center, Richmond, VA 23219, United States

**Kunal Patel, Brian J Strife,** Division of Interventional Radiology, Virginia Commonwealth University Medical Center, Richmond, VA 23219, United States

**Corresponding author:** Nikki Duong, MD, Academic Fellow, Division of Gastroenterology, Hepatology, and Nutrition, Virginia Commonwealth University Medical Center, 1200 E Broad Street, Richmond, VA 23219, United States. nduong91@gmail.com

## Abstract

### *BACKGROUND*

Transjugular intrahepatic portosystemic shunt (TIPS) is used to treat complications of portal hypertension, such as ascites and variceal bleeding (VB). While liver doppler ultrasound (DUS) is used to assess TIPS patency, trans-shunt venography (TSV) is the gold standard.

### *AIM*

To determine the accuracy of DUS to assess TIPS dysfunction and for need for revision.

### *METHODS*

Retrospective review of patients referred for TIPS revision from 2008-2021. Demographics, DUS parameters at baseline and at the DUS preceding TIPS revision, TSV data were collected. Receiver operating characteristics curves, sensitivity, specificity, performance for doppler to predict need for revision were performed. Univariate and multivariate analyses were used to predict clinical factors associated with need for TIPS revision.

### *RESULTS*

The cohort consisted of 89 patients with cirrhosis (64% men, 76% white, 31% alcohol as etiology); median age 59 years. Indication for initial TIPS were VB (41%), refractory ascites (51%), and other (8%). TIPS was revised in 44%. On univariate analysis, factors associated with need for TIPS revision were male ($P$ = 0.03), initial indication for TIPS ($P$ = 0.05) and indication for revision ($P$ = 0.01).

Revision of TIPS was associated with lower mortality (26% *vs* 46%) and significantly lower rates of transplant (13% *vs* 24%; $P = 0.1$). In predicting need for TIPS revision, DUS has a 40% sensitivity, 45% specificity, PPV 78%, and NPV 14%. The most accurate location for shunt velocity measure was distal velocity (Area under the curve: 0.79; $P = 0.0007$).

*CONCLUSION*
DUS has poor overall sensitivity and specificity in predicting need for TIPS revision. Non-invasive methods of predicting TIPS dysfunction are needed since those needing TIPS revision had better survival.

**Key Words:** Transjugular intrahepatic portosystemic shunt; Doppler ultrasound; Portal hypertension

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**Core Tip:** Transjugular intrahepatic portosystemic shunt (TIPS) is used to treat complications of portal hypertension, however methods to assess TIPS patency are highly variable. Herein, we present a retrospective review of patients referred for TIPS revision from 2008-2021, and demonstrate that doppler ultrasound has poor overall sensitivity and specificity in predicting need for TIPS revision. Non-invasive methods of predicting TIPS dysfunction are needed since those needing TIPS revision had better survival.

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## INTRODUCTION

Complications of cirrhosis can include ascites and variceal bleeding due to portal hypertension. When ascites and variceal bleeding are refractory to diuretics and endoscopic therapy, respectively, transjugular intrahepatic portosystemic shunt (TIPS) can be considered. Since 1989, TIPS has been used for complications of portal hypertension with high clinical and technical success rates[1]. Formerly, bare metal stents were used and propone to dysfunction from narrowing[2]. However, patency rates have increased over the past 20 years due to the advent of available expandable polytetraflouroethylene (ePTFE) covered stents[3]. With ePTFE stents, TIPS patency rates have improved significantly, with studies showing 93% and 75.9% patency at 1 and 3 years, respectively[4]. However, TIPS dysfunction, including occlusion, stenosis, and encephalopathy still occur and are potentially deleterious complications.

Though there are no guidelines to suggest optimal timing of TIPS surveillance or thresholds for shunt dysfunction, clinical symptoms such as recurrence of ascites or variceal bleeding should prompt investigation. TIPS dysfunction due to stenosis is defined as greater than 50% reduction in lumen diameter on angiography or porto-systemic gradient (PSG), above 12 mmHg[5]. Currently, the gold standard is trans-shunt venography (TSV); however, this test is costly and invasive. Though isotope studies, computed tomography (CT), magnetic resonance imaging have been used as non-invasive methods of evaluating TIPS, doppler ultrasound (DUS) is the most widely accepted method[1]. The direction of blood flow can be cephalic (toward the heart) *vs* caudal (away from the heart) with the side of the TIPS closest to the heart termed the distal, cephalic, or hepatic vein end, whereas the proximal side has been deemed caudal, or portal vein end.

Though easily accessible, the utility of DUS to assess need to TIPS revision is poorly defined. The variability of results could be explained by the absence of a consensus definition of shunt dysfunction, differences in doppler measurements and the small number of patients reported in these case series. To our knowledge, there are limited prospective studies assessing the accuracy of DUS for assessing TIPS dysfunction. Although several studies have attempted to identify the optimal cut-off for TIPS dysfunction, there remains a significant amount of variability in terms of accuracy. Some argue that a lower limit of normal shunt velocity should be used. On the contrary, assuming that focal stenosis could lead to higher velocities at the stenotic level (a.k.a. Bernoulli's principle), one could seek an upper limit of normal as well. Additionally, others have used the main portal vein velocity, or the difference between the maximum and minimum peak intra-stent velocities as indicators of malfunction[6-9].

To address this gap in knowledge, our aim was to determine the accuracy of DUS in assessing the need for TIPS revision using clinical and predictive factors.

## MATERIALS AND METHODS

A retrospective study at a tertiary academic medical center that performs liver transplantation was performed under IRB approval. Adult patients from January 2008 to January 2021 who underwent TIPS revision were identified and reviewed. The patient's electronic medical records were reviewed for demographic, clinical, and radiologic information at the time of TIPS revision. Of 100 patients identified, 11 were excluded; 9 for TIPS revision for worsening hepatic encephalopathy not based on DUS, and 2 for incomplete data. Therefore, 89 subjects were included in the final analysis.

Information on demographics (age, race), indication for initial TIPS (recurrent ascites or variceal bleeding, abnormal DUS), Model for End-Stage Liver Disease (MELD) at time of TIPS placement and at time of revision, DUS parameters (proximal, mid, distal velocities), TSV specificities (mm dilation, PSG before and after TIPS revision), presence or absence of stenosis, need for intervention, and clinical outcomes (death, transplant) were included.

Baseline TIPS patency at our institution was assessed by performing DUS 2-4 wk after TIPS placement, 6 and 12 mo after TIPS placement, and thereafter, assessed at routine HCC screening surveillance intervals. Additionally, assessment of TIPS patency was pursued if there are clinical signs of portal hypertension (*i.e.,* recurrent ascites or variceal bleeding). The abnormal flow rates during TSV that led to a venography study are reported as "at revision," whereas the baseline flow rates from the penultimate doppler preceding the venography are reported as "pre-TIPS baseline." The normal range of flow is 90-190 cm/second; any gradient change of greater than 50 cm/second across the stent is considered abnormal and concerning for stenosis. TIPS venographic abnormalities included shunt occlusion, shunt stenosis, and/or elevation of the portosystemic gradient above 12mmHg. If present, the shunt was revised.

Descriptive statistics were used to describe the cohort. Mean (standard deviation, SD) and medians (interquartile range, IQR) were used for normalized and non-normalized data, respectively and compared by student's t-test or Wilcoxon rank sum. Proportions were compared by chi-square or Fisher's exact test as appropriate. Receiver operating characteristics curves, sensitivity, specificity, and performance of DUS to predict need for TIPS revision were performed. Univariate and multivariate analyses were used to predict clinical factors associated with need for TIPS revision.

## RESULTS

The cohort consisted of 89 patients with cirrhosis (64% men, 76% white), with age range from 51-62 (median age of 59 years). The etiology of liver disease was alcohol (31%), hepatitis C virus (16%), non-alcoholic steatohepatitis (20%), other (30%). Indication for initial TIPS were refractory variceal bleeding (41%), refractory ascites (51%), and other causes (8%) (Table 1).

The mean MELD at initial TIPS was 16.6 (SD: 6.1), PSG 15.5 mmHg (millimeters of mercury) (SD: 4.5) pre-TIPS and 6.17 mmHg (SD: 2.54), post-TIPS. The mean TIPS diameter was 8.41 (SD: 0.91) mm. The median of days to TIPS revision was 311 (54-661).

TIPS revision was prompted by either 1) clinical factors such as recurrent ascites (23%), or 2) an abnormal flow noted on the doppler ultrasound which was performed as part of our patency assessment protocol in clinic. Therefore, referral to interventional radiology for TIPS assessment were due to high doppler velocity (indicative of early TIPS dysfunction) in 23%, low velocity suggestive of late dysfunction in 51%, or clinical factors such as recurrent ascites in 23% (Table 2). Overall, 82% of the dopplers had abnormal flow. Fourty-four percent had true stenosis that required revision of TIPS; however, during venography, 56% of patients who were referred for revision, had widely patent TIPS.

MELD at TIPS revision was 15.5 (SD: 6.8). Among those undergoing TIPS revision ($n$ = 39) followed a median 1503 (IQR 663-2491) days, 13% underwent subsequent liver transplant and 26% died, therefore, the transplant free survival was 61%. Those that underwent TIPS revision had higher transplant free survival (Figure 1) ($P$ = 0.14).

On univariate analysis, factors associated with need for TIPS revision were male gender ($P$ = 0.026), initial indication for TIPS ($P$ = 0.05) and indication for TIPS revision ($P$ = 0.006). On multivariate analysis, only gender was associated with TIPS revision ($P$ = 0.023). While TIPS flow in the proximal TIPS at the baseline doppler was lower in the revision group than in the non-revision group ($P$ = 0.04), TIPS flow was lower at the time of revision at all parts of the stent (all $P$ < 0.01).

DUS has a 40% sensitivity, 45% specificity, PPV 78%, and NPV 14% of predicting TIPS stenosis or occlusion requiring intervention. In order to calculate these statistical values, we compared whether or not the DUS was abnormal *vs* if TIPS revision was performed by radiology. The most accurate location for shunt velocity measure was distal velocity [Area under the curve (AUC): 0.79; $P$ = 0.0007] (Figure 2), compared to proximal (AUC 0.65) and mid (AUC 0.71) velocities (Tables 3 and 4). A distal flow value of 114 cm/s or less had 77% sensitivity, 70% specificity, PPV 60%, NPV 84% for predicting need for revision.

**Table 1 Characteristics of the cohort**

| Characteristics | Mean (SD) | Median (IQR) |
|---|---|---|
| Age ($n$ = 89) | 56.5 (9.8) | 59 (51-62) |
| % male | 64 | |
| % White/Black/other | 76/18/3 | |
| Etiology of Liver disease % | | |
| EtOH | 31 | |
| HCV | 16 | |
| NASH | 20 | |
| Other | 30 | |
| Indication for TIPS % | | |
| VB | 41 | |
| Refractory ascites | 51 | |
| other | 8 | |
| MELD at initial TIPS | 16.6 (6.1) | |
| PSG before TIPS mmHg | 15.5 (4.5) | 15 (12.5-18) |
| PSG after TIPS mmHg | 6.17 (2.54) | 6 (4-8) |
| TIPS dilation mm | 8.41 (0.91) | |
| TIPS revision (d) | 514 (670) | 311 (54-661) |
| Indication for revision (% doppler) | 74 | |
| MELD at revision | 17.3 (6.8) | |
| Doppler abnormal % | 82 | |
| High vel/low vel/clinical % | 36/31/30 | |
| Doppler flow at revision Doppler prox cm/s | 122 (58) | 127 (77-169) |
| Doppler flow at revision doppler mid | 135 (73) | 140 (77-185) |
| Doppler flow at revision Doppler distal | 141 (103) | 122 (57-205) |
| TIPS occluded % | 10 | |
| Doppler flow pre-TIPS baseline prox cm/s | 125 (43) | 122 (100-146) |
| Doppler flow pre-TIPs baseline mid | 133 (42) | 140 (109-161) |
| Doppler flow pre-TIPS baseline distal | 128 (52) | 128 (89-155) |
| Change prox | 45 (36) | 36 (12-80) |
| % change prox | -0.01 (.47) | -0.03 (-0.253-0.312) |
| Change mid | 55 (50) | 45.5 (16.9-74.2) |
| % change mid | 0.11 (0.66) | 1 (-20-33) |
| Change distal | 69 (76) | 48.7 (20-92) |
| % change distal | 0.1 (0.89) | -12% (-45-38) |
| PSG pre TIPS revision mmHg | 14 (12) | 12 (9-15) |
| PSG after TIPS revision mmHg | 8.32 (3.7) | 8 (6-10) |
| Outcome (Alive/LT/Dead) % | 49/13/37 | |
| TIPS stenosis (Y) % | 43% | |
| TIPS revised | 44% | |

SD: Standard deviation; IQR: Interquartile range; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; TIPS: Transjugular intrahepatic

portosystemic shunt; VB: Variceal bleeding; MELD: Model for End-Stage Liver Disease; PSG: Porto-systemic gradient.

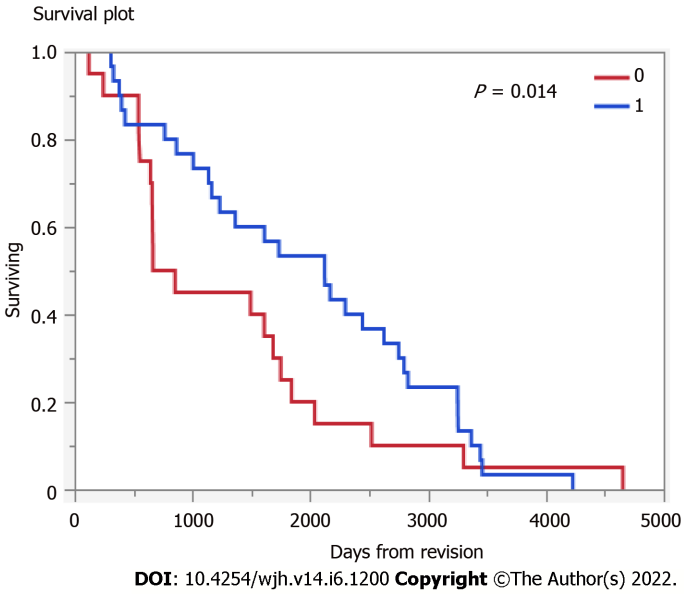


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**Figure 1 Transplant free survival (blue: Transjugular intrahepatic portosystemic shunt revision; red: Did not undergo transjugular intrahepatic portosystemic shunt revision).**

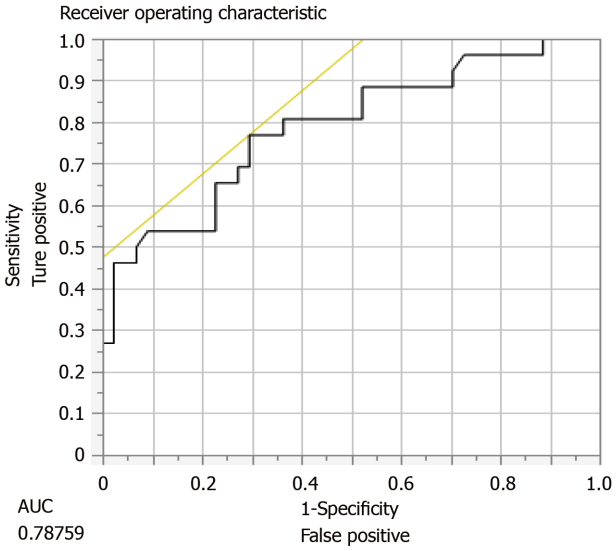


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**Figure 2 Sensitivity and specific of distal velocity in need for transjugular intrahepatic portosystemic shunt revision.**

## DISCUSSION

This study of patients referred for TIPS revision over a 13-year period found that DUS overall has poor sensitivity and specificity for predicting TIPS dysfunction. However, distal velocity seemed to be the most accurate location for determining shunt velocity in this study. Those that underwent TIPS revision were found to have higher transplant free survival.

We observed that significantly more men required TIPS revision than women. We did not observe a statistically significant difference in regards to MELD score or PSG. This is in contrast to prior studies by Brants *et al*[3] who define TIPS dysfunction as an occluded shunt, increase in PSG > 12 mmHg, or stenosis of at least 50% of the shunt diameter[3]. In addition, at the time of revision, patients who needed a TIPS TSV had lower MELD scores, perhaps suggesting that our revision group had fewer decompensations than the non-revision group which could have influenced the survival outcomes of

**Table 2 Comparison of those who underwent transjugular intrahepatic portosystemic shunt revision**

| Characteristic | Yes revision (*n* = 39) | No revision (*n* = 50) | *P* value |
|---|---|---|---|
| Age | 56.7 (12) | 57.3 (7.6) | |
| % male/female | 51/49 | 74/26 | 0.0266 |
| % White/Black/other | 72/23/5 | 80/18/2 | |
| Etiology of liver disease % | | | 0.08 |
| EtOH | 26 | 36 | |
| HCV | 11 | 20 | |
| NASH | 21 | 20 | |
| Indication for TIPS % | | | 0.05 |
| VB | 53 | 50 | |
| Refractory ascites | 34 | 46 | |
| MELD at initial TIPS | 15.8 (6.5) | 17.2 (5.8) | |
| PSG before TIPS mmHg | 15.9 (5.1) | 15.1 (4.0) | |
| PSG after TIPS mmHg | 5.89 (3.1) | 6.3 (2.1) | |
| TIPS dilation mm | 8.6 (1.04) | 8.2 (0.76) | |
| Days to TIPS revision | 572 (740) | 466 (612) | |
| Indication for revision (% doppler) | 84 | 59 | 0.006 |
| MELD at revision | 15.5 (6.8) | 18.7 (6.5) | 0.03 |
| Revision indication | 84 [flow issue] | 66 [clinical] | .04 |
| Doppler abnormal % | 78 [> 5% change proximal flow] | 86 [< 5% change] | 0.39 |
| High vel/low vel/clinical % | 23/51/23 | 46/16/36 | 0.0028 |
| DF at revision Doppler prox cm/s | 103 (64) | 134 (21) | 0.0356 |
| DF at revision Doppler mid | 109 (92) | 151 (52) | 0.017 |
| DF at revision Doppler distal | 86.6 (72) | 174 (105) | 0.0003 |
| TIPS occluded % | 23 | 2 | 0.0019 |
| DF pre-TIPS baseline prox cm/s | 112 (50) | 133 (36) | 0.044 |
| DF pre-TIPs baseline mid | 125 (52) | 140 (32) | 0.16 |
| DF pre-TIPS baseline distal | 117 (58) | 136 (45) | 0.13 |
| Change prox | 42 (37) | 47 (36) | |
| % change prox | -0.11 (0.42) | 0.10 (0.49) | |
| Change mid | 69 (69) | 46 (30) | 0.1 |
| % change mid | -0.057 (0.69) | 0.16 (0.51) | 0.17/.05 (Wilcoxon) |
| Change distal | 62.9 (51) | 73 (89) | |
| % change distal | -0.20 (0.76) | 0.32 (0.92) | 0.021/0.0014 (Wilcoxan) |
| PSG pre TIPS revision mmHg | 15.5 (6.1) | 13.1(14.8) | |
| PSG after TIPS revision mmHg | 8.11 (4.3) | 8.46 (3.3) | |
| Outcome (Alive/LT/Dead) % | 61/13/26 | 40/14/46 | 0.1 |
| TIPS stenosis (Y) % | 100 | 46 | < 0.0001 |

DF: Doppler flow; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; TIPS: Transjugular intrahepatic portosystemic shunt; VB: Variceal bleeding; MELD: Model for End-Stage Liver Disease; PSG: Porto-systemic gradient.

**Table 3 Area under the curve based on intra-transjugular intrahepatic portosystemic shunt velocity**

| TIPS velocity | Area under the curve |
|---|---|
| Proximal flow velocity | 0.65 |
| Mid flow velocity | 0.71 |
| Distal flow velocity | 0.79 ($P$ = 0.0007) |

TIPS: Transjugular intrahepatic portosystemic shunt.

**Table 4 Performance of doppler ultrasound in predicting need for transjugular intrahepatic portosystemic shunt revision**

| Performance characteristic | |
|---|---|
| Sensitivity | 40% |
| Specificity | 45% |
| Negative predictive value | 14% |
| Positive predictive value | 78% |

this study. As such, we found that revision of TIPS was associated with lower mortality (26% *vs* 46%) and significantly lower rates of transplant (13% *vs* 24%; $P$ = 0.1).

While DUS is accessible and non-invasive to detect TIPS dysfunction, studies have shown that DUS is inaccurate and variable in detecting TIPS dysfunction[10]. Much of the established literature has examined bare metal stents alone; however, less is known about the accuracy of DUS in the evaluation of covered stents. *In vitro* model such as DUS has its limitations in accuracy compared to an *in vivo* model because gradient measurements are multifactorial including resistance through the TIPS, resistance through hepatic parenchyma, and presence of collateral vessels[9].

Many factors can influence interpretation of doppler ultrasound. Because the stent is a three-dimensional structure that may not be located within a given plane, an area of focal stenosis could be incorrectly assessed[10]. Inherent to its technique, ultrasound is affected by operator experience. For instance, if only the intravascular portions are assessed, rather than the intraparenchymal segments, a TIPS may be mislabeled as patent. Finally, clinically factors such as obesity, ascites, breathing patterns could impact the ultrasound examination.

To date, there is a lack of well-designed multi-center trials that prospectively explore the accuracy of DUS and clinical factors in predicting TIPS dysfunction. The currently available results are inconsistent and variable due to the absence of a consensus definition of shunt dysfunction, differences in doppler measurements, and the small number of patients included in these series. Because stenosis can lead to a decreased velocity and slower flow, some studies have identified a lower limit of normal for peak shunt velocity, whereas, others have explored an upper limit of normal assuming that focal stenosis would lead to elevated velocities at the stenosis level.

A study of 43 patients using a mean portal vein velocity of < 30 cm/sec and a distal shunt velocity of < 90 cm/sec and > 220 cm/sec, Kanterman *et al*[5], reported a 94% sensitivity and 72% specificity if either parameter was abnormal. This study is in keeping with our results where a distal flow of < 114 cm/sec predicted need for TIPS revision with a 70% specificity. However, Chong *et al*[6] used a lower threshold, 50 cm/sec, which was 100% sensitive and 93% specific for predicting TIPS stenosis. This was based only on a series of 28 patients[1,6]. In our study, at the time of TIPS revision, the velocities at all portions of the stent were significantly decreased, however, the distal shunt velocity outperformed the proximal and mid shunt velocities. Though, a study by Benito *et al*[1] of 105 patients found that a middle shunt velocity threshold of 98cm/sec had the highest receiver operating characteristic with a 46% sensitivity and 79% specificity.

TIPS patency rates have increased over the past 20 years since the introduction of covered ePTFE stents, as compared to bare metal stents. Our study only included patients with ePTFE stents. This is in contrast to a study by Engstrom *et al*[11] where peak shunt velocities from covered and bare metal TIPS showed comparable sensitivities when using either depressed or elevated velocity criteria. However, they reported that a depressed velocity was more specific in covered TIPS, whereas, an elevated velocity was more specific in bare metal TIPS.

Our study is limited due to its retrospective design and lack of predefined DUS criteria to define TIPS dysfunction. In our analysis, we considered the normal range of velocity flow to be 90-190 cm/second, with an abnormal flow to be greater than 50 cm/second increase from previous ultrasound. Furthermore, our small sample size limits the generalizability of our findings. Although previous studies have included the main portal vein velocities, we chose to only focus on clinical parameters and

TIPS velocities.

Although the gold standard for assessment of TIPS function is venography with portosystemic pressure gradient measurements, this procedure remains invasive and can be cost-prohibitive. Recently, color-doppler ultrasound, spleen and liver stiffness measurements *via* point shear wave elastography have shown promise in potentially serving as non-invasive methods to assess for dysfunction[12-16]. Helical CT angiography may also play a role, although future studies are needed to validate these findings[17]. However, these newer methods are not widely available and have not been used to assess TIPS dysfunction.

In summary, if TIPS is placed in the carefully selected patient, it could be life-saving. However, an important consideration is TIPS stenosis that could lead to recurrence of hepatic decompensation. Therefore, an inexpensive, non-invasive, and accurate screening method for early detection of TIPS stenosis is needed. In this study, distal velocity may be able to predict TIPS stenosis with acceptable accuracy while improving transplant free survival rates. However, multi-center prospective studies with a larger cohort are needed to confirm these findings.

## CONCLUSION

DUS has poor overall sensitivity and specificity in predicting need for TIPS revision. Non-invasive methods of predicting TIPS dysfunction are needed since those needing TIPS revision had better survival.

## ARTICLE HIGHLIGHTS

### *Research background*

Portal hypertension as a result of cirrhosis can lead to complications such as variceal bleeding and ascites. Refractory variceal bleeding or ascites can be treated with Transjugular intrahepatic portosystemic shunt (TIPS), an expandable polytetrafluoroethylene covered stent used to decrease portal pressures. However, a complication of this procedure is stent stenosis.

### *Research motivation*

There are currently no guidelines to assist providers in ensuring TIPS patency. Our study aims to assess the accuracy of doppler ultrasound in predicting need for TIPS revision, compared to trans-shunt venography (TSV) as the gold standard.

### *Research objectives*

To determine the accuracy of doppler ultrasound to assess TIPS dysfunction and for need for revision.

### *Research methods*

Retrospective chart review of patients referred for TIPS revision from 2008-2021 at a tertiary medical center. We collected demographical data, doppler ultrasound (DUS) parameters at baseline and at the DUS preceding TIPS revision, TSV data were collected. Receiver operating characteristics curves, sensitivity, specificity, performance for doppler to predict need for revision were performed.

### *Research results*

The cohort consisted of 89 patients with cirrhosis (64% men, 76% white, 31% alcohol as etiology); median age 59 years. TIPS was revised in 44%. On univariate analysis, factors associated with need for TIPS revision were male ($P$ = 0.03), initial indication for TIPS ($P$ = 0.05) and indication for revision ($P$ = 0.01). Revision of TIPS was associated with lower mortality (26% *vs* 46%) and significantly lower rates of transplant (13% *vs* 24%; $P$ = 0.1). In predicting need for TIPS revision, DUS has a 40% sensitivity, 45% specificity, PPV 78%, and NPV 14%. The most accurate location for shunt velocity measure was distal velocity (AUC 0.79; $P$ = 0.0007).

### *Research conclusions*

DUS has poor overall sensitivity and specificity in predicting need for TIPS revision.

### *Research perspectives*

Future research should include multi-center prospective trials using our proposed cut-off of a distal shunt velocity of less than 114 cm/second, to determine if this is the optimal cut-off to predict need for TIPS revision.

## FOOTNOTES

**Author contributions:** Duong N and Sterling RK contributed to the design of the study, data analysis, and major edits; Duong N, Healey M and Patel K contributed to data collection; Strife B contributed to major edits of the manuscript; all authors have read and approve the final manuscript.

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

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**Country/Territory of origin:** United States

**ORCID number:** Nikki Duong 0000-0002-3141-6485; Marcus Healey 0000-0003-2179-2935; Kunal Patel 0000-0001-5281-133X; Brian J Strife 0000-0003-0151-6538; Richard K Sterling 0000-0002-8637-2475.

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ORIGINAL ARTICLE

Observational Study

# Gut dysbiosis and body composition in cirrhosis

Roman Maslennikov, Vladimir Ivashkin, Aliya Alieva, Elena Poluektova, Anna Kudryavtseva, George Krasnov, Maria Zharkova, Yuri Zharikov

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**Roman Maslennikov, Vladimir Ivashkin, Aliya Alieva, Elena Poluektova, Maria Zharkova,** Department of Internal Medicine, Gastroenterology and Hepatology, Sechenov University, Moscow 119435, Russia

**Roman Maslennikov, Vladimir Ivashkin, Elena Poluektova, Anna Kudryavtseva,** Scientific Community for Human Microbiome Research, Moscow 119435, Russia

**Roman Maslennikov,** Department of Internal Medicine, Consultative and Diagnostic Center No. 2, Moscow City Health Department, Moscow 107564, Russia

**Anna Kudryavtseva, George Krasnov,** Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia

**Anna Kudryavtseva, George Krasnov,** Laboratory of Postgenomic Research, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia

**Yuri Zharikov,** Department of Anatomy, Sechenov University, Moscow 119435, Russia

**Corresponding author:** Roman Maslennikov, MD, PhD, Assistant Professor, Professor, Department of Internal Medicine, Gastroenterology and Hepatology, Sechenov University, Pogodinskaya st, 1, Moscow 119435, Russia. mmmm00@yandex.ru

## Abstract

### *BACKGROUND*

Gut dysbiosis and changes in body composition (*i.e.*, a decrease in the proportion of muscle mass and an increase in extracellular fluid) are common in cirrhosis.

### *AIM*

To study the relationship between the gut microbiota and body composition in cirrhosis.

### *METHODS*

This observational study included 46 patients with cirrhosis. Stool microbiome was assessed using 16S rRNA gene sequencing. Multifrequency bioelectrical impedance analysis was performed to assess body composition in these patients.

### *RESULTS*

An increase in fat mass and a decrease in body cell mass were noted in 23/46 (50.0%) and 15/46 (32.6%) patients, respectively. Changes in the gut microbiome

were not independently associated with the fat mass percentage in cirrhosis. The abundance of *Bacteroidaceae* ($P$ = 0.041) and *Eggerthella* ($P$ = 0.001) increased, whereas that of *Erysipelatoclostridiaceae* ($P$ = 0.006), *Catenibacterium* ($P$ = 0.021), *Coprococcus* ($P$ = 0.033), *Desulfovibrio* ($P$ = 0.043), *Intestinimonas* ($P$ = 0.028), and *Senegalimassilia* ($P$ = 0.015) decreased in the gut microbiome of patients with body cell mass deficiency. The amount of extracellular fluid increased in 22/46 (47.6%) patients. Proteobacteria abundance ($P$ < 0.001) increased, whereas Firmicutes ($P$ = 0.023), Actinobacteria ($P$ = 0.026), Bacilli ($P$ = 0.008), *Anaerovoraceceae* ($P$ = 0.027), *Christensenellaceae* ($P$ = 0.038), *Eggerthellaceae* ($P$ = 0.047), *Erysipelatoclostridiaceae* ($P$ = 0.015), *Erysipelotrichaceae* ($P$ = 0.003), *Oscillospiraceae* ($P$ = 0.024), *Rikenellaceae* ($P$ = 0.002), *Collinsella* ($P$ = 0.030), *Hungatella* ($P$ = 0.040), *Peptococcaceae* ($P$ = 0.023), *Slackia* ($P$ = 0.008), and *Senegalimassilia* ($P$ = 0.024) abundance decreased in these patients. Patients with clinically significant ascites ($n$ = 9) had a higher abundance of Proteobacteria ($P$ = 0.031) and a lower abundance of Actinobacteria ($P$ = 0.019) and Bacteroidetes ( $P$ = 0.046) than patients without clinically significant ascites ($n$ = 37).

***CONCLUSION***

Changes in the amount of body cell mass and extracellular fluid are associated with changes in the gut microbiome in cirrhosis patients.

**Key Words:** Dysbiosis; Microbiome; Microbiota; Gut-Liver axis; Sarcopenia; Malnutrition; Cirrhosis

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**Core Tip:** The abundance of *Bacteroidaceae* and *Eggerthella* increased, whereas that of *Erysipelatoclostridiaceae*, *Catenibacterium*, *Coprococcus*, *Desulfovibrio*, *Intestinimonas*, and *Senegalimassilia* decreased in the gut microbiome of patients with body cell mass deficiency. Proteobacteria abundance was increased, whereas Firmicutes, Actinobacteria, Bacilli, *Christensenellaceae*, *Anaerovoraceceae*, *Eggerthellaceae*, *Erysipelatoclostridiaceae*, *Erysipelotrichaceae*, *Oscillospiraceae*, *Peptococcaceae*, *Rikenellaceae, Collinsella*, *Hungatella*, *Slackia*, and *Senegalimassilia* abundance decreased in cirrhosis patients with excess extracellular fluid.

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## INTRODUCTION

Cirrhosis is the final stage of chronic liver diseases. However, it is not limited to lesions in this organ but is also associated with a decrease in muscle mass (sarcopenia) and water accumulation in the body. The pathogenesis of sarcopenia in cirrhosis is complex, and it is assumed that changes in composition of the gut microbiota (gut dysbiosis) and small intestinal bacterial overgrowth (SIBO) play important roles in its development[1-5]. It is believed that these disorders of the gut microbiota promote bacterial translocation (the penetration of bacteria and their components into body tissues) and hyperammonemia, which increase protein catabolism and levels of myostatin, a protein that inhibits muscle growth[2].

Water retention in cirrhosis has also been suggested to be associated with disorders of the gut microbiota and occurs in response to bacterial translocation-induced vasodilation[6]. This leads to hypotension and compensatory fluid retention to maintain normal blood pressure levels. Although these relationships have been established with respect to SIBO[7,8], there are no studies on such associations with gut dysbiosis.

In addition, the gut microbiota status is known to be associated with disorders of lipid metabolism, leading to an increase in fat content in the body[9].

Bioelectrical impedance analysis is a method used for the complex assessment of body composition and is based on measurements of capacitive and active resistance of the human body. These can be used to identify fat and lean (free-fat) mass. The latter is represented by body cell mass, consisting mainly of musculoskeletal mass, and extracellular mass, comprised mainly of extracellular fluid. Although fat is located within cells, it and body cell mass are conditionally considered to be different components of the body in this analysis. Fat is practically non-conductive. Cells are capacitors (*i.e.*, an electrolyte solution surrounded by a dielectric membrane) and give rise to the capacitive component of resistance, while free extracellular fluid contributes to the active resistance. Therefore, it is possible to assess body

composition (amount of fat, body cell mass, and extracellular fluid) by analyzing the capacitive and active components of resistance of the body[10-13].

Although recent publications have reported the associations of some taxa of the gut microbiome with sarcopenia diagnosed by computed tomography[4,5], no studies have investigated the relations between the gut microbiome and all three main body components (fat, cells, and extracellular fluid) in cirrhosis.

The aim of the present study was to assess the relationship between the gut microbiota and body composition in cirrhosis.

## MATERIALS AND METHODS

### Patients

In this observational study, 97 patients with cirrhosis were consecutively admitted to the Department of Hepatology of the Clinic for Internal Medicine, Gastroenterology and Hepatology at Sechenov University (Moscow, Russia) and screened for participation. The procedures were explained to potential participants, and written informed consent was obtained before enrollment. The study was approved by the Ethics Committee of Sechenov University in accordance with the Declaration of Helsinki.

The inclusion criteria were diagnosis of cirrhosis verified by histological examination or clinical, biochemical, and ultrasound findings, and age between 18 and 70 years. The exclusion criteria included use of lactulose, lactitol, or other prebiotics, probiotics, antibiotics, or metformin in the past 6 wk, alcohol consumption in the past 6 wk, or diagnosis of inflammatory bowel disease, cancer, or any other serious disease. The exclusion criteria were specifically selected to remove the influence of these factors on the composition of the gut microbiota. Of the original 97 patients screened for inclusion, 46 were enrolled in the study and 51 were excluded (Figure 1).

In addition, 14 healthy persons were examined.

### Gut microbiome analysis

The gold standard for studying the composition of the gut microbiota is analysis of the gut microbiome that is a cumulative genome of gut bacteria.

A stool sample was obtained from each patient and placed in a sterile disposable container the morning after admission and immediately frozen at -80°C[14].

Total DNA was isolated using the AmpliPrime DNA-sorb-AM kit (NextBio, Moscow, Russia) for clinical specimens, according to the manufacturer's protocol. The isolated DNA was stored at -20°C. For qualitative and quantitative assessment of the isolated DNA we used NanoDrop 1000 equipment (Thermo Fisher Scientific, Waltham, MA, United States). The 16S library preparation was carried out according to the protocol of 16S Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA, United States), which is recommended for Illumina MiSeq sample prep. The first round of amplification of V3-V4 16S rDNA variable regions was performed using the following primers: forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG) and reverse (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC). These primers are aimed at the amplification of bacterial (more than 90%) but not archaeal (less than 5%) rRNA genes. The amplification program (Applied Biosystems 2720 Thermal Cycler, Foster City, CA, United States) was as follows: (1) 95°C for 3 min; (2) 30 cycles: 95°C for 30 s; 55°C for 30 s; 72°C for 30 s; (3) 72°C for 5 min; and (4) 4°C.

The derived amplicons were purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, United States) beads according to the manufacturer's protocol. The second amplification round was used for double-indexing samples with a combination of specific primers. The amplification program was as follows: (1) 95°C for 3 min; (2) 8 cycles: 95°C for 30 s; 55°C for 30 s; 72°C for 30 s; (3) 72°C for 5 m; and (4) 4°C.

The purification of PCR products was also carried out using Agencourt AMPure XP. The concentration of the derived 16S rDNA libraries was measured using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, United States) using QuantiT™ dsDNA High-Sensitivity Assay Kit. The purified amplicons were mixed equimolarly according to the derived concentration values. Quality of the libraries was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States) and Agilent DNA 1000 Kit. Sequencing was carried out on a MiSeq machine (Illumina) using the MiSeq Reagent Kit v2 (paired-end reads, 2 × 300 nt).

First, forward and reverse reads were merged using MeFiT and CASPER[15]. For most samples more than 99% reads were successfully merged. Non-merged reads were excluded. Next, the merged reads were analyzed by the DADA2 package (a part of the Bioconductor project) for R[16] in order to infer RSV (ribosomal sequence variants). The analysis included the following steps: (1) Primer sequences were removed using Cutadapt; (2) Reads were filtered by quality; (3) Error distribution models were derived based on read quality profiles; (4) Sequencing errors were estimated and corrected; (5) RSV sequences were obtained; and (6) Chimeric RSVs were eliminated. Next, taxonomic annotation of the derived RSVs was performed with the DADA2 package using the Silva (version 138) 16S reference sequence database[17].

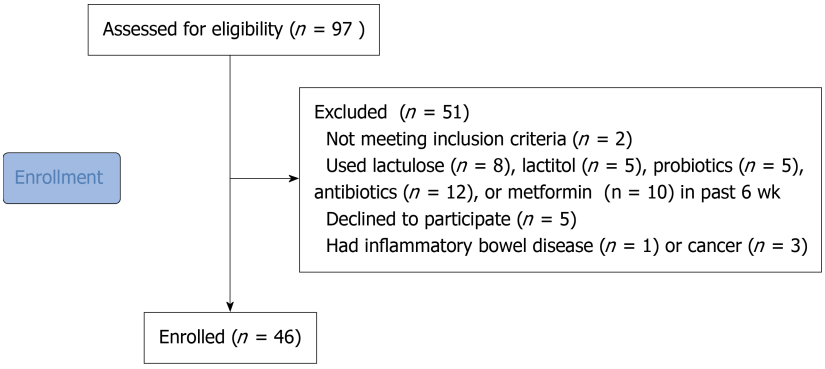


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**Figure 1 CONSORT 2010 flow diagram.**

### *Bioelectrical impedance analysis*

Bioelectrical impedance analysis was performed on the day after patient admission in the morning, to ensure that the patient had an empty stomach. The MEDASS device (Russia) was used for this purpose in accordance with the manufacturer's instructions.

The measurement was carried out by passing an alternating current with frequencies of 5 and 50 kHz through the patient. Conduction originates almost entirely due to the extracellular fluid in the presence of constant current. With an alternating current, the intracellular fluid also contributes to current conduction, depending on its frequency. The manufacturer's software provides the values for fat and body cell mass and total and extracellular fluid based on these values of conduction and patient's age, sex, height, and weight. This software also calculated individual norms for each patient based on his/her anthropometric data, age, sex, and the results of a local population study. Patients with a fat mass value above the upper limit of their individual norm were included in the group of patients with excess fat mass, and those with body cell mass below the lower limit of their individual norm were included in the group of patients with cell mass deficiency. Similarly, patients with an amount of extracellular fluid higher than the upper limit of their individual norm were included in the group of patients with excess extracellular fluid.

The underlying principle of this analysis and the methods used for calculating the indicators have been described in detail in previous publications[10,11].

We used the ratio of body cell mass to free-fat mass to assess body cell mass and the ratio of extracellular fluid to total fluid to quantitate the amount of extracellular fluid. This method ensured minimal influence of values of fat and body cell mass and extracellular fluid on each other.

### *Statistical analysis*

Statistical analysis was performed with STATISTICA 10 (StatSoft Inc., Tulsa, OK, United States). The data are presented as medians (interquartile ranges). The abundance of taxa in the gut microbiome is presented as a percentage. Differences between continuous variables were assessed with the Mann-Whitney test. Fisher's exact test was used to assess the differences between categorical variables. Correlations between variables were computed using Spearman's rank correlation. If the compared groups differed in age, sex or severity of cirrhosis, multivariate regression analysis was performed. $P$ values ≤ 0.05 were considered statistically significant.

## RESULTS

The characteristics of the patients enrolled in the study are listed in Table 1.

The amount of extracellular fluid and the fat mass were higher but the body cell mass was lower in patients with cirrhosis than in healthy individuals. The abundance of Bacteroidetes, Proteobacteria, and Bacilli was higher, but the abundance of Firmicutes and Clostridia was lower in the gut microbiome of these patients than in that of healthy individuals (Table 2).

The fat mass was increased in 23/46 (50.0%) patients. The abundance of Bacteroidetes, Desulfobacteria, *Barnesiellaceae*, *Coriobacteriaceae*, *Eggerthellaceae*, *Marinifilaceae*, *Bilophila, Senegalimassilia, Slackia,* and *Parasutterella* was higher in the gut microbiome of these patients. On the other hand, the abundance of *Clostridiaceae, Odoribacter*, and *Veillonella* was decreased in the gut microbiome of these patients (Table 3).

The proportion of fat mass in total body mass showed a positive correlation with the abundance of Bacteroidetes, Desulfobacteria, *Coriobacteriaceae*, *Barnesiellaceae*, *Bilophila*, *Collinsella*, *Megamonas*, *Parasutterella*, and *Slackia* and a negative correlation with the abundance of *Clostridiaceae*, *Campylobacter*, and

**Table 1 Characteristics of the enrolled patients**

| Parameter | Value |
|---|---|
| Age, yr | 55 [43-61] |
| Male/Female | 18/28 |
| Etiology: | |
| Alcohol | 15 (32.6%) |
| Hepatitis C virus | 5 (10.9%) |
| Primary biliary cholangitis | 4 (8.7%) |
| Primary sclerosing cholangitis | 2 (4.3%) |
| Autoimmune hepatitis | 5 (10.9%) |
| Metabolic-associated liver disease | 4 (8.7%) |
| Wilson disease | 3 (6.5%) |
| Mixed | 3 (6.5%) |
| Cryptogenic | 5 (10.9%) |
| Red blood cells, $10^{12}$/L | 4.1 [3.5-4.8] |
| White blood cells, $10^{9}$/L | 4.9 [3.1-6.3] |
| Platelets, $10^{9}$/L | 105 [76-150] |
| Serum albumin, g/L | 37 [33-41] |
| Serum total bilirubin, μmol/L | 28 [16-56] |
| Prothrombin (Quick test), % | 70 [60-89] |
| Ascites: grade 2-3, *n* (%) | 9 (19.6%) |
| Esophageal varices: grade 2-3, *n* (%) | 17 (36.9%) |
| Spleen length, cm | 15.4 [13.1-17.1] |
| Portal vein diameter, mm | 13.0 [11.0-14.2] |
| Hepatic encephalopathy, *n* (%) | 15 (32.5%) |
| Child-Pugh class: A/B/C | 14/21/11 |

*Veillonella* (Table 4).

Since the groups under comparison differed with respect to age, sex, and severity of cirrhosis, we performed a multivariate regression analysis and found that these changes in the gut microbiome were not independent factors affecting the percentage of fat mass in the total body mass of these patients.

The body cell mass was decreased in 15/46 (32.6%) patients. The abundance of *Bacteroidaceae* and *Eggerthella* increased in the gut microbiome of these patients, whereas that of *Erysipelatoclostridiaceae, Catenibacterium*, *Coprococcus*, *Desulfovibrio*, *Intestinimonas*, and *Senegalimassilia* decreased (Table 5).

The proportion of body cell mass correlated positively with the abundance of *Barnesiellaceae*, *Erysipelatoclostridiaceae*, *Anaerotruncus*, *Catenibacterium*, *Oscillospira*, and *Senegalimassilia*, whereas a negative correlation with abundance of *Bacteroidaceae* and *Veillonella* was observed (Table 4).

The amount of extracellular fluid increased in 22/46 (47.6%) patients. The abundance of Proteobacteria was increased in the gut microbiome of these patients. However, the abundance of Firmicutes, Bacilli, *Anaerovoraceceae*, *Christensenellaceae*, *Eggerthellaceae*, *Erysipelatoclostridiaceae*, *Erysipelotrichaceae*, *Oscillospiraceae*, *Peptococcaceae*, *Rikenellaceae*, *Actinobacteria*, *Collinsella*, *Hungatella*, *Slackia*, and *Senegalimassilia* was decreased in the gut microbiome of these patients (Table 6).

The proportion of extracellular fluid in total body fluid in these patients was positively correlated with the abundance of Proteobacteria and *Bilophila*, and negatively correlated with that of Firmicutes, Bacilli, and Clostridia in the gut microbiome (Table 4).

There was no significant difference between these groups of patients in terms of the indices of microbiota biodiversity (Shannon, Chao1, ACE—Figure 2)[18], and no significant correlation was found between the latter and indicators of body composition.

A comparison of the gut microbiome at the phylum level between patient groups is represented in Figure 3.

**Table 2 Comparison of the main characteristics, body composition, and the abundance of major taxa of the gut microbiome between patients with cirrhosis and healthy persons**

| | Patients with cirrhosis (*n* = 46) | Healthy persons (*n* = 14) | *P* |
|---|---|---|---|
| Age, yr | 55 [43-61] | 51 [41-63] | 0.484 |
| Male/Female | 18/28 | 3/11 | 0.187 |
| Body mass index, kg/m$^2$ | 27.0 [23.6-30.1] | 21.1 [19.7-26.0] | 0.002 |
| Fat mass, % | 34.7 [28.1-43.5] | 24.5 [20.7-31.2] | 0.002 |
| Free-fat mass, % | 65.3 [56.5-71.9] | 75.5 [68.8-79.3] | 0.002 |
| Body cell mass, % | 32.4 [28.0-36.5] | 44.5 [38.4-46.0] | < 0.001 |
| (Body cell mass)/(free-fat mass) | 0.50 [0.46-0.55] | 0.58 [0.55-0.60] | < 0.001 |
| Extracellular fluid, % | 20.1 [17.4-21.4] | 18.6 [16.8-19.3] | 0.044 |
| Total fluid, % | 47.9 [42.0-53.3] | 51.7 [46.8-52.9] | 0.238 |
| (Extracellular fluid)/(total fluid) | 0.41 [0.40-0.43] | 0.36 [0.36-0.37] | < 0.001 |
| Phase angle, ° | 5.3 [4.9-6.3] | 7.0 [6.2-7.3] | < 0.001 |
| Firmicutes | 38.6 [27.7-52.9] | 90.8 [85.7-94.1] | < 0.001 |
| Bacteroidetes | 38.6 [26.6-58.5] | 5.9 [4.7-8.1] | < 0.001 |
| Proteobacteria | 5.5 [2.3-10.6] | 0.4 [0.1-0.5] | < 0.001 |
| Clostridia | 36.2 [24.8-50.0] | 89.3 [86.7-91.0] | < 0.001 |
| Bacilli | 0.4 [0.2-1.1] | 0.1 [0.0-0.2] | < 0.001 |

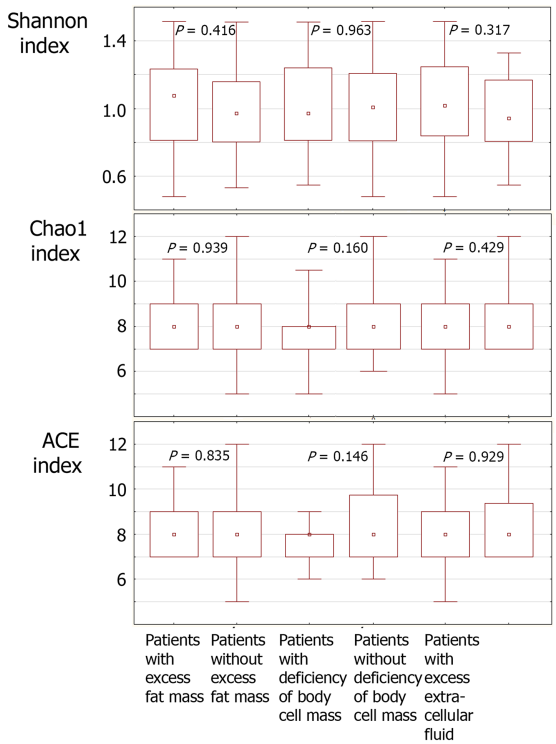


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**Figure 2 Comparison of gut microbiota biodiversity indices between the patient groups.**

Patients with clinically significant ascites (stages 2 and 3 according to the classification of the International Club of Ascites; *n* = 9) had a higher abundance of Proteobacteria [17.3 (7.9-23.2)% *vs* 5.03 (2.26-7.93)%; *P* = 0.031] and a lower abundance of Actinobacteria [0.11 (0.09-0.66)% *vs* 1.04 (0.36-3.89)%; *P* = 0.019] and Bacteroidetes [35.2 (12.9-37.6)% *vs* 43.2 (29.4-60.3)%; *P* = 0.046] in their gut microbiome than patients without clinically significant ascites (*n* = 37).

**Table 3 Patients grouped with respect to excess fat mass**

| | Patients with excess fat mass (*n* = 23) | Patients without excess fat mass (*n* = 23) | *P* |
|---|---|---|---|
| Age, yr | 58 [49-62] | 45 [39-59] | 0.033 |
| Male/Female | 3/20 | 15/8 | < 0.001 |
| Body mass index, kg/m$^2$ | 29.7 [27.0-35.6] | 23.6 [22.0-27.1] | < 0.001 |
| Body fat, % | 43.5 [37.6-47.3] | 28.1 [23.1-31.2] | < 0.001 |
| Free-fat mass, % | 56.5 [52.7-62.4] | 71.9 [68.8-76.9] | < 0.001 |
| Body cell mass, % | 29.6 [25.6-33.9] | 34.9 [31.2-40.6] | 0.001 |
| (Body cell mass)/(free-fat mass) | 0.51 [0.45-0.56] | 0.49 [0.46-0.53] | 0.231 |
| Extracellular fluid, % | 17.6 [16.7-18.8] | 21.1 [20.4-22.4] | < 0.001 |
| Total fluid, % | 42.0 [38.5-45.8] | 52.6 [50.4-56.3] | < 0.001 |
| (Extracellular fluid)/(total fluid) | 0.42 [0.41-0.43] | 0.40 [0.39.2-0.41] | 0.005 |
| Phase angle, ° | 5.4 [5.2-6.6] | 5.1 [4.6-5.8] | 0.093 |
| Red blood cells, $10^{12}$/L | 4.5 [3.9-4.8] | 3.9 [3.3-4.6] | 0.097 |
| White blood cells, $10^9$/L | 5.0 [3.6-6.3] | 4.7 [2.9-5.7] | 0.465 |
| Platelets, $10^9$/L | 104 [77-150] | 106 [72-150] | 0.945 |
| Serum albumin, g/L | 37.6 [34.4-42.9] | 34.4 [31.3-38.1] | 0.030 |
| Serum total bilirubin, μmol/L | 20.1 [13.5-36.9] | 46.6 [19.1-77.9] | 0.037 |
| Prothrombin (Quick test), % | 75 [69-92] | 64 [40-80] | 0.022 |
| Ascites: grade 2-3 | 1 (4.3%) | 8 (34.8%) | 0.011 |
| Esophageal varices: grade 2-3 | 8 (34.8%) | 9 (39.1%) | 0.500 |
| Spleen length, cm | 15.3 [13.0-17.2] | 15.7 [13.1-17.1] | 0.759 |
| Portal vein diameter, mm | 12.7 [11.0-14.2] | 13.0 [11.0-14.4] | 0.803 |
| Hepatic encephalopathy | 4 (17.4%) | 9 (39.1%) | 0.095 |
| Child-Pugh score | 7 [6-9] | 9 [7-12] | 0.018 |
| Bacteroidetes | 46.1 [33.0-61.0] | 35.2 [18.3-44.1] | 0.039 |
| *Barnesiellaceae* | 0.92 [0.43-2.04 | 0.04 [0.00-0.11] | 0.016 |
| *Marinifilaceae* | 0.66 [0.21-1.03] | 0.17 [0.00-0.49] | 0.006 |
| Desulfobacteria | 0.55 [0.29-1.53] | 0.18 [0.01-0.45] | 0.032 |
| *Bilophila* | 0.36 [0.04-1.30] | 0.04 [0.00-0.18] | 0.016 |
| *Coriobacteriaceae* | 0.09 [0.04-0.56] | 0.03 [0.00-0.06] | 0.004 |
| *Eggerthellaceae* | 0.08 [0.03-0.20] | 0.03 [0.01-0.06] | 0.028 |
| *Senegalimassilia* | 0.01 [0.00-0.05] | 0.00 [0.00-0.00] | 0.040 |
| *Slackia* | 0.01 [0.00-0.05] | 0.00 [0.00-0.00] | 0.004 |
| *Parasutterella* | 0.01 [0.00-0.19] | 0.00 [0.00-0.00] | 0.015 |
| *Odoribacter* | 0.10 [0.03-0.24] | 0.26 [0.16-0.52] | 0.047 |
| *Veillonella* | 0.01 [0.00-0.07] | 0.16 [0.01-0.82] | 0.012 |
| *Clostridiaceae* | 0.01 [0.00-0.05] | 0.07 [0.00-0.29] | 0.041 |

Only significant changes in the gut microbiome are indicated.

The abundance of Firmicutes was decreased in patients with excess extracellular fluid regardless of the presence of clinically significant ascites, while a decrease in the abundance of Bacteroidetes occurred only in those patients with excess extracellular fluid who had clinically significant ascites (Figures 4 and 5). The abundance of Proteobacteria progressively increased, and the abundance of Actinobacteria

**Table 4 Significant correlations between the volumes of the body components and the taxa of the gut microbiome**

| | Fat mass | Body cell mass | Extracellular fluid |
|---|---|---|---|
| Bacteroidetes | $r = 0.329$; $P = 0.026$ | NS | NS |
| Desulfobacteria | $r = 0.347$; $P = 0.018$ | NS | NS |
| Firmicutes | NS | NS | $r = -0.386$; $P = 0.008$ |
| Proteobacteria | NS | NS | $r = 0.320$; $P = 0.031$ |
| Bacilli | NS | NS | $r = -0.378$; $P = 0.009$ |
| Clostridia | NS | NS | $r = -0.305$; $P = 0.039$ |
| *Bacteroidaceae* | NS | $r = -0.294$; $P = 0.047$ | NS |
| *Barnesiellaceae* | $r = 0.291$; $P = 0.049$ | $r = 0.332$; $P = 0.024$ | NS |
| *Clostridiaceae* | $r = -0.326$; $P = 0.027$ | NS | NS |
| *Coriobacteriaceae* | $r = 0.319$; $P = 0.031$ | NS | NS |
| *Erysipelatoclostridiaceae* | NS | $r = 0.310$; $P = 0.036$ | NS |
| *Anaerotruncus* | NS | $r = 0.338$; $P = 0.022$ | NS |
| *Bilophila* | $r = 0.383$; $P = 0.009$ | NS | $r = 0.294$; $P = 0.048$ |
| *Campylobacter* | $r = -0.404$; $P = 0.005$ | NS | NS |
| *Catenibacterium* | NS | $r = 0.306$; $P = 0.040$ | NS |
| *Collinsella* | $r = 0.319$; $P = 0.031$ | NS | NS |
| *Megamonas* | $r = 0.337$; $P = 0.022$ | NS | NS |
| *Oscillospira* | NS | $r = 0.375$; $P = 0.010$ | NS |
| *Parasutterella* | r = 0.365; $P = 0.013$ | NS | NS |
| *Senegalimassilia* | ns | $r = 0.379$; $P = 0.009$ | NS |
| *Slackia* | $r = 0.439$; $P = 0.002$ | NS | NS |
| *Veillonella* | $r = -0.308$; $P = 0.037$ | $r = -0.294$; $P = 0.047$ | NS |

NS: Not significant.

progressively decreased in the transition from patients without excess extracellular fluid to patients with excess extracellular fluid but without clinically significant ascites, and further to patients with clinically significant ascites (Figures 4 and 5).

## DISCUSSION

Gut dysbiosis is common in cirrhosis and is associated with the development of hepatic encephalopathy, lower serum albumin and cholinesterase levels, systemic inflammation, and poorer short- and long-term prognosis[19-21]. The aim of the present study was to assess the relationship between the gut microbiota and body composition in patients with cirrhosis.

The changes in body composition and the gut microbiome with cirrhosis in our study were mostly consistent with earlier findings[1,6,19-21].

Although malnutrition is typical in patients with cirrhosis, half of the patients enrolled in the present study had excess fat mass. This can be explained by the fact that 30% of the included patients had compensated cirrhosis (class A Child-Pugh score), while severe cirrhosis (Child-Pugh class C), for which malnutrition was most characteristic, was observed in less than a quarter of the patients. The inclusion of a small percentage of patients with severe cirrhosis is both a disadvantage and an advantage in our study, as we included patients with varying degrees of cirrhosis severity, which enabled a more generalized analysis.

Cirrhosis was less severe in patients with excess fat mass. In terms of the taxa of gut microbiota, the increased abundance of Bacteroidetes in these patients was the most significant change. However, obesity in patients without cirrhosis is associated with a decrease in the abundance of Bacteroidetes[22,23]. The change in abundance of Bacteroidetes in cirrhosis is controversial: studies have reported its decrease[24-26], increase[27], and non-significant changes[19]. One study reported an increase in

**Table 5 Patients grouped with respect to body cell mass deficiency**

| | Patients with body cell mass deficiency (*n* = 15) | Patients without body cell mass deficiency (*n* = 31) | *P* |
|---|---|---|---|
| Age, yr | 56 [46-63] | 49 [39-61] | 0.331 |
| Male/Female | 4/11 | 14/17 | 0.190 |
| Body mass index, kg/m$^2$ | 27.0 [23.8-29.0] | 27.1 [23.2-30.9] | 0.806 |
| Body fat, % | 33.6 [29.8-42.7] | 36.3 [27.3-44.9] | 0.656 |
| Free-fat mass, % | 66.4 [57.3-70.2] | 63.7 [55.1-72.7] | 0.656 |
| Body cell mass, % | 29.0 [25.6-31.9] | 34.7 [30.0-37.1] | 0.002 |
| (Body cell mass)/(free-fat mass) | 0.45 [0.41-0.46] | 0.53 [0.49-0.56] | < 0.001 |
| Extracellular fluid, % | 20.4 [17.7-21.1] | 19.6 [17.0-21.8] | 0.648 |
| Total fluid, % | 48.6 [42.0-51.5] | 47.1 [41.0-53.3] | 0.926 |
| (Extracellular fluid)/(total fluid) | 0.42 [0.41-0.43] | 0.41 [0.39-0.43] | 0.223 |
| Phase angle, ° | 4.5 [4.2-4.6] | 5.8 [5.2-6.5] | < 0.001 |
| Red blood cells, $10^{12}$/L | 3.8 [3.5-4.6] | 4.2 [3.6-4.8] | 0.211 |
| White blood cells, $10^9$/L | 4.1 [3.0-7.2] | 5.1 [3.3-5.9] | 0.159 |
| Platelets, $10^9$/L | 116 [77-170] | 101 [72-142] | 0.211 |
| Serum albumin, g/L | 34.1 [29.3-37.3] | 37.6 [33.3-42.4] | 0.028 |
| Serum total bilirubin, μmol/L | 46.6 [18.7-66.2] | 22.3 [15.0--54.6] | 0.314 |
| Prothrombin (Quick test), % | 71 [54-92] | 70 [60-86] | 0.981 |
| Ascites: grade 2-3 | 4 (26.7%) | 5 (16.1%) | 0.320 |
| Esophageal varices: grade 2-3 | 4 (26.7%) | 13 (41.9%) | 0.251 |
| Spleen length, cm | 15.8 [13.4-17.0] | 15.3 [13.0-17.2] | 0.864 |
| Portal vein diameter, mm | 13.8 [11.0-14.4] | 12.7 [11.0-14.2] | 0.695 |
| Hepatic encephalopathy | 7 (46.7%) | 8 (25.8%) | 0.141 |
| Child-Pugh score | 9 [7-11] | 7 [6-9] | 0.092 |
| *Bacteroidaceae* | 22.7 [6.8-40.8] | 4.0 [1.4-20.5] | 0.041 |
| *Eggerthella* | 0.01 [0.00-0.03] | 0.00 [0.00-0.00] | 0.001 |
| *Erysipelatoclostridiaceae* | 0.02 [0.01-0.08] | 0.11 [0.04-0.25] | 0.006 |
| *Coprococcus* | 0.24 [0.06-0.68] | 0.68 [0.16-1.26] | 0.033 |
| *Intestinimonas* | 0.00 [0.00-0.03] | 0.03 [0.01-0.07] | 0.028 |
| *Desulfovibrio* | 0.00 [0.00-0.01] | 0.02 [0.00-0.38] | 0.043 |
| *Catenibacterium* | 0.00 [0.00-0.00] | 0.00 [0.00-0.20] | 0.021 |
| *Senegalimassilia* | 0.00 [0.00-0.00] | 0.00 [0.00-0.03] | 0.015 |

Only significant changes in the gut microbiome are indicated.

Bacteroidetes abundance in compensated cirrhosis, which decreased further to attain normal levels with decompensation[28]. Patients with excess fat mass had less severe cirrhosis and were older than patients without excess fat mass. Multivariate regression analysis established that the age and Child-Pugh score, but not the gut microbiome status, significantly determined the level of fat mass in patients with cirrhosis, thereby resolving this contradiction.

Patients with body cell mass deficiency who were considered to have sarcopenia accounted for one third of the included patients. They also had another sign of malnutrition (namely, hypoalbuminemia), although they did not show significant differences in the values of other biomarkers of liver failure

**Table 6 Patients grouped with respect to excess extracellular fluid**

| | Patients with excess extracellular fluid (*n* = 22) | Patients without excess extracellular fluid (*n* = 24) | *P* |
|---|---|---|---|
| Age, yr | 53 [39-61] | 57 [44-62] | 0.545 |
| Male/Female | 10/12 | 8/16 | 0.300 |
| Body mass index, kg/m$^2$ | 28.1 [24.2-31.2] | 26.8 [22.9-29.1] | 0.129 |
| Body fat, % | 32.4 [24.5-44.9] | 36.7 [30.4-42.7] | 0.391 |
| Free-fat mass, % | 67.6 [55.1-75.5] | 63.3 [57.3-69.6] | 0.391 |
| Body cell mass, % | 33.6 [28.0-40.6] | 32.2 [28.2-35.2] | 0.545 |
| (Body cell mass)/(free-fat mass) | 0.50 [0.45-0.55] | 0.49 [0.47-0.55] | 0.921 |
| Extracellular fluid, % | 20.9 [17.7-22.6] | 18.5 [17.2-20.4] | < 0.001 |
| Total fluid, % | 50.8 [41.2-55.9] | 45.9 [42.0-50.9] | 0.169 |
| (Extracellular fluid)/(total fluid) | 0.43 [0.40-43.3] | 0.41 [0.39-0.42] | 0.042 |
| Phase angle, ° | 5.4 [4.6-6.3] | 5.2 [4.9-6.3] | 0.879 |
| Red blood cells, $10^{12}$/L | 4.2 [3.4-4.8] | 4.0 [3.6-4.6] | 0.991 |
| White blood cells, $10^9$/L | 4.6 [2.4-6.3] | 5.2 [3.4-6.2] | 0.419 |
| Platelets, $10^9$/L | 89 [72-113] | 124 [76-158] | 0.082 |
| Serum albumin, g/L | 34.5 [30.4-41.3] | 37.5 [34.3-40.7] | 0.113 |
| Serum total bilirubin, μmol/L | 39.4 [18.5-66.2] | 24.8 [15.4-44.5] | 0.684 |
| Prothrombin (Quick test), % | 61 [40-86] | 76 [70-91] | 0.012 |
| Ascites: grade 2-3 | 9 (40.9%) | 0 | 0.001 |
| Esophageal varices: grade 2-3 | 9 (40.9%) | 8 (33.3%) | 0.410 |
| Spleen length, cm | 16.7 [14.8-18.2] | 14.4 [12.4-16.4] | 0.021 |
| Portal vein diameter, mm | 13.5 [11.0-15.0] | 12.5 [11.0-14.0] | 0.180 |
| Hepatic encephalopathy | 10 (45.5%) | 5 (20.8%) | 0.050 |
| Child-Pugh score | 9 [6-12] | 7 [6-9] | 0.088 |
| Proteobacteria | 8.93 [5.58-22.60] | 2.84 [1.61-5.66] | < 0.001 |
| Firmicutes | 31.4 [26.6-44.6] | 43.6 [33.4-58.7] | 0.023 |
| *Oscillospiraceae* | 4.43 [1.57-8.46] | 8.33 [5.00-12.9] | 0.024 |
| *Rikenellaceae* | 0.98 [0.03-1.78] | 2.83 [0.85-5.51] | 0.002 |
| Actinobacteria | 0.56 [0.11-1.43] | 1.21 [0.42-5.93] | 0.026 |
| Bacilli | 0.24 [0.15-0.52] | 0.54 [0.34-2.1] | 0.008 |
| *Christensenellaceae* | 0.12 [000-0.43] | 0.43 [0.07-2.59] | 0.038 |
| *Collinsella* | 0.04 [0.01-0.05] | 0.10 [0.02-0.24] | 0.030 |
| *Eggerthellaceae* | 0.04 [0.01-0.06] | 0.08 [0.03-0.20] | 0.047 |
| *Erysipelatoclostridiaceae* | 0.04 [0.00-0.13] | 0.10 [0.05-0.41] | 0.015 |
| *Erysipelotrichaceae* | 0.01 [0.00-0.03] | 0.05 [0.01-0.11] | 0.003 |
| *Anaerovoraceceae* | 0.01 [0.00-0.06] | 0.04 [0.01-0.11] | 0.027 |
| *Hungatella* | 0.00 [0.00-0.00] | 0.01 [0.00-0.04] | 0.040 |
| *Slackia* | 0.00 [0.00-0.00] | 0.00 [0.00-0.04] | 0.008 |
| *Peptococcaceae* | 0.00 [0.00-0.00] | 0.00 [0.00-0.02] | 0.023 |
| *Senegalimassilia* | 0.00 [0.00-0.00] | 0.01 [0.00-0.04]; | 0.024 |

Only significant changes in the gut microbiome are indicated.

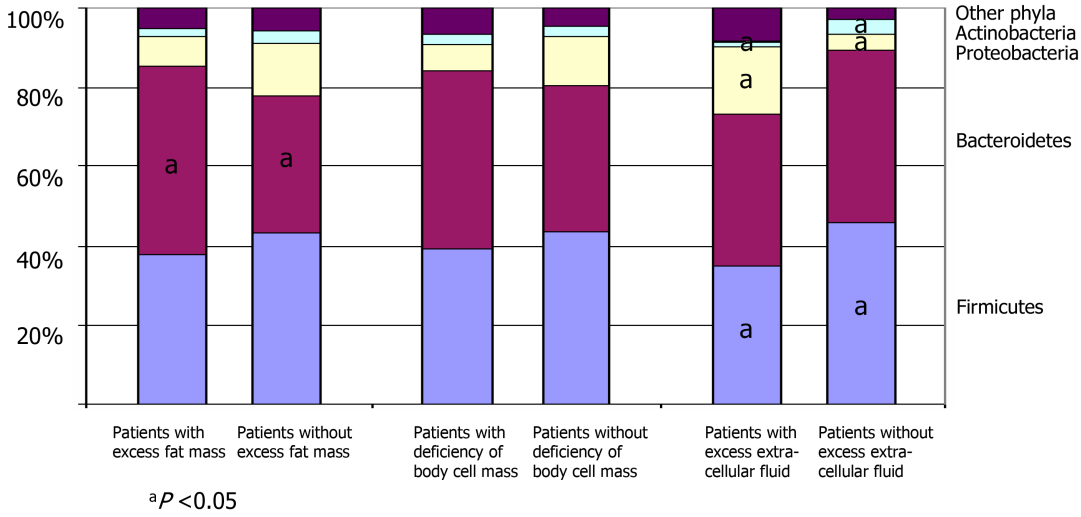


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**Figure 3 Comparison of gut microbiome at the phylum level between the patient groups.**

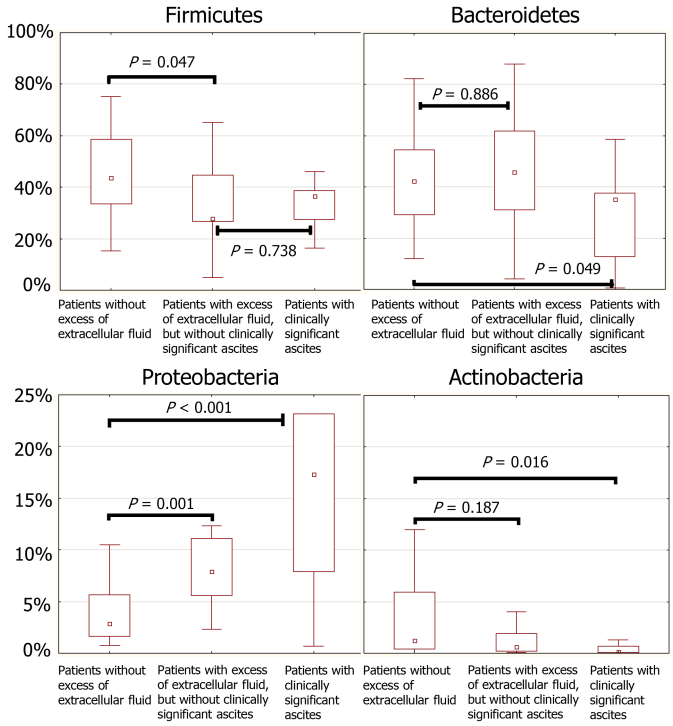


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**Figure 4 Abundance of the main phyla in the gut microbiome of patients without excess extracellular fluid, patients with excess extracellular fluid but without clinically significant ascites, and patients with clinically significant ascites.**

(serum bilirubin and prothrombin) and portal hypertension (clinically significant ascites and spleen length) compared to patients with normal body cell mass. Patients grouped with respect to body cell mass deficiency did not show significant differences in the gut microbiome at the level of higher taxa (phyla), although the abundance of *Bacteroidaceae* was higher in patients with body cell mass deficiency. These patients also had increased abundance of *Eggerthella*, which is considered a biomarker of fragility [29,30]. These findings are consistent with recent studies of the gut microbiome in cirrhosis patients with sarcopenia[4,5]. However, body cell mass deficiency in cirrhosis patients was found to be associated with a decrease in the abundance of *Coprococcus*, *Intestinimonas*, *Catenibacterium*, and *Barnesiellaceae* in our study, which was not reported in these earlier studies[4,5]. A decrease in the abundance of the butyrate-producing *Coprococcus* has been reported in hemodialysis patients with sarcopenia[31]. *Intestinimonas* produces butyrate and vitamin B12, and is involved in the metabolism of bile acids and glucose in hosts[32-34]. *Catenibacterium* is associated with the development of insulin resistance in morbid

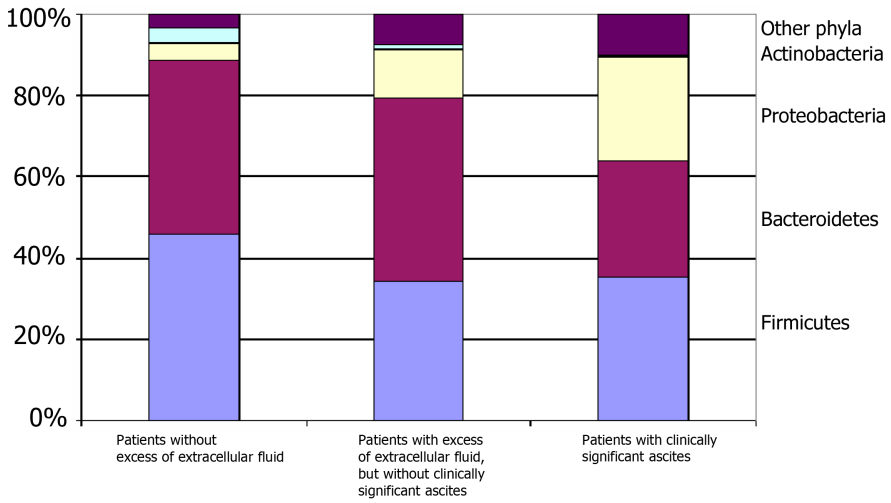


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**Figure 5 Comparison of gut microbiome at the phylum level between the groups of the patients without excess extracellular fluid, patients with excess extracellular fluid but without clinically significant ascites, and patients with clinically significant ascites.**

obesity[35]; thus, it is quite possible that a decrease in its content in the gut microbiome is associated with malnutrition. Decreased abundance of *Barnesiellaceae* and increased abundance of *Veillonella* in the gut microbiome, found in our study in patients with body cell mass deficiency, have previously been described in the general cohort of sarcopenic patients[36], but not in earlier investigations of sarcopenia in cirrhosis patients[4,5].

The pathophysiology of sarcopenia in cirrhosis has started to attract more attention[37]. The present study is the third publication to describe the changes in the gut microbiome in this condition. The major findings from all three publications partially correspond with each other, but there are also some differences between them, which highlights the need for further studies of these relationships. We did not obtain a significant correlation between the main taxa responsible for bacterial translocation (Proteobacteria and Bacilli) and a decrease in the body cell mass. This diminishes the plausibility of the hypothesis of their relationship. Unfortunately, we were unable to investigate blood levels of myostatin and ammonia and the correlations between them, the body cell mass, and taxa of the gut microbiome. Thus, the exact mechanisms of the effects of gut microbiota on muscle mass in cirrhosis should be established by further research.

An increase in the content of extracellular fluid in the body was accompanied by the frequent development of clinically significant ascites and splenomegaly. Among the large number of taxa that changes were associated with an increase in the content of extracellular fluid in patients with cirrhosis, the most important changes were an increase in the abundance of Proteobacteria and a decrease in that of Firmicutes.

An increase in the abundance of Proteobacteria and other taxa belonging to this phylum was previously described in patients with cirrhosis compared to healthy individuals in most studies[19,24,26-28,38-43]. Proteobacteria have an active endotoxin, which is believed to be associated with the development of systemic inflammation, vasodilation, and subsequent compensatory accumulation of extracellular fluid in cirrhosis[20]. Despite multiple reviews touching upon this aspect, the present study is the first to prove that an increased abundance of Proteobacteria in the gut microbiome is indeed associated with the accumulation of extracellular fluid in patients with cirrhosis.

Firmicutes are mainly represented by the class of autochthonous strict anaerobes Clostridia and the class of facultative anaerobes Bacilli. Among the Bacilli, there are many opportunistic species associated with endogenous infections in cirrhosis[44]. The abundance of Clostridia and Bacilli changes with the progression of cirrhosis: while the former decreases, the latter increases[19,28]. Therefore, the net change in the abundance of Firmicutes in cirrhosis has been reported to increase in some studies[25] and decrease in others[41]. The association of increased extracellular fluid content with decreased abundance of beneficial Clostridia was expected, but the observed association with the decreased abundance of harmful Bacilli was surprising. However, Bacilli, unlike Proteobacteria, do not produce endotoxin. Therefore, it seems that it is endotoxin, and not other factors of bacterial pathogenicity, that plays a major role in the accumulation of extracellular fluid in patients with cirrhosis.

Our study showed that fluid retention developed before the development of clinically significant ascites. At the same time, there was a further increase in the abundance of Proteobacteria with a decrease in the abundance of Bacteroidetes in patients with clinically significant ascites. We observed a stepwise change in the gut microbiome at the phylum level with an increase in the content of extracellular fluid in the body: First Proteobacteria displace Firmicutes, and then they override

Bacteroidetes (Figure 5).

The strengths of the present study are that these findings represent the first comprehensive report on the relationship between gut microbiota and changes in body composition in cirrhosis, and the first confirmation that an increased abundance of Proteobacteria is associated with increased extracellular fluid in patients with cirrhosis. In addition, this study is one of the few works that have investigated the relationship between the gut microbiome and sarcopenia in patients with cirrhosis.

The limitation of our study lies in its small sample size, although this did not prevent us from obtaining significant results.

## CONCLUSION

In conclusion, we have shown that the various body components are differently associated with changes in the gut microbiome in cirrhosis. The amount of fat mass does not depend on its composition, the amount of body cell mass is associated with changes in the abundance of its minor taxa, and the amount of extracellular fluid is associated with changes in the abundance of the main taxa of the gut microbiome (Proteobacteria, Firmicutes, and Bacteroidetes).

## ARTICLE HIGHLIGHTS

### *Research background*

Gut dysbiosis and changes in body composition (*i.e.*, a decrease in the proportion of muscle mass and an increase in extracellular fluid) are common in cirrhosis.

### *Research motivation*

To study the relationship between the gut microbiota and body composition in cirrhosis.

### *Research objectives*

To study the relationship between the gut microbiota and various body components in cirrhosis.

### *Research methods*

This observational study included 46 patients with cirrhosis. Stool microbiome was assessed using 16S rRNA gene sequencing. Multifrequency bioelectrical impedance analysis was performed to assess body composition in these patients.

### *Research results*

The abundance of *Bacteroidaceae* and *Eggerthella* increased, whereas that of *Coprococcus*, *Erysipelatoclostridiaceae*, *Intestinimonas*, *Desulfovibrio*, *Catenibacterium*, and *Senegalimassilia* decreased in the gut microbiome of patients with body cell mass deficiency. Proteobacteria abundance was increased, whereas Firmicutes, *Oscillospiraceae*, *Rikenellaceae*, *Actinobacteria*, Bacilli, *Christensenellaceae*, *Collinsella*, *Eggerthellaceae*, *Erysipelatoclostridiaceae*, *Erysipelotrichaceae*, *Anaerovoraceceae*, *Hungatella*, *Slackia*, *Peptococcaceae*, and *Senegalimassilia* abundance decreased in cirrhosis patients with excess extracellular fluid.

### *Research conclusions*

Changes in the amount of body cell mass and extracellular fluid are associated with changes in the gut microbiome in cirrhosis patients.

### *Research perspectives*

Further studies are required to establish the mechanisms underlying the influence of the gut microbiota on the value of body cell mass.

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## FOOTNOTES

**Author contributions:** Ivashkin V contributed to the research idea; Ivashkin V and Maslennikov R designed the study; all authors participated in the research and analyzed the data; Maslennikov R wrote the draft; all authors edited the draft; and Maslennikov R is the guarantor.

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**Institutional review board statement:** The present study was approved by the Ethics Committee of Sechenov University.

**Informed consent statement:** The study procedures were explained to potential participants, and written informed consent was obtained before enrollment.

**Conflict-of-interest statement:** The research was supported by Biocodex Microbiota Foundation: National Research Grant Russia 2019. No other conflicts of interest.

**Data sharing statement:** Data can be provided upon request by e-mail to the corresponding author.

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

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**Country/Territory of origin:** Russia

**ORCID number:** Roman Maslennikov 0000-0001-7513-1636; Vladimir Ivashkin 0000-0002-6815-6015; Aliya Alieva 0000-0002-7606-2246; Elena Poluektova 0000-0002-9038-3732; Anna Kudryavtseva 0000-0002-3722-8207; George Krasnov 0000-0002-6493-8378; Maria Zharkova 0000-0001-5939-1032; Yuri Zharikov 0000-0001-9636-3807.

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ORIGINAL ARTICLE

Observational Study

# Prevalence of nonalcoholic fatty liver disease and its association with age in patients with type 2 diabetes mellitus

Ryosuke Yamane, Kentaro Yoshioka, Kazuhiko Hayashi, Yuko Shimizu, Yuki Ito, Komei Matsushita, Michiyo Yoshizaki, Go Kajikawa, Taro Mizutani, Atsuko Watarai, Kosuke Tachi, Hidemi Goto

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**Ryosuke Yamane, Kazuhiko Hayashi, Yuko Shimizu, Yuki Ito, Komei Matsushita, Michiyo Yoshizaki, Go Kajikawa, Taro Mizutani, Kosuke Tachi, Hidemi Goto,** Department of Gastroenterology and Hepatology, Federation of National Public Service Personnel Mutual Aid Associations Meijo Hospital, Nagoya 460-0001, Aichi, Japan

**Kentaro Yoshioka,** Center for Liver Diseases, Federation of National Public Service Personnel Mutual Aid Associations Meijo Hospital, Nagoya 460-0001, Aichi, Japan

**Atsuko Watarai,** Department of Diabetes and Endocrinology, Federation of National Public Service Personnel Mutual Aid Associations Meijo Hospital, Nagoya 460-0001, Aichi, Japan

**Corresponding author:** Kentaro Yoshioka, MD, PhD, Director, Center for Liver Diseases, Federation of National Public Service Personnel Mutual Aid Associations Meijo Hospital, 1-3-1 Sannomaru, Naka-ku, Nagoya 460-0001, Aichi, Japan. kyoshiok@fujita-hu.ac.jp

## Abstract

### *BACKGROUND*

Type 2 diabetes mellitus (T2DM) is a risk factor for nonalcoholic fatty liver disease (NAFLD).

### *AIM*

To determine the prevalence and clinical correlates of NAFLD in a large cohort of patients with T2DM.

### *METHODS*

Four hundred thirty-seven participants with T2DM who consulted at Meijo Hospital from April 2019 to September 2020 and underwent computed tomography (CT) were assessed. The mean age was 74 ± 13 years, and 269 were men. Hepatic attenuation minus splenic attenuation ($CT_{L-S}$) less than 1 Hounsfield unit was considered fatty liver. NAFLD was defined as fatty liver in the absence of significant alcohol consumption and hepatitis virus infection. A multiple logistic regression was used to assess the independent factors associated with NAFLD.

### *RESULTS*

NAFLD was identified in 25.2% of the participants. Young age (odds ratio [OR] = −0.945; 95% confidence interval [CI]: 0.922–0.969), higher hemoglobin levels (OR = 1.501, 95%CI: 1.278–1.764), lower high-density lipoprotein (HDL) cholesterol

levels (OR = 0.971, 95%CI: 0.953–0.989), and the absence of dialysis (OR = 0.109, 95%CI: 0.014–0.856) were independent predictors of NAFLD.

*CONCLUSION*

NAFLD was detected with CT in 25.2% of the participants. NAFLD was associated with younger age, higher hemoglobin levels, lower HDL cholesterol levels, and an absence of dialysis.

**Key Words:** Age; Computed tomography; Dialysis; Hemoglobin; Nonalcoholic fatty liver disease; Type 2 diabetes mellitus

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**Core Tip:** Type 2 diabetes mellitus (T2DM) is a risk factor for nonalcoholic fatty liver disease (NAFLD). The prevalence of NAFLD by computed tomography (CT) has been reported in a few studies. The clinical correlates of NAFLD are often ambiguous. We determined the prevalence and clinical correlates of NAFLD determined by CT in a large cohort of patients with T2DM. The prevalence of NAFLD by CT was 25.2%. NAFLD was associated with younger age, higher hemoglobin levels, lower HDL cholesterol levels, and an absence of dialysis.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) frequently coexists with type 2 diabetes mellitus (T2DM). Both synergistically increase adverse outcomes[1,2]. NAFLD, T2DM, and obesity are epidemiologically correlated, but their causal interrelationships remain incompletely understood. Liu *et al*[3] proposed the hypothesis of disease subphenotyping in which genetically-driven NAFLD promotes T2DM and central obesity but protects against overall obesity. In contrast, genetically-driven T2DM and obesity increase the NAFLD risk.

A meta-analysis showed that the prevalence of NAFLD in patients with T2DM was 56% with ultrasonography (US) and proton magnetic resonance spectroscopy (MRI)[4]. The prevalence of NAFLD in patients with T2DM was highest in Europe (68%), but varies widely depending on the population. Three studies from Japan using US reported that the prevalence of NAFLD was 31%, 69%, and 61%[5-7]. The sensitivity of diagnosing NAFLD varies with the method. The prevalence of NAFLD in patients with T2DM detected by computed tomography (CT) was 10% in the United States[8], 22% in Turkey[9], and 27% in Japan[10].

In patients with T2DM, NAFLD is associated with an increased risk of overall death[11] but not with liver-related deaths[8]. Meanwhile, in NAFLD patients, T2DM is associated with advanced liver fibrosis [12] and increased mortality related to liver-related deaths[13]. NAFLD in patients with T2DM is associated with an increased risk of cardiovascular disease[7,9,10,14]. However, some reports have denied this association[8,15-17]. A positive association between NAFLD and nephropathy in patients with T2DM has been reported in some studies[18,19], while others did not find an association[20-22]. The association of NAFLD with cardiovascular risk and chronic kidney disease in the general population was reported to start in childhood[23,24].

We studied the prevalence of NAFLD in patients with T2DM in our hospital using CT and determined the factors associated with NAFLD in patients with T2DM.

## MATERIALS AND METHODS

### *Study population*

Data of patients with T2DM were retrieved from the hospital database. There were 724 Japanese diabetic patients who consulted at the Federation of National Public Service Personnel Mutual Aid Associations Meijo Hospital from April 2019 to September 2020. We excluded participants who had chronic hepatitis B ($n$ = 6) and C ($n$ = 28) infections, no assessment of hepatitis B surface antigen and

hepatitis C virus antibody ($n$ = 105), and an alcohol intake ≥ 20 g/d ($n$ = 33). We also excluded 115 participants who did not have an abdominal CT examination; thus, 437 participants (269 men and 168 women) were included in the analysis (Figure 1). The mean age was 74 ± 13 years. There were 322 patients treated with oral hypoglycemic agents, 32 with insulin, one with a glucagon-like peptide-1 receptor agonist, and 82 with diet and exercise. All patients had more than a year with T2DM.

The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committee of Federation of National Public Service Personnel Mutual Aid Associations Meijo Hospital (Approval No. 166). Written informed consent was waived because the data were analyzed anonymously based on information stored in the hospital database.

### Abdominal CT examinations

A non-enhanced CT was performed using either a 16-section multidetector scanner (Aquilion 16; Canon Medical Systems, Tochigi, Japan), a 64-section multidetector scanner (Aquilion 64; Canon Medical Systems, Tochigi, Japan), or an 80-section multidetector scanner (Aquilion Prime SP/iEdition; Canon Medical Systems, Tochigi, Japan). The CT indications were to screen for diseases in the chest and abdomen in 430 patients. The CT was performed to investigate liver diseases in seven patients. Nine and three regions of interest were positioned at the liver or spleen, respectively, to avoid macroscopic vessels. Median hepatic or splenic attenuation values were obtained. Hepatic attenuation minus splenic attenuation ($CT_{L-S}$) less than 1 Hounsfield unit was considered fatty liver[25].

### Statistical analysis

Student's t-test was used to analyze differences in continuous variables between two groups. Categorical variables were compared with the Chi-squared or Fisher's exact test. Linear regression was performed to assess the relationship between two variables. Multiple logistic regression was performed to determine the independent factors associated with the presence of NAFLD. $P$ values < 0.05 were considered significant. All analyses were performed with StatFlex version 6.0 for Windows (StatFlex, Osaka, Japan).

## RESULTS

Fatty liver was detected in 110 of 437 patients (25.2%). It was significantly associated with male gender ($P$ = 0.005), younger age ($P$ < 0.001), greater height ($P$ < 0.001) and weight ($P$ < 0.001), higher body mass index (BMI) ($P$ < 0.001), blood parameters [higher white blood cell counts ($P$ = 0.021) and higher hemoglobin ($P$ < 0.001)], altered liver function tests [higher albumin ($P$ < 0.001), higher total bilirubin ($P$ = 0.015), aspartate aminotransferase ($P$ = 0.014), alanine aminotransferase ($P$ < 0.001), and gamma glutamyl transpeptidase ($P$ < 0.001)], kidney function [lower creatinine ($P$ = 0.012) and higher estimated glomerular filtration rate (eGFR) ($P$ = 0.004)], metabolic status [higher total cholesterol ($P$ = 0.012), lower high density lipoprotein (HDL) cholesterol ($P$ = 0.039), higher triglycerides ($P$ < 0.001), and higher low density lipoprotein (LDL) cholesterol ($P$ = 0.001)], a lower fibrosis-4 (FIB-4) index ($P$ = 0.005), and non-hypertensive ($P$ = 0.013) and non-dialysis patients ($P$ = 0.003) (Table 1). Fatty liver also tended to be associated with higher HbA1c ($P$ = 0.066).

Multivariate logistic regression was used to elucidate the independent factors associated with fatty liver. Gender, age, BMI, HbA1c, white blood cell count, hemoglobin, albumin, eGFR, total cholesterol, HDL cholesterol, triglyceride, LDL cholesterol, hypertension, and dialysis therapy were analyzed. Age ($P$ < 0.001), hemoglobin level ($P$ < 0.001), HDL cholesterol level ($P$ = 0.002), and absence of dialysis ($P$ = 0.035) were independent factors associated with fatty liver (Table 2).

The patients were stratified according to age, hemoglobin level, and HDL cholesterol level, and the association with fatty liver was assessed. The prevalence of fatty liver significantly decreased with increasing age ($P$ < 0.001) (Figure 2A), significantly increased as hemoglobin levels increased ($P$ < 0.001) (Figure 2B), and was significantly higher in patients with HDL cholesterol < 70 mg/dL (29.9%) than patients with HDL cholesterol ≥ 70 mg/dL (13.8%) ($P$ = 0.012) (Figure 2C).

## DISCUSSION

The prevalence of NAFLD in patients with T2DM detected by CT was 25.2%. NAFLD was associated with younger age, higher hemoglobin levels, lower HDL cholesterol levels, and the absence of dialysis.

A previous meta-analysis found that the prevalence of NAFLD in patients with T2DM was 56% with US or proton magnetic resonance spectroscopy[4]. The prevalence of NAFLD in patients with T2DM detected by CT was 10% in the United States[8], 22% in Turkey[9], and 27% in Japan[10]. Lee *et al*[26] reported that the sensitivity of US (92%) for detecting more than 30% hepatic steatosis was higher than CT (64%). The prevalence of NAFLD in this study is comparable to the reported prevalence in patients with T2DM diagnosed by CT in Japan. In a preliminary study of 179 subjects who attended health

**Table 1 Demographics of subjects**

| Variables | ALL (*n* = 437) | Fatty liver (*n* = 110) | Non-fatty liver (*n* = 327) | *P* value |
|---|---|---|---|---|
| Gender (Male/Female) | 269/168 | 80/30 | 189/138 | 0.005 |
| Age (yr) | 74.0 ± 13.0 | 64.7 ± 13.0 | 77.1 ± 11.0 | < 0.001 |
| Height (cm) | 159.4 ± 10.2 | 163.2 ± 10.0 | 158.2 ± 9.9 | < 0.001 |
| Body weight (kg) | 61.3 ± 14.5 | 70.0 ± 16.6 | 58.5 ± 12.5 | < 0.001 |
| BMI (kg/m$^2$) | 24.0 ± 4.9 | 26.0 ± 4.7 | 23.3 ± 4.7 | < 0.001 |
| HbA1c (%) | 7.0 ± 1.2 | 7.2 ± 1.2 | 6.9 ± 1.2 | 0.066 |
| Blood glucose (mg/dl) | 164 ± 80 | 170 ± 116 | 161 ± 63 | 0.297 |
| White blood cells (/μL) | 6618 ± 2299 | 7056 ± 1649 | 6470 ± 2463 | 0.021 |
| Hemoglobin (g/dL) | 12.6 ± 2.4 | 14.2 ± 2.1 | 12.0 ± 2.2 | < 0.001 |
| Platelets (10$^4$/μL) | 20.7 ± 7.0 | 20.4 ± 5.5 | 20.8 ± 7.4 | 0.615 |
| Prothrombin time (%) | 89.6 ± 22.3 | 89.2 ± 23.6 | 89.7 ± 22.1 | 0.895 |
| Albumin (g/dL) | 3.7 ± 0.7 | 3.9 ± 0.6 | 3.6 ± 0.6 | < 0.001 |
| Total bilirubin (mg/dL) | 0.76 ± 0.50 | 0.86 ± 0.49 | 0.72 ± 0.49 | 0.015 |
| ALP (U/L) | 266 ± 156 | 247 ± 80 | 272 ± 176 | 0.182 |
| AST (U/L) | 25.3 ± 22.8 | 29.0 ± 20.2 | 23.0 ± 23.4 | 0.014 |
| ALT (U/L) | 23.5 ± 20.8 | 35.0 ± 27.4 | 19.0 ± 16.1 | < 0.001 |
| GGT (U/L) | 46.2 ± 63.1 | 68.0 ± 74.4 | 38.0 ± 56.6 | < 0.001 |
| Creatinine (mg/dL) | 1.71 ± 2.10 | 1.27 ± 1.58 | 1.85 ± 2.22 | 0.012 |
| eGFR (mL/min) | 52.0 ± 29.2 | 58.9 ± 21.9 | 49.6 ± 30.9 | 0.004 |
| Total cholesterol (mg/dL) | 181 ± 50 | 193 ± 64 | 177 ± 43 | 0.012 |
| HDL cholesterol (mg/dL) | 53 ± 17 | 50 ± 15 | 54 ± 17 | 0.039 |
| Triglyceride (mg/dL) | 160 ± 179 | 228 ± 299 | 134 ± 87 | < 0.001 |
| LDL cholesterol (mg/dL) | 101 ± 36 | 111 ± 40 | 96 ± 33 | 0.001 |
| FIB-4 index | 2.20 ± 1.56 | 1.84 ± 1.08 | 2.32 ± 1.67 | 0.005 |
| Cirrhosis (%) | 8 (1.8) | 1 (0.9) | 7 (2.1) | 0.405 |
| Hepatocellular carcinoma (%) | 7 (1.6) | 1 (0.9) | 6 (1.8) | 0.504 |
| Cerebrovascular accident (%) | 45 (10) | 9 (8.2) | 36 (11) | 0.399 |
| Cardiovasculart disease (%) | 137 (31) | 29 (26) | 108 (33) | 0.193 |
| Dyslipidemia (%) | 205 (47) | 53 (48) | 152 (46) | 0.758 |
| Hypertension (%) | 270 (62) | 57 (51) | 213 (65) | 0.013 |
| Dialysis (%) | 45 (10) | 3 (2.7) | 42 (12) | 0.003 |

Values are expessed as mean ± SD. Statistical analysis are conducted using the chi-squared test, Fisher's exact test, or Student's *t* test. ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanin aminotransferase; BMI: Body mass index; GGT: Gamma glutamyl transpeptidase; eGFR: Estimated glomelular filtration rate; HDL cholesterol: High density lipoprotein cholesterol; LDL cholesterol: Low density lipoprotein cholesterol; FIB-4 index: Fibrosis-4 index.

screening in our hospital (120 males and 59 females; 53.7 ± 10.8 years), CT detected fatty liver in 40 (22%). Thus, the prevalence of NAFLD in T2DM is higher than in the general population considering older age.

The prevalence of NAFLD significantly decreased with increasing age in this study. In contrast, Targher *et al*[14] reported that the prevalence of NAFLD detected by US in patients with T2DM increased with age; 65% among patients aged 40 to 59 and 75% among those aged 60 and older. The difference in this study compared to Targher's regarding the association of NAFLD and age may be attributed to the difference in the mean age of the participants (74 *vs* 64 years, respectively). By combining Targher's results and this study, it is suggested that the prevalence of NAFLD increases with

**Table 2 Multivariate analysis for factors associated with fatty liver**

| Variables | Odds ratio | 95% confidence interval | *P* value |
|---|---|---|---|
| Age | 0.945 | 0.922-0.969 | < 0.001 |
| Hemoglobin | 1.501 | 1.278-1.764 | < 0.001 |
| HDL cholesterol | 0.971 | 0.953-0.989 | 0.002 |
| Dialysis | 0.109 | 0.014-0.856 | 0.035 |

HDL cholesterol: High density lipoprotein cholesterol.

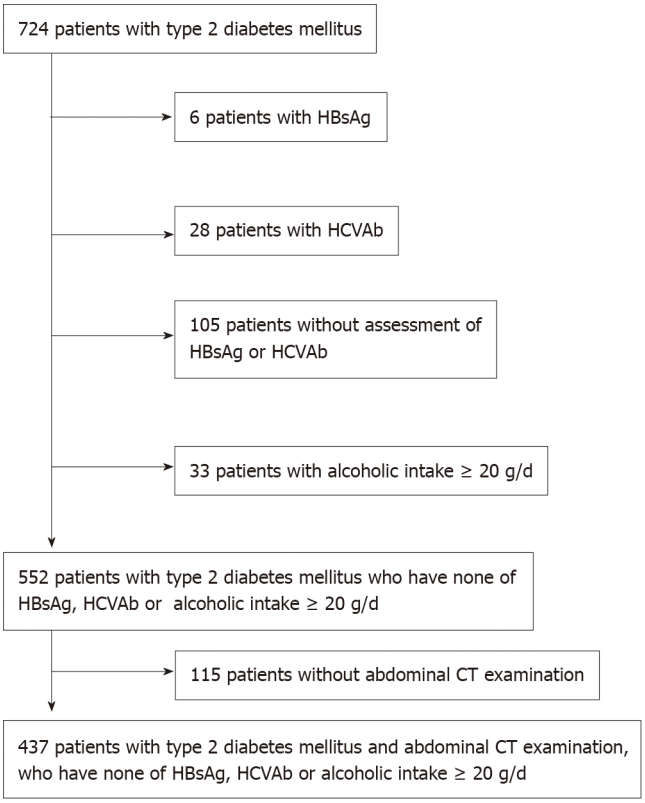


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**Figure 1 The participants in the present study consisted of 724 Japanese type 2 diabetic patients who consulted at Meijo Hospital from April 2019 to September 2020.** We excluded participants who had chronic hepatitis B ($n$ = 6) and C ($n$ = 28) infection, no assessment of hepatitis B surface antigen (HBsAg) and hepatitis C virus antibody ($n$ = 105), and alcohol intake ≥ 20 g/d ($n$ = 33). We also excluded 115 participants with no abdominal computed tomography examinations. The final analysis included 437 participants (269 men and 168 women). HCVAb: Hepatitis C virus antibody; HBsAg: Hepatitis B surface antigen; CT: Computed tomography.

age until about 60 years (as in Targher's study) and then decreases with age, as shown in this study. An "inverted U curve," in which the prevalence of NAFLD reaches a peak in late adulthood and decreases afterward has been reported in the general population[27-29]. However, a meta-analysis observed a consistent increase in the NAFLD prevalence across all age groups[30].

Poor nutritional status is more common in older people[31]. Age-related changes in appetite, health problems, and social problems predispose older adults to less food intake. Poor nutritional status in older people is also common in patients with T2DM[32]. The decreasing prevalence of NAFLD with age shown in the present study may be attributed to poor nutritional status. Age significantly negatively correlated with BMI in this study ($r$ = −0.32, $P$ < 0.001, data not shown). A high BMI is associated with NAFLD[28] and a low BMI is associated with poor nutritional status[33]. BMI was associated with NAFLD in this study by univariate analysis but not by multivariate analysis.

In this study, lower hemoglobin values were associated with a lower prevalence of NAFLD. Anemia is common in the elderly and an indicator of poor nutritional status[34]. Therefore, the lower prevalence of NAFLD in patients with lower hemoglobin values may be attributed to the poor nutritional status of these patients. Hemoglobin values also significantly positively correlated with BMI ($r$ = 0.21, $P$ < 0.001, data not shown).

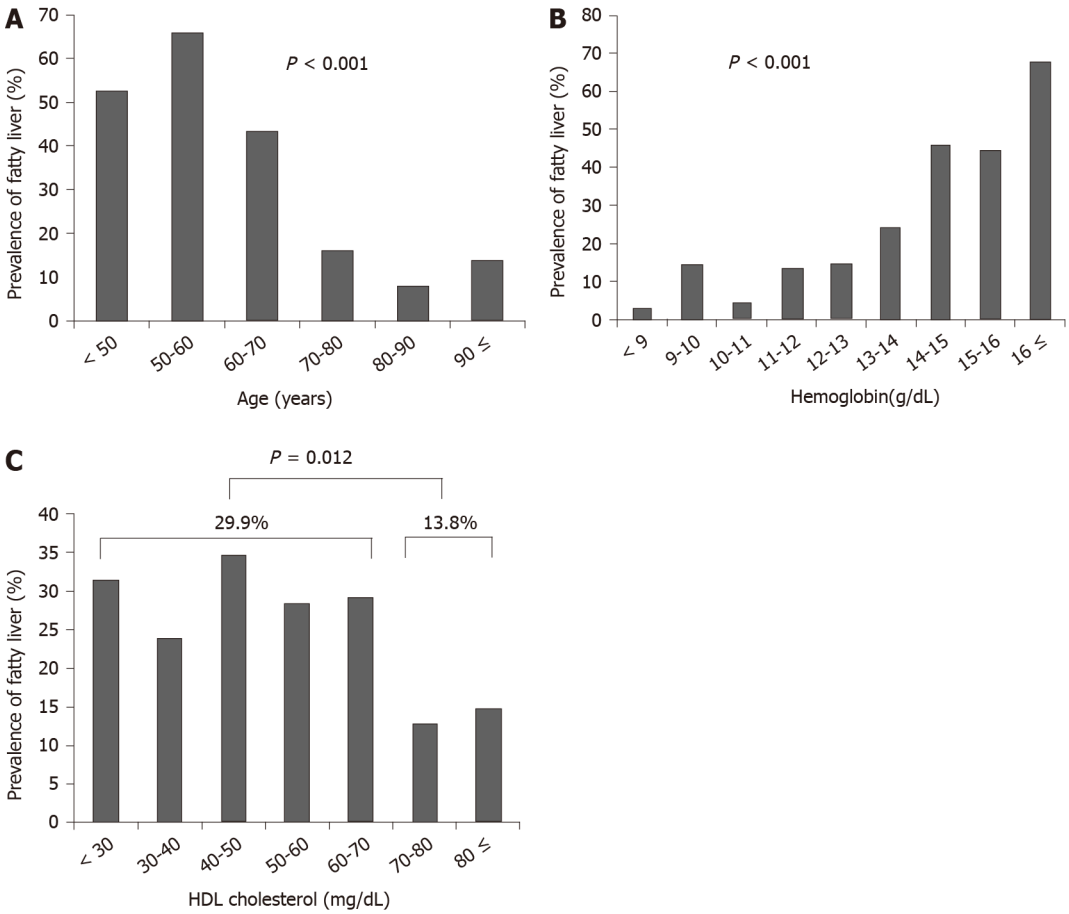


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**Figure 2 The patients were stratified according to age, hemoglobin level, and high-density lipoprotein cholesterol level, and the association with fatty liver was assessed.** A: The prevalence of fatty liver significantly decreased with age ($P < 0.001$); B: The prevalence of fatty liver significantly increased as hemoglobin increased ($P < 0.001$); C: The prevalence of fatty liver was significantly higher in patients with high-density lipoprotein cholesterol of < 70 mg/dL (29.9%) than those with HDL cholesterol ≥ 70 mg/dL (13.8%) ($P = 0.012$). HDL: High-density lipoprotein

Higher HDL cholesterol values were associated with a lower prevalence of NAFLD. This association between HDL cholesterol and NAFLD has previously been reported[28].

Dialysis treatment was associated with a lower prevalence of NAFLD. Diabetic nephropathy affects approximately 25% of patients with T2DM and is the leading cause of renal failure. Two studies reported that NAFLD is inversely associated with nephropathy in patients with T2DM, similar to this study[20,21]; however, some studies have reported a positive or no association[18,19,22]. The difference in the studied populations may cause this discrepancy. When the study population was not limited to patients with T2DM, a meta-analysis showed a positive association between NAFLD and chronic kidney disease[35]. However, in patients with T2DM, this association is still ambiguous.

This study did not find an association between NAFLD and cardiovascular disease. This association has been previously reported[7,9,10,14]; however, other studies deny it[8,15-17]. Higher HDL cholesterol values associated with a lower prevalence of NAFLD in this study reduce the risk of cardiovascular disease[36]. Thus a follow-up study may reveal an association between NAFLD and cardiovascular disease.

In this study, the number of patients with cirrhosis or hepatocellular carcinoma (HCC) was small, and cirrhosis and HCC were not associated with NAFLD. In a follow-up study of patients with T2DM (mean, 10.9 years), Adams *et al*[11] reported that 5 of 116 patients with NAFLD and none of 221 patients without NAFLD died from liver-related causes. Dunn *et al*[8] reported that NAFLD was not associated with liver-related outcomes (transplant, HCC, or encephalopathy) in patients with T2DM in a five-year retrospective cohort study. Further studies are needed to assess these associations.

There are three limitations to this study. First, it is a cross-sectional study. There may be a question of whether NAFLD decreases with age. Poor nutritional status in older people may be one reason why NAFLD decreases with age. It is also possible that we assessed a certain subpopulation of T2DM with a low risk of NAFLD and survival until older age, while the patients with a high risk of NAFLD dropped out until older age because of complications. Thus, the temporal association of NAFLD with the factors assessed in this study has to be clarified by prospective cohort studies. Second, fatty liver was diagnosed by CT in this study. The sensitivity of MRI, US, and CT for detecting a fatty liver of 5% or higher is 77%-

80%, 53%-62%, and 50% compared with liver biopsy[37]. However, a liver biopsy is invasive and has a risk of severe complications. Thus noninvasive modalities, such as US, CT, and MRI, have been commonly used to detect fatty liver. MRI is expensive and scarce. The disadvantage of US is its subjective nature. The high liver iron content increases CT Hounsfield units and may obliterate the diagnosis of fatty liver. However, CT is widely available in Japan, and the diagnosis is objective. Thus, CT is a promising modality for diagnosing fatty liver. Third, the present study was performed in a single hospital. The prevalence found has to be reevaluated in multicenter studies.

## CONCLUSION

The prevalence of NAFLD in patients with T2DM detected by CT was 25.2%. NAFLD was associated with age, hemoglobin level, HDL cholesterol level, and the absence of dialysis treatment.

## ARTICLE HIGHLIGHTS

### *Research background*

Type 2 diabetes mellitus (T2DM) is an established risk factor for the development of nonalcoholic fatty liver disease (NAFLD). Both synergistically increase adverse outcomes.

### *Research motivation*

The prevalence of NAFLD assessed by computed tomography (CT) was reported only in a few studies. The clinical correlates of NAFLD are often ambiguous.

### *Research objectives*

To determine the prevalence and clinical correlates of NAFLD assessed by CT in a large cohort of T2DM patients.

### *Research methods*

Four hundred thirty-seven participants with T2DM who consulted at Meijo Hospital from April 2019 to September 2020 and underwent CT were assessed.

### *Research results*

The prevalence of NAFLD as detected by CT was 25.2% in T2DM patients, and NAFLD was associated with a younger age, higher hemoglobin levels, lower high density lipoprotein cholesterol levels, and absence of dialysis treatment.

### *Research conclusions*

The prevalence of NAFLD in T2DM is higher than in the general population considering older age and decreases with age.

### *Research perspectives*

The association of NAFLD with age has to be clarified by prospective cohort studies.

## FOOTNOTES

**Author contributions:** All authors contributed to the conception and design of this study; Yamane R and Yoshioka K performed data collection and analysis; Yamane R wrote the first draft of the manuscript; All the authors commented the previous versions of the manuscript and read and approved the final manuscript.

**Institutional review board statement:** The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committee of Federation of National Public Service Personnel Mutual Aid Associations Meijo Hospital (Approval No. 166).

**Informed consent statement:** Written informed consent was waived because the data were analyzed anonymously based on information in the hospital database.

**Conflict-of-interest statement:** Kentaro Yoshioka is a consultant to Sanwa Kagaku Kenkyusho Co., Ltd. and received a research grant from Sumitomo Dainippon Pharma Co., Ltd. and scholarship grants from Otsuka Pharmaceutical Co., Ltd., and AbbVie GK. The remaining authors declare no conflict of interest.

**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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**Country/Territory of origin:** Japan

**ORCID number:** Ryosuke Yamane 0000-0001-5603-7791; Kentaro Yoshioka 0000-0002-5352-9851; Kazuhiko Hayashi 0000-0002-7919-6071; Yuko Shimizu 0000-0002-3753-0123; Yuki Ito 0000-0002-3094-1472; Komei Matsushita 0000-0003-1519-5376; Michiyo Yoshizaki 0000-0003-2638-1369; Go Kajikawa 0000-0001-7125-1534; Taro Mizutani 0000-0003-4969-0519; Atsuko Watarai 0000-0002-6463-9984; Kosuke Tachi 0000-0001-6464-7194; Hidemi Goto 0000-0001-8389-1416.

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SYSTEMATIC REVIEWS

# Factors early in life associated with hepatic steatosis

Sabrina Xin Zi Quek, Eunice Xiang-Xuan Tan, Yi Ping Ren, Mark Muthiah, Evelyn Xiu Ling Loo, Elizabeth Huiwen Tham, Kewin Tien Ho Siah

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**Sabrina Xin Zi Quek, Eunice Xiang-Xuan Tan, Mark Muthiah, Kewin Tien Ho Siah,** Division of Gastroenterology and Hepatology, Department of Medicine, National University Hospital, Singapore 119228, Singapore

**Eunice Xiang-Xuan Tan, Mark Muthiah,** National University Centre for Organ Transplantation, National University Health System, Singapore 119228, Singapore

**Eunice Xiang-Xuan Tan, Mark Muthiah, Kewin Tien Ho Siah,** Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

**Yi Ping Ren,** Department of Medicine, National University Hospital, Singapore 119228, Singapore

**Evelyn Xiu Ling Loo, Elizabeth Huiwen Tham,** Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore; Khoo Teck Puat-National University Children's Medical Institute, National University Hospital, National University Health System, Singapore 119228, Singapore

**Evelyn Xiu Ling Loo,** Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research, Singapore 117609, Singapore

**Corresponding author:** Kewin Tien Ho Siah, MBBS, MRCP, Assistant Professor, Doctor, Division of Gastroenterology and Hepatology, Department of Medicine, National University Hospital, 1E Kent Ridge Road, Singapore 119228, Singapore. kewin_siah@nuhs.edu.sg

## Abstract

### *BACKGROUND*

The rise in prevalence of non-alcoholic fatty liver disease (NAFLD) mirrors the obesity epidemic. NAFLD is insidious but may gradually progress from simple steatosis to steatohepatitis, fibrosis and cirrhosis and/or hepatocellular carcinoma. Intervention strategies to ameliorate developmental programming of NAFLD may be more efficacious during critical windows of developmental plasticity.

### *AIM*

To review the early developmental factors associated with NAFLD.

### *METHODS*

Databases MEDLINE *via* PubMed, and EMBASE and Reference Citation Analysis were searched and relevant publications up to April 30, 2021 were assessed. Original research studies that included risk factors associated with early development of NAFLD in human subjects were included. These factors include:

Maternal factors, intrauterine and prenatal factors, post-natal factors, genetic and ethnic predisposition, childhood and adolescence environmental factors. Studies were excluded if they were review articles or animal studies, case reports or conference abstracts, or if NAFLD was not clearly defined and assessed radiologically.

***RESULTS***

Of 1530 citations identified by electronic search, 420 duplicates were removed. Of the 1110 citations screened from title and abstract, 80 articles were included in the final analysis. Genetic polymorphisms such as patatin-like phospholipase domain-containing protein 3 (PNPLA3) and membrane-bound O-acyltransferase domain-containing protein 7 (MBOAT7) were associated with increased risk of NAFLD. Familial factors such as maternal obesogenic environment and parental history of hepatic steatosis was associated with offspring NAFLD. Longer duration of exclusive breastfeeding in infancy was associated with a lower risk of developing NAFLD later in life while metabolic dysfunction and/or obesity in adolescence was associated with increased risk of NAFLD. Studies relating to socioeconomic factors and its association with NAFLD reported confounding results.

***CONCLUSION***

Maternal metabolic dysfunction during pregnancy, being exclusively breastfed for a longer time postnatally, diet and physical activity in childhood and adolescence are potential areas of intervention to decrease risk of NAFLD.

**Key Words:** Epidemiology; Natural history; Obesity; Fatty liver; Developmental

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**Core Tip:** Prevalence of non-alcoholic fatty liver disease (NAFLD) in adolescents has more than doubled in the last two decades, with its downstream complications placing an increasing burden on healthcare systems globally. The aim of this study is to review the early developmental factors associated with NAFLD and potentially identify areas where intervention can be made to halt the progress to steatohepatitis, fibrosis and cirrhosis and/or hepatocellular carcinoma which may develop later in life.

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## INTRODUCTION

The global prevalence of non-alcoholic fatty liver disease (NAFLD) is approaching 30%, in keeping with the growing obesity epidemic[1]. With rising obesity rates worldwide, the prevalence of NAFLD is set to increase markedly in the near future. The pathogenesis of NAFLD has been considered to be a "multi-hit" disease, where epigenetic, genetic, and environmental factors may interplay to cause progressive disease. The initial hit in this multifactorial process may be early in life – during pre-conception, in-utero, infancy and early childhood[2]. For example, the predictive adaptive response hypothesis has been proposed to explain the phenomenon where poor conditions during childhood increase the risk of metabolic diseases later in life[3,4]. Being aware of factors associated with early development of NAFLD allows physicians to alter the natural course of disease progression, be it through lifestyle or pharmacological interventions. This is made even more important as the mainstay of treatment of NAFLD at present is weight loss, which is often difficult to achieve and sustain[5]. Interventional strategies to ameliorate the developmental programming of NAFLD may thus potentially be more efficacious during the critical windows of developmental plasticity, negating the need for strategies to reverse NAFLD later in life. This systematic review aims to review the early developmental factors associated with NAFLD. These factors include maternal and paternal factors, intrauterine factors, postnatal factors such as breastfeeding, lifestyle factors in adolescence including sleep, physical activity, nutrition and presently still non-modifiable factors such as genetic polymorphisms.

## MATERIALS AND METHODS

### Search strategy and data extraction

The study was carried out using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standards (Supplementary Table 1). A comprehensive search of databases and conference proceedings to identify all relevant studies up to April 30, 2021, was performed. The following electronic databases were searched: Medline *via* PubMed, Embase, and Reference Citation Analysis. We use both text words and medical subject heading terms. The literature search strategy was adapted to suit each database. Our study was only restricted to full text articles in English language. For example, on PubMed, we used the combination of the following medical subject heading terms: "non-alcoholic fatty liver disease" and "Risk" and "maternal-fetal relations" or "maternal nutritional physiological phenomena" or "maternal exposure" or "maternal-fetal exchange" or "maternal behavior" or "obesity, maternal" or "maternal age" or "maternal inheritance" or "maternal health" or "educational status" or "pregnancy complications, infectious" or "gestational weight gain" or "perinatal care" or "prenatal nutritional physiological phenomena" or "prenatal care" or "prenatal education" or "prenatal exposure delayed effects" or "prenatal diagnosis" or "embryonic and fetal development" or "growth and development" or "fetal weight" or "pregnancy" or "fetal therapies" or "parents" or "parent-child relations" or "paternal exposure" or "paternal behavior" or "paternal age" or "nutrition assessment" or "child nutrition disorders" or "nutrition disorders" or "infant nutrition disorders" or "diet, food, and nutrition" or "nutritional physiological phenomena" or "fetal nutrition disorders" or "child nutrition sciences" or "nutritional status" or "nutritional sciences" or "adolescent nutritional physiological phenomena" or "exercise".

The methods for data collection and analysis were based on the Cochrane Handbook of Systematic Reviews for Interventions. Corresponding authors were contacted through electronic mail for clarification when required. Literature search and data review was performed using a case report form independently by pairs of investigators (SQ, ET, RYP) and each article was independently inspected to verify that they met the pre-specified inclusion criteria. Duplicates were excluded. Discordance during the data extraction process was resolved by consensus between the two reviewers and or consultation with a third and senior investigator (ET, KS). The study selection process is summarized in Figure 1.

### Inclusion and exclusion criteria

Original research studies that included risk factors associated with early development of NAFLD in human subjects were included in this study. The risk factors of particular interest included: maternal factors, intrauterine and prenatal factors, post-natal factors, genetic and ethnic predisposition, childhood and adolescence environmental factors – such as (but not limited to) nutrition and physical activity. Studies were excluded if they were review articles or animal studies, case reports or conference abstracts, or if NAFLD was not clearly defined in the study. Definition of hepatic steatosis in this review was based on hepatic ultrasound, computed tomography or magnetic resonance imaging.

## RESULTS

### Search results

Of 1530 citations identified by electronic search, 420 duplicates were removed. Of the 1110 citations screened from title and abstract, 957 articles were excluded. Full text review of the 150 potentially relevant citations was performed to assess eligibility for inclusion into study and 70 reports were excluded. 80 articles were included in the final analysis (Figure 1).

### Maternal, paternal factors and intrauterine factors

Seven articles included assessment of maternal, paternal and intrauterine factors on the risk of development of NAFLD. Maternal diabetes and pre-natal obesity was associated with increased odds of offspring NAFLD[6] (Supplementary Table 1). This was also reported in Patel *et al*[7]'s study of 1215 patients, where maternal diabetes was found to be associated with offspring NAFLD, with an adjusted odds ratio (OR) of 6.74 (95%CI 2.47-18.40). Moreover, a higher pre-pregnancy body mass index (BMI) was associated with greater odds of fatty liver in offspring, even when adjusted for confounding factors, with adjusted OR 2.72 (95%CI 1.20-6.15, $P < 0.001$). Similarly, in a study from Australia[6], which studied NAFLD in adolescents at the age of 17 years, prevalence of NAFLD was associated with maternal obesity and maternal weight gain ≥ 6.0 kg by 18$^{th}$ week of gestation. These studies suggest that the in-utero environment can be associated with development of NAFLD later in life. On the contrary, a smaller study by Rajindrajith *et al*[8] reported in 499 adolescents that maternal or paternal history of metabolic syndrome was not associated with NAFLD.

Parental diagnosis of fatty liver was also associated with NAFLD in offspring. Long *et al*[9] reported that a larger proportion of patients with parental history of hepatic steatosis was diagnosed with hepatic steatosis (21.3% *vs* 12.6%, $P = 0.004$). After adjustment for confounding factors such as insulin resistance,

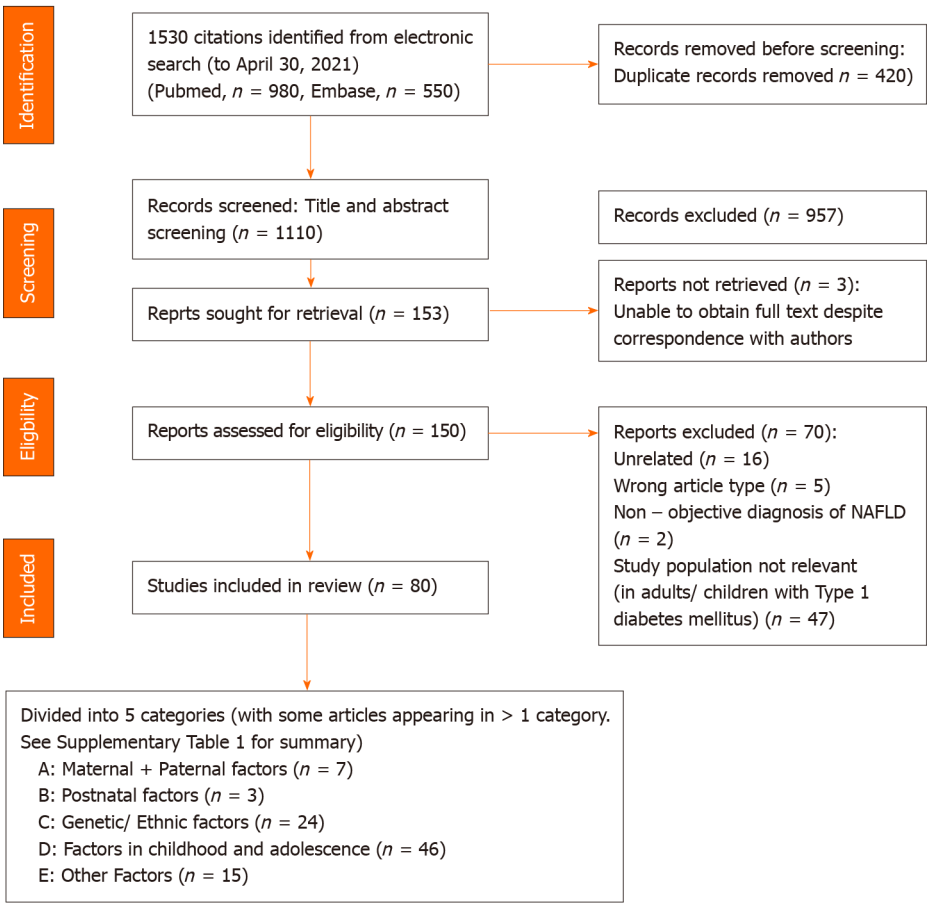


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**Figure 1 PRISMA flow diagram.**

BMI, age and gender, the odds of hepatic steatosis in an individual remained increased in individuals with at least one parent diagnosed with hepatic steatosis. Similar findings were reported in a study from India[10], which included 99 subjects. The authors reported that presence of NAFLD in one and two parents increased the odds of an offspring being diagnosed with NAFLD by 3.9 and 6.7 times respectively. Long and colleagues found that parental history of hepatic steatosis was only a significant risk factor for NAFLD among subjects without cardiometabolic risk factors: (16.1% *vs* 5.2%, $P < 0.001$) in those with no cardiometabolic coexisting disease compared to (30.3% *vs* 28.5%, $P = 0.78$) in patients with cardiometabolic risks[9].

Malnutrition pre- and postnatally was reported in two studies to be associated with prevalence of NAFLD in offsprings[6,11,12]. A study from China studied 8752 patients and classified them into various categories in relation to the Great Chinese famine in 1959-196112. They analysed the prevalence of various metabolic diseases in relation to famine exposure. In that study, authors found that the prevalence of NAFLD was higher in participants born around the time of the Great Chinese famine; prenatally (born 1960-1961) and postnatally (born 1957-1958) exposed subjects, compared to those who were non-exposed (born 1963-1964): Prevalence 23.0%, 22.9%, 17.3% respectively ($P = 0.02$). Consequently, the adjusted OR of offspring NAFLD in prenatally and postnatally exposed compared to non-exposed women for NAFLD were 2.42 (95%CI 1.14-5.16) and 1.61 (95%CI 1.29-2.01) respectively ($P < 0.05$).

Other socioeconomic factors at time of birth may be associated with NAFLD development however there is heterogeneity in available literature. For example, Ayonrinde *et al*[11] reported that lower socioeconomic status at birth was significantly associated with increased risk of NAFLD in male adolescents residing in Australia. On the other hand, a study from Sri Lanka by Rajindrajith *et al*[8] that studied 499 adolescents found that parental highest education and family income were not associated with NAFLD in adolescents.

### *Postnatal factors*

Five studies examined postnatal factors associated with NAFLD (Supplementary Table 2). Duration of breastfeeding was negatively associated with NAFLD. Rajindrajith *et al*[8] reported more subjects who had NAFLD were breastfed less than four months, compared to non-NAFLD subjects (33.3% *vs* 17.1%, $P = 0.02$). A similar finding was reported from an Australian cohort, where exclusive breastfeeding in

excess of six months was associated with decreased odds of NAFLD development at adolescence (OR 0.64, 95%CI 0.43-0.94, $P$ = 0.02)[11] which persisted after adjustment for adolescent dietary patterns. Moreover, breastfeeding without supplementary milk for ≥ 6 mo compared with < 6 mo was associated with decreased prevalence of severe steatosis (3.5% *vs* 7.7%, $P$ = 0.005). Additionally, some studies[13,14] have also reported that a more rapid increase in weight in early life is associated with NAFLD. However, these studies have not been included in this systematic review, as the diagnosis of hepatic steatosis was not determined by imaging a priori.

### *Factors in childhood and adolescence*

45 studies reported factors in childhood and adolescence that are associated with NAFLD (Supplementary Table 3). We have further divided this section into obesity as defined by BMI, other anthropometric factors, metabolic dysfunction, dietary, physical activity and sleep, and menarche in females.

**Obesity (BMI) and changes in weight:** A BMI > 25 kg/m$^2$ in Sri Lankan adolescents was shown by Rajindrajith *et al*[8] in a study to be significantly associated with NAFLD. Adolescents with NAFLD also had a higher amount of total body fat ($P$ < 0.001) and subcutaneous fat ($P$ < 0.001) than those without NAFLD. Studies from China also supported these findings[15,16]. For example, Yan *et al*[16], in a cohort study of 1350 subjects, reported that overweight or obese children were more likely to develop NAFLD in adulthood: males and females OR 2.49 (95%CI 1.51-4.11) and OR 3.34 (95%CI 1.77-6.29) respectively, $P$ < 0.001. However, in a cohort of 242 adolescents undergoing bariatric surgery of which 59% had NAFLD, Shulman *et al*[17] showed that the presence of more severe stages of NAFLD on liver biopsy was not associated with BMI in this obese cohort.

It has been widely reported in adult NAFLD literature that weight gain was strongly associated with NAFLD. Interestingly, Virtue *et al*[18] found that a gain in BMI from 7 to 13 years of age conferred an increased risk of NAFLD and suggested that this increased risk may be irrespective of initial or attained BMI. In this large study population of 244464 children, adjusting for BMI z-score at age 7 years, the hazard ratio of adult NAFLD was 1.15 (95%CI 1.05-1.26) and 1.12 (95%CI 1.02-1.23) per 1-unit gain in BMI z-score in males and females, respectively. Importantly, the increased odds of development of NAFLD seen in obese children may potentially be ameliorated by obtaining a normal BMI by adulthood [16].

**Other anthropometric/body fat measurements:** Obese adolescents with NAFLD may have a different composition compared to those without NAFLD – with greater fat body mass and visceral fat compared to those without NAFLD[19]. Besides waist circumference, other measurements such as subcutaneous adipose tissue (SCAT), intra-abdominal adipose tissue (IAAT), and anthropometric measurements including suprailiac skinfold thickness and neck circumference were shown to be associated with NAFLD. Silveira *et al*[20] studied 182 obese sedentary children and adolescents and showed that a higher IAAT but not SCAT was positively associated with NAFLD. In another study of adolescent girls, suprailiac skinfold thickness was found to be independently associated with NAFLD (OR 1.14, 95%CI 1.08-1.20, $P$ < 0.001)[21]. Peña-Vélez *et al*[22] reported neck circumference was larger in NAFLD pediatric patients compared to those without NAFLD ($P$ < 0.001) and this was found to be an independent risk factor in multivariate analysis (OR 1.172, 95%CI 1.008-1.362, $P$ = 0.038).

**Metabolic dysfunction:** Metabolic syndrome was shown to be associated with NAFLD, highlighted in four studies. In a study by Rajindrajith *et al*[8] consisting of 499 adolescents, 8.2% with NAFLD living in an urban Sri Lankan community, more children with NAFLD were to found to have metabolic derangements as compared to those without (85.8% *vs* 26.3% in controls, $P$ < 0.0001). In this study, children with NAFLD had a significantly higher waist circumference, homeostatic model assessment for insulin resistance (HOMA-IR) result and hypertriglyceridemia. These results were mirrored by other studies from Poland, Egypt and Italy[23-25]. In the study by Prokopowicz *et al*[23], which studied 108 obese hospitalized children in Poland, fasting insulin concentration was the strongest independent factor associated with NAFLD. Alkassabany *et al*[24] reported that the odds for NAFLD increased in school children aged 6 to 18 years with an increased number of components of metabolic syndrome. When there were three or more components of metabolic syndrome, the OR of NAFLD was 158.3 (95%CI 87.4-202.9, $P$ < 0.05)[24] as compared to school children without any components of the metabolic syndrome.

Most studies that investigated associations of insulin resistance and NAFLD reported a positive association with a few exceptions[19,21,26-28]. Pre-diabetes and diabetes was associated with a two-fold increased risk in non-alcoholic steatophepatitis (NASH)[25]. In a group of 520 obese children in China, it was also reported that high levels of fasting c-peptide was independently associated with NAFLD[29]. In another study, insulin like growth factor 1 (IGF-1) standard deviation scores (IGF-1 SDS) was significantly lower in adolescents with NAFLD as compared to the control group (OR 0.727, 95%CI 0.559-0.946, $P$ = 0.017)[27]. In contrast, Jimenez-Rivera *et al*[28] reported in their study of 97 obese children that while median triglyceride (TG) level was higher in those with NAFLD (1.5 ± 0.9 *vs* 1.1 ± 0.5 mmol/L, $P$ = 0.01), other factors such as HOMA-IR, hyperlipidemia and BMI were not associated with NAFLD. NAFLD was more prevalent in girls with polycystic ovarian syndrome (PCOS) compared to

girls without (37.5% *vs* 15.1%, $P$ = 0.003), and PCOS was found to be independently predictive of NAFLD (OR 2.99, 95%CI 1.01-8.82, $P$ = 0.048)[22].

**Dietary:** NAFLD is predominantly a condition associated with net caloric excess. Thus it is unsurprising that total calorie intake was found to be significantly higher in overweight children with NAFLD compared to similarly overweight children without NAFLD by (approximately 250 kcal per day)[30]. Dissecting down to the type of macronutrient composition and its association with NAFLD, the total intake of carbohydrates trended higher in overweight children with NAFLD than those without (approximately 120 kcal per day). In particular, intakes of fructose[31] and glucose were higher in overweight children with NAFLD than those without, whereas intake of fat protein and fiber was similar. Similarly, Félix *et al*[32] also showed that high amounts of refined carbohydrates in diet was independently associated with the presence of NAFLD (OR 2.17, 95%CI 1.05-6.82, $P$ = 0.038). Mosca *et al*[31] studied 271 biopsy-proven NAFLD obese children and found that fructose consumption in this group of patients was independently associated with NASH (OR 1.612, 95%CI 1.25-1.86, $P$ = 0.001) after adjustment for confounders[31]. In a study from India performed on 242 undergraduate students, soft drink consumption was associated with NAFLD. The prevalence of NAFLD was 75% in the group who consumed ≥ 2 soft drinks per day, 16% in the group that consumed 1 soft drink per day and 4% in the group that consumed < 1 soft drink per day ($P$ = 0.001). Importantly, Siddiqi and colleagues found no differences in baseline metabolic risks in those who consumed diet soft drinks which are often marketed as a healthier alternative, *vs* regular soft drinks in that study[33].

A Western diet, which is generally characterized by high intakes of take-away foods, red and processed meats, full-fat dairy products, fried potatoes, refined sugars and soft drinks, has been shown to be associated with a higher risk of NAFLD[34]. Liu *et al*[35] compared different types of diets (Chinese *vs* Western *vs* High Energy) of 1639 participants in Shandong Province, China. After adjustment for confounders, those with traditional Chinese dietary pattern had the lowest risk (OR 0.726, 95%CI 0.383-0.960, $P$ < 0.05), while the Western dietary pattern was associated with an increased risk of NAFLD (OR 1.197, 95%CI 1.013-1.736, $P$ < 0.01). On the contrary, in a study of 1170 adolescents in Australia, neither a Western dietary nor healthy dietary pattern at age 14 was associated with NAFLD at 17 years of age. Only in a subgroup of obese adolescents was a Western dietary pattern at 14 years significantly associated with NAFLD at age 17 years (OR 1.45, 95%CI 1.05-2.00, $P$ = 0.03). Nevertheless, this became insignificant when it was further adjusted for duration of breastfeeding and maternal obesity[11].

The Mediterranean diet is beneficial in the treatment of NAFLD and has been included in guidance for adult NAFLD[36]. However, this has not been widely studied in pediatric literature. Della Corte *et al*[37] studied 243 obese children and performed liver biopsy on 100 out of 166 children who had hepatic steatosis on ultrasound. In that study, it was found that low adherence to Mediterranean diet was significantly higher in patients with NASH compared with patients without NASH or hepatic steatosis. Similar findings were reported in two other studies[38,39]. Moreover, poor adherence to Mediterranean diet was also correlated with liver damage, liver inflammation and fibrosis[37].

Docosahexaenoic (DHA) supplementation was studied in a double blind, parallel group placebo-controlled randomized trial by Pacifico *et al*[40]. The final analysis at 6 mo showed that liver fat was reduced by 53.4% (95%CI 33.4-73.4) in the group that received DHA supplementation, as compared with 22.6% (95%CI 6.2-39.0) in the placebo group ($P$ = 0.04). Other metabolic factors such as fasting insulin and TG were also significantly reduced in the group treated with DHA supplementation.

**Physical activity and sleep:** A sedentary lifestyle was shown to be associated with an increased risk of NAFLD. Nier *et al*[30] compared normal weight healthy children with overweight children with NAFLD in Southern Germany aged 5 to 9 years. Normal weight children on average spent 140 min per day compared to overweight children with NAFLD who spent approximately 215 min per day on sedentary activities (*i.e.* handcrafting, drawing, reading, watching television or playing video games). While the previous study may be confounded by participants' BMI, Félix *et al*[32] also reported that a sedentary lifestyle was a significant independent predictive factor of NAFLD in a study population that only included obese children (OR 3.35, 95%CI 1.97-11.76, $P$ = 0.006). In another study by Trovato and colleagues, on multiple regression sleep shortage and wearing over-sized clothing was shown to be associated with NAFLD[39].

**Menarche:** Ryu *et al*[41] conducted a cross sectional study involving 76415 women, 9601 of whom were diagnosed with NAFLD and an inverse association between age of menarche and development of NAFLD was seen. The prevalence ratios for NAFLD for early menarche and menarche at 12, compared with menarche at 13 years were: 1.26 (95%CI 1.14-1.39) and 1.04 (95%CI 0.97-1.12) respectively, whereas prevalence ratio of menarche at 14, 15, and 16 to 18 years compared with menarche at 13 years were 0.94 (95%CI 0.89-1.00), 0.91 (95%CI 0.86-0.97), and 0.83 (95%CI 0.78-0.89), respectively ($P$ < 0.001). Even adjusting for other variables, the association between time of menarche and NAFLD persisted. Similarly, Mueller *et al*[42] reported in their study of 1214 women that one-year earlier menarche was associated with higher prevalence of NAFLD at young adulthood even after adjustment for body weight (RR 1.15, 95%CI 1.07-1.24, $P$ < 0.05).

### Genetic and ethnic factors associated with NAFLD

**Gene polymorphisms:** Several gene polymorphisms were found to be associated with NAFLD and have been extensively reported in adult NAFLD (Supplementary Table 4). Patatin-like phospholipase domain-containing protein 3 (PNPLA3) is the most widely reported. Other polymorphisms reported in the included studies in this review include: Transmembrane 6 Superfamily Member 2 (TM6SF2), heme-oxygenase-1 (HO-1), and membrane-bound O-acyltransferase domain-containing protein 7 (MBOAT7).

PNPLA3 polymorphisms and its association with NAFLD in children and adolescents were reported in several studies[25,43]. A study of 230 Italian children with obesity reported that homozygous PNPLA3 carriers (GG genotype) showed the highest risk of NAFLD (OR 14.9, 95%CI: 4.3-51.5, $P$ < 0.001). In a study comprising 1010 adolescents, Grandone *et al*[43] found that the E167K allele of TM6SF2 gene was associated with hepatic steatosis ($P$ < 0.0001). Interestingly in subjects homozygous for the PNPLA3 148M allele, carrying this rare variant of TM6SF2 showed increased odds of 12.2 (CI 3.8-39.6, P ≤ 0.001) to have raised alanine transaminase (ALT) as compared to the remaining patients[43]. This is further substantiated by Zusi and colleagues[44], who found in their cohort of 514 obese children and adolescents that genetic variants in TM6SF2 rs58542926, Glucokinase regulatory protein (GCKR) rs1260326, PNPLA3 rs738409, and Elongation of Very Long chain fatty acids-2 (ELOVL2) rs2236212 were significantly associated with a higher risk of NAFLD. These genetic variants in TM6SF2, GCKR and PNPLA3 were also found to be independently associated with NAFLD.

Multiple studies also reported associations with the MBOAT7 gene. A study by Di Sessa *et al*[45] found that carriers of the MBOAT7 T allele showed both significantly higher ALT and Pediatric NAFLD Fibrosis Index (PNFI) values compared to non-carriers. The MBOAT7 rs641738 variant was also found to exert an additive effect with PNPLA3 and TM6SF2 variants on NAFLD risk in obese children. Other variants in the MBOAT7 gene were found not to be associated NAFLD, such as with the rs641738 single nucleotide polymorphism (SNP) as reported by Lin *et al*[46]. Additionally, Di Costanzo *et al*[25] reported no association identified between hepatic fat content and MBOAT7 genotypes. In addition, the HO-1 gene promoter polymorphism was found to play an important role in the development of NAFLD. Chang *et al*[47] reported that patients 6 to 17 years with L alleles to the HO-1 gene were at higher risk of developing pediatric NAFLD (OR 18.84, 95%CI 1.45-245.22, $P$ = 0.025). SNPs analysed and found not to be associated with risk of NAFLD include rs62064119, rs2297508, rs11868035 and rs13306741 in the sterol regulatory element binding protein 1c (SREBP1c) gene as reported by Peng *et al*[48].

**Ethnic factors associated with NAFLD in adolescence and adulthood:** Few ethnic factors were also found to be associated with NAFLD. In a study conducted by Younossi *et al*[49] in the US, 11613 participants aged 20 or older were enrolled and assessed according to four major racial or ethnic groups: non-Hispanic whites, non-Hispanic blacks, Hispanics, and ''Other,'' (Aleut, Eskimo, American Indian, Asian, or Pacific Islander). Multivariate analysis showed that NASH was independently associated with being Hispanic, having a younger age, and having components of metabolic syndrome such as hypertension ($P$ < 0.05). On the other hand, smaller studies such as Lee *et al*[50] from Canada found in a study of 57 children analysing age, ethnicity, total body fat, fat-free mass, visceral fat, abdominal subcutaneous fat, and cardiorespiratory fitness, 'visceral fat' was the only factor to be independently associated with increased odds of having hepatic steatosis (OR 1.12, 95%CI 1.04-1.21, $P$ = 0.003).

### Gut microbiota

NAFLD is associated with gut microbiota dysbiosis, which has been associated with increased intestinal permeability (Supplementary Table 5). Circulating zonulin levels, a known mediator of intestinal permeability and modulating intracellular tight junctions is increased in children with NAFLD. Circulating zonulin is also significantly correlated with histological severity of hepatic steatosis[51]. Lipopolysaccharide-binding protein (LBP), possible surrogates of intestinal barrier function, were found to be significantly higher in overweight children with NAFLD than in those without[30]. In another study from Romania, Belei and colleagues investigated small intestinal bacterial overgrowth (SIBO) by glucose hydrogen breath test in 445 children[52]. NAFLD was detected in 28 of 47 (59.5%) of the SIBO positive obese group, compared to only 8 of 78 (10.2%) of the SIBO negative obese group ($P$ < 0.001) and 0/120 (0%) non-obese group ($P$ < 0.001).

### Miscellaneous factors

Other markers that have been studied in relation to NAFLD include serum 25-Hydroxyvitamin D levels uric acid levels. Two studies reported the association of serum 25-hydroxyvitamin D and NAFLD (Supplementary Table 5). The first study conducted in West Australia involved 994 adolescents, where vitamin D concentrations were measured at ages 14 and 17 years and liver ultrasonography was done at 17 years to diagnose NAFLD. In the cohort, 16% ($n$ = 156) had NAFLD, of which 51% and 17% had insufficient or deficient vitamin D status respectively[53]. Lower serum vitamin D concentrations at 17 years were significantly associated with NAFLD (independent of BMI and insulin resistance OR 0.74, 95%CI 0.56-0.97, $P$ = 0.029). The second study conducted in Turkey[54] reported 87 obese adolescents ($n$ = 42 with NAFLD) also showed an association of serum vitamin D and NAFLD. The group of adolescents with NAFLD had significantly lower measurements of serum vitamin D than the non-NAFLD group (29.5 ± 18.4 *vs* 41.0 ± 17.9 ng/mL, $P$ < 0.001).

Four studies described the association of serum uric acid levels and NAFLD. Two studies reported uric acid to be independently associated with histologically more advanced NAFLD after adjustment for measured confounders[27,31]. On the contrary, in a cross-sectional study including 129 obese children and adolescents, authors found no significant association between high levels of uric acid and NAFLD. Instead, in that study, uric acid levels was only associated with the presence of metabolic syndrome and age range. However, the severity of NAFLD was not further characterised in this study[55].

## DISCUSSION

Prevalence of NAFLD in adolescents has more than doubled in the last two decades[56]. Without early intervention, young patients with NAFLD can also develop steatohepatitis and finally decompensated cirrhosis and/or hepatocellular carcinoma later in life. Here, we have reported several studies which suggest that a maternal obesogenic environment has been associated with NAFLD in offspring. Lifestyle factors in early childhood and adolescence such as a sedentary lifestyle, sleep deficit and/or a high calorie, high carbohydrate diet are also factors associated with hepatic steatosis; while a longer period of exclusive breastfeeding, a Mediterranean diet and DHA supplementation appears to be protective. In the studies looking at gene polymorphisms, we also see that even among those who are obese and have metabolic risk factors, there are gene polymorphisms (PNPLA3, TM6SF2, HO-1 and MBOAT7) that increase the risk of NAFLD.

NAFLD may be the first silent manifestation of metabolic syndrome and predictive of other metabolic diseases, which contribute to adverse health outcomes. Metabolic syndrome and NAFLD is often linked to nutrient excess and obesity, though not all that are obese are metabolically unhealthy and vice versa. The adipose tissue expandability hypothesis by Virtue and Puig suggests that capacity to store lipids by expanding adipose tissue is variable in different individuals. When capacity is reached, adipose tissue then gets stored in ectopic tissues like the muscle and liver. Increase in visceral adipose tissue appears to be associated with metabolic disorders[17]. This concept suggests that instead of obesity in general, fat distribution, adipose tissue functionality and presence of insulin resistance are the likely key drivers of metabolic syndrome and NAFLD[18], which often exist as a continuum.

At present, there are no current FDA approved pharmacological agents to treat NAFLD. A 7%-10% weight loss is the first line treatment for adult NAFLD, which is often challenging, and difficult to maintain. Developmental plasticity is the ability where a given genotype may produce different phenotypes in response to different environments[57,58]. An exposure to a suboptimal condition during critical period of developmental programming can result in a diseased state. It is thought that the programmable windows of human obesity, which is tightly associated with NAFLD, may exist during periods of greatest weight velocity[59]. Thus, it is imperative to also target these modifiable factors associated with NAFLD early in life.

A maternal obesogenic environment has been associated with NAFLD in offspring *via* various potential mechanisms, associated with maternal insulin resistance, including inflammation, hormones and fetal hypoxia[2,60,61]. In animal model studies, it was shown that a maternal obesogenic diet was associated with fetal fatty liver in absence of fetal or maternal adiposity[62]. This is suggestive that maternal circulating hormones, lipids or cytokines could result in hepatic steatosis in-utero[63-65]. Other studies show that exposure to maternal obesogenic diet in early life was associated with an increased expression of hepatic transcription factor SREBP1c and its co-activators in offspring[66,67]. Furthermore, animal studies support that exposure to an obesogenic diet in early life may have long lasting consequences during these periods of developmental plasticity. Studies in mice and macaques have shown that in offsprings exposed to these diets, weaning to standard control diet does not completely reverse NAFLD, even in the absence of obesity in offspring[68,69].

While maternal obesity has been widely reported to be associated with NAFLD, poor nutrition in the form of undernourishment in-utero has also been associated with NAFLD in offspring[70-73]. This hypothesis was explored by Zheng *et al*[12] as summarized in the results section earlier. However, the mechanisms which potentiate metabolic dysfunction and/or NAFLD in the offspring, remains unclear. In murine studies, exposure to undernourishment in the in-utero environment increased activation of *de novo* lipogenesis is observed in parallel with the occurrence of NAFLD[74]. This was also associated with increased carbohydrate responsive element binding protein and SREBP1c expression at both transcriptional and protein levels.

Ex-utero, breastfeeding may confer some protective effects against NAFLD later in life. Breast milk contains high levels of oligosaccharides, which are complex sugars with substantial prebiotic effects for desired gut microbial growth. Additionally, it is also a rich source of long chain polyunsaturated fatty acids that has been reported to suppress de novo lipogenesis *via* inhibition of SREBP-1c[75,76]. However, these hypothesized mechanisms have yet to be clearly proven in the setting of NAFLD as protection against NAFLD.

While we present a comprehensive review of early developmental factors of NAFLD, which is not widely reported, our study is not without limitations. Firstly, there are only seven studies looking at maternal, paternal and postnatal factors associated with NAFLD. Secondly, most of the studies included

in this systematic review are cross-sectional or cohort studies, and thus an inherent limitation of such studies is its inability to prove causation. For example, in most studies that reported in-utero factors, genetic polymorphisms, a major confounding factor, was not concurrently studied. Furthermore, post-natal factors such as lifestyle factors in childhood and adolescence have been associated with NAFLD. In particular, a high calorie, high refined carbohydrate and/or Western diet have been associated with NAFLD and adherence to Mediterranean diet was protective. Hepatic steatosis develops when the rate of fatty acid uptake and synthesis is greater than the rate that the liver can oxidise and export fatty acid [77]. It is difficult to establish if such dietary choices directly affect hepatic lipid metabolism independent of obesity, as obese individuals have more free fatty acid being released from adipose tissues with increase delivery and uptake into the liver. Nevertheless, this is an inherent limitation in any observational cross sectional study. Fourthly, only patients with imaging findings of hepatic steatosis were included, and we were unable to choose studies that only included patients who had secondary causes for hepatic steatosis and other liver diseases excluded. However, we expect that the proportion of patients who have viral hepatitis would be low[78]. Lastly, NAFLD is an umbrella term consisting of simple steatosis, steatohepatitis and/or fibrosis. Those with NASH and/or fibrosis are at highest risk of cardiovascular mortality and liver related morbidity. In this review, only 5 studies further graded severity of NAFLD by histology and reported factors associated with NAFLD severity grade, thus limiting the reporting of factors associated with severe NAFLD.

In spite of these limitations, our review serves as a useful overview and identifies areas in which further interventional studies can be considered. Measures to ameliorate the developmental programming of NAFLD should be introduced in early life, during times of developmental plasticity to elicit the most effective benefits. Some potential areas for further studies would be maternal control of metabolic dysregulation, maternal dietary intake during gestation and breastfeeding for example. Furthermore, studies in lean individuals with NAFLD are needed and would help to identify risk factors without the confounder of BMI.

## CONCLUSION

In summary, our systematic review summarizes the current available literature on early developmental factors associated with hepatic steatosis. Maternal in utero environment, breastfeeding and nutritional, physical and genetic factors are associated with NAFLD. This time period in early life is potentially a time of developmental plasticity and may be a window of opportunity for early intervention to alter the natural course of this increasingly common and potentially debilitating disease.

## ARTICLE HIGHLIGHTS

### *Research background*

Prevalence of non-alcoholic fatty liver disease (NAFLD) in adolescents has more than doubled in the last two decades, with its downstream complications placing an increasing burden on healthcare systems globally.

### *Research motivation*

At present, there is a paucity of treatment options NAFLD. In line with the developmental origins of heath and disease (DOHaD) concept, we hope to identify factors in early life where possible intervention can be instituted to prevent the development of NAFLD later in life.

### *Research objectives*

To review the early developmental factors associated with NAFLD and potentially identify areas where intervention can be made to halt the progress to steatohepatitis, fibrosis and cirrhosis and/or hepato-cellular carcinoma which may develop later in life.

### *Research methods*

Original research studies that included risk factors associated with early development of NAFLD in human subjects were identified from databases MEDLINE *via* PubMed, and EMBASE and relevant publications up to April 30, 2021 were assessed.

### *Research results*

Genetic polymorphisms, familial factors such as maternal obesogenic environment and parental history of hepatic steatosis was associated with offspring NAFLD. Longer duration of exclusive breastfeeding in infancy was associated with a lower risk of developing NAFLD later in life while metabolic dysfunction and/or obesity in adolescence was associated with increased risk of NAFLD.

### *Research conclusions*

Our systematic review summarizes the current available literature on early developmental factors associated with hepatic steatosis. Maternal in utero environment, breastfeeding and nutritional, physical and genetic factors are associated with NAFLD.

### *Research perspectives*

Maternal metabolic dysfunction during pregnancy, being exclusively breastfed for a longer time postnatally, diet and physical activity in childhood and adolescence are potential areas where research and interventions can be explored to prevent the development of NAFLD. Studied in lean individuals with NAFLD are needed and would help to identify risk factors without the confounder of BMI.

## FOOTNOTES

**Author contributions:** Tan EXX, Quek SXZ, Kewin STH contributed to study design; Tan EXX, Quek SXZ, Ren YP contributed to data acquisition and data analysis; Kewin STH contributed to study concept and study supervision; All authors contributed to manuscript drafting; Tan EXX, Quek SXZ contributed equally to this manuscript.

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**Country/Territory of origin:** Singapore

**ORCID number:** Sabrina Xin Zi Quek 0000-0001-5377-1528; Eunice Xiang-Xuan Tan 0000-0002-3757-4986; Yi Ping Ren 0000-0003-1241-4537; Mark Muthiah 0000-0002-9724-4743; Evelyn Xiu Ling Loo 0000-0001-7690-3191; Elizabeth Huiwen Tham 0000-0003-1037-6143; Kewin Tien Ho Siah 0000-0002-2795-5101.

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META-ANALYSIS

# Efficacy and safety of sofosbuvir/velpatasvir with or without ribavirin in hepatitis C genotype 3 compensated cirrhosis: A meta-analysis

Jing Hong Loo, Wen Xin Flora Xu, Jun Teck Low, Wei Xuan Tay, Le Shaun Ang, Yew Chong Tam, Prem Harichander Thurairajah, Rahul Kumar, Yu Jun Wong

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**Jing Hong Loo, Wen Xin Flora Xu, Jun Teck Low, Wei Xuan Tay, Le Shaun Ang, Prem Harichander Thurairajah,** Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119077, Singapore

**Yew Chong Tam,** Education Resource Center, Medical Board, Singapore General Hospital, Singapore 100059, Singapore

**Prem Harichander Thurairajah,** Department of Gastroenterology and Hepatology, National University Hospital, Singapore 119077, Singapore

**Rahul Kumar, Yu Jun Wong,** Department of Gastroenterology and Hepatology, Changi General Hospital, Singapore 529889, Singapore

**Rahul Kumar, Yu Jun Wong,** Duke-NUS Medicine Academic Clinical Program, Singapore 100059, Singapore

**Corresponding author:** Yu Jun Wong , FRCP, MD, Consultant Physician-Scientist, Department of Gastroenterology and Hepatology, Changi General Hospital, Singhealth, 2, Simei Street 3, Singapore 529889, Singapore. eugene.wong.y.j@singhealth.com.sg

## Abstract

***BACKGROUND***

Hepatitis C virus (HCV) is a leading cause of liver cirrhosis and hepatocellular carcinoma globally. Sofosbuvir/velpatasvir (SOF/VEL) is an effective pan-genotypic direct-acting antiviral combination for treatment of chronic HCV infection. While the addition of ribavirin (RBV) to SOF/VEL improved sustained virological response (SVR12) in genotype 3 (GT3) decompensated cirrhosis patients, the benefits of RBV in GT3 compensated cirrhosis patients receiving SOF/VEL remains unclear.

***AIM***

To evaluate the efficacy and safety of SOF/VEL, with or without RBV in GT3 compensated cirrhosis patients.

***METHODS***

We searched four electronic databases (PubMed/Medline, Embase, Cochrane Library and Web of Science) from inception up to June 2021 using both free text and MeSH terms. There was no restriction on language, geography, publication

dates and publication status (full text or abstracts). All GT3 compensated cirrhosis patients treated with 12 wk of SOF/VEL, with or without RBV, were included, regardless of age, gender or prior treatment experience. The primary outcome was sustained virological response 12-wk post-treatment (SVR12). The secondary outcome was treatment-related adverse events, as defined by symptomatic anemia requiring transfusion or a drop in hemoglobin beyond 2 g/dL. The pooled relative risk (RR), 95%CI and heterogeneity ($I^2$) were estimated using Review Manager version 5.3.

***RESULTS***

From 1752 citations, a total of seven studies (2 randomized controlled trials, 5 cohort studies) with 1088 subjects were identified. The SVR12 was similar in GT3 compensated cirrhosis patients, regardless of the use of RBV, for both the intention-to-treat RR 1.03, 95%CI: 0.99-1.07; $I^2$ = 0%) and the per-protocol analysis (RR: 1.03, 95%CI: 0.99-1.07; $I^2$ = 48%). The overall pooled rate of treatment-related adverse events was 7.2%. Addition of RBV increased the pooled risk of treatment-related adverse events in GT3 compensated cirrhosis patients receiving SOF/VEL (RR: 4.20, 95%CI: 1.29-13.68; $I^2$ = 0%). Subgroup analysis showed that RBV was associated with a higher SVR12 in GT3 compensated cirrhosis patients with baseline resistance-associated substitutions. However, addition of RBV did not significantly increase the SVR12 among treatment-experienced GT3 compensated cirrhosis patients.

***CONCLUSION***

Ribavirin was not associated with higher SVR12 in GT3 compensated cirrhosis patients receiving SOF/VEL. Our findings suggest a limited role for RBV as routine add-on therapy to SOF/VEL in GT3 compensated cirrhosis patients.

**Key Words:** Direct-acting antiviral; Hepatitis C; Cirrhosis; Sofosbuvir/velpatasvir

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**Core Tip:** Ribavirin (RBV) as routine add-on therapy was not associated with higher sustained virological response in genotype 3 (GT3) compensated cirrhosis patients receiving sofosbuvir/velpatasvir (SOF/VEL), except in the subgroup of patients with baseline resistance-associated substitution mutation. As RBV is associated with a higher risk of treatment-related adverse event, RBV as routine add-on therapy to SOF/VEL should be reconsidered among compensated GT3 cirrhosis patients.

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## INTRODUCTION

Hepatitis C virus (HCV) is an important cause of liver cirrhosis and hepatocellular carcinoma, affecting 71 million people globally[1]. Genotype 3 (GT3) is the second most common HCV genotype worldwide and is responsible for up to 30% of global HCV infections, especially in South and Central Asia[2,3]. GT3 HCV is associated with a higher incidence of liver steatosis[4], fibrosis progression[5] and liver cirrhosis [6]. GT3 HCV infection was also associated with poorer prognosis with an 80% increased risk of hepatocellular carcinoma[6] and 17% increased risk of all-cause mortality compared to other HCV genotypes [7].

The introduction of direct-acting antiviral (DAA) therapy has significantly improved the treatment success for HCV infection, thus providing a simplified approach for global HCV elimination. The improvement in treatment outcome was observed since the first generation of DAA, albeit to a lesser degree among GT3 HCV patients with cirrhosis or prior treatment experience[8,9]. Because of the poorer treatment response among GT3 HCV patients treated with DAA, GT3 HCV infection was considered the difficult-to-treat population. Currently, there are two approved pan-genotypic DAA regimens available, namely sofosbuvir and velpatasvir (SOF/VEL), as well as glecaprevir and pibrentasvir. While both regimens are highly efficacious with sustained virological response 12-wk post-treatment (SVR12) rates beyond 95% in most scenarios, only SOF/VEL is approved to treat decompensated HCV cirrhosis patients[10,11].

The potential of ribavirin (RBV) as add-on therapy to SOF/VEL to improve SVR12 in HCV patients remains an area of interest. Ribavirin, a guanosine nucleoside analog, has been used in HCV treatment regimens since the pre-DAA era. It is postulated that RBV interferes with viral replication by direct and indirect means. RBV directly inhibits viral mRNA polymerase by binding to the nucleotide binding site of the enzyme and indirectly, by inducing error prone mutagenesis and promoting T-helper-type-1-mediated immune responses[12]. The addition of RBV to a SOF/VEL regimen improves SVR rates where there is pre-existing baseline NS5A Y93H resistance-associated substitutions (RAS). The ASTRAL-3 study reported an SVR of 97% *vs* 84% in patients with or without baseline RAS[13]. Indeed, American Associated for the Study of Liver Disease (AASLD) guidelines recommend adding RBV for compensated GT3 cirrhosis with baseline RAS or decompensated HCV cirrhosis, regardless of genotype [14]. While the use of RBV significantly increases the SVR12 in decompensated cirrhosis receiving SOF/VEL[15], the benefit of RBV remains controversial among GT3 compensated cirrhosis patients. A Spanish randomized controlled trial had demonstrated a comparable SVR12 among GT3 compensated cirrhosis patients treated with SOF/VEL, regardless of the use of RBV[16].

In routine clinical practice, the application of pretreatment RAS testing for patients with GT3 compensated cirrhosis is often limited by their cost and availability. Moreover, such a strategy should be balanced with the need for closer monitoring for adverse events from RBV such as anemia[17]. In order to address these gaps, we performed a systematic review and meta-analysis to compare the efficacy and safety of RBV in GT3 compensated cirrhosis patients treated with SOF/VEL.

## MATERIALS AND METHODS

### Eligibility and search strategy

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline for data extraction and reporting[18]. All potential literature was identified from a comprehensive search of four electronic databases, namely PubMed/Medline, Embase, Cochrane and Web of Science, from initiation up to 1 June 2021, with the help of a medical librarian. There was no restriction on language, geography, publication dates and publication status (full text and abstract). The search keywords included a combination of "sofosbuvir", "velpatasvir", "ribavirin", and "hepatitis C" using both the free text and MeSH terms as detailed in Supplementary Table 1. Additionally, a relevant search by Reference Citation Analysis (https://www.referencecitationanalysis.com) was conducted. All GT3 compensated cirrhosis patients treated with 12 wk of SOF/VEL, with or without RBV, were included, regardless of age, gender or prior treatment experience. References of all included studies were manually searched for additional studies. We also included grey literature from abstracts published in major conferences from 2015 to 2020.

### Study selection

In this meta-analysis, we included all studies that met the following inclusion criteria: (1) Studies that evaluated patients with hepatitis C GT3 compensated cirrhosis; (2) studies that evaluated the efficacy or safety of SOF/VEL, with or without RBV; and (3) reported SVR12, and/or treatment-related adverse events as study outcomes. We excluded case reports, case series, review articles, editorials, guidelines, and animal or pediatric studies. Two authors independently performed the initial screening of titles and abstracts during the primary search. The full texts of all relevant studies were extracted and reviewed. Any discrepancy in the article selection was resolved by consensus and discussion with a third coauthor.

### Data extraction

The data from each study were independently extracted by two authors from the included studies using a predefined standardized form. The data extracted included study design, sample size, demographic of study participants, GT3 subtypes, coinfection with human immunodeficiency virus (HIV), baseline RAS, history of prior treatment, SVR12, and treatment-related adverse events. Treatment-related adverse events were defined as symptomatic anemia requiring transfusion or a drop in hemoglobin > 2 g/dL due to RBV. Corresponding authors were contacted in the event of any missing information.

### Data synthesis and analysis

We used Review Manager Software version 5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) to perform our meta-analysis. The effect measures were presented as relative risk ratio (RR) and their respective 95%CI. The meta-analysis was analyzed using the random-effects model as the *a priori* model. $P < 0.05$ was considered to be statistically significant. The statistical heterogeneity was evaluated using Cochran's $Q$ test and $I^2$ statistics[19]. We defined substantial heterogeneity across the study when $P$ was < 0.10 in the Cochran $Q$ test and $I^2 > 50\%$. Publication bias of the primary outcome was assessed based on funnel plot symmetry.

Prespecified subgroup analyses were performed based on study design [randomized controlled trial (RCT) *vs* non-RCT] and publication status (full text *vs* abstracts). Because non-RCT and abstracts are more susceptible to selection and recall bias, we also performed sensitivity analyses to estimate the effect size by the serial exclusion of individual studies and using a fixed-effect model to assess the reliability of our findings.

### Risk of bias assessment

We used the Cochrane Risk of Bias 2.0 tool to assess randomized studies based on sequence generation, allocation concealment, performance bias, detection bias and reporting bias[20]. The Newcastle–Ottawa Scale was used to assess cohort studies based on selection, comparability and exposure[21]. Based on a total score ≥ 7, 4-6 or ≤ 3, each cohort study was classified as low, moderate or high risk of bias, respectively. Two authors independently assessed the risk of bias of all included studies. All discrepancy in risk of bias assessment was resolved by consensus with a third coauthor.

## RESULTS

### Search results and population characteristics

A total of 1752 citations were identified using our search strategy (Supplementary Figure 1). After removing duplicates and the title screen, we included a total of 69 studies for full-text review. Sixty-two studies were excluded for the following reasons: decompensated cirrhosis as study population ($n$ = 6); intervention did not involve SOF/VEL and RBV ($n$ = 42); and no comparison of outcomes by genotype ( $n$ = 14). Finally, seven studies fitted our inclusion criteria, as shown in the PRISMA flowchart (Supplementary Figure 1).

### Characteristics and quality of studies

Seven studies, including 1,088 subjects (506 in the SOF/VEL with RBV group and 582 in the SOF/VEL without RBV group), were included in the final analysis. Five studies were published as full manuscripts[16,22-25], and two were published as abstracts[26,27]. The patient characteristics of all included studies are summarized in Table 1. The proportion of patients with GT3a and GT3b subtype was 99.5% and 0.5%, respectively[15]. The pooled rate of HIV coinfection was 13.0% (35/269)[16,23]. Overall, the proportion of subjects with baseline NS5A RASs mutation and prior treatment history was 6.4% (17/264) and 39.6% (127/321), respectively. The proportion of patients with baseline RAS mutation and prior treatment were comparable between the intervention and control groups[16,23]. Four studies had a low risk of bias (Supplementary Figure 2 and Supplementary Table 2). Three studies have a moderate risk of bias due to concerns over the severity of liver disease between intervention and control groups[24,26,27].

### SVR12

All seven studies (1088 subjects) reported SVR12 in GT3 compensated cirrhosis patients treated with SOF/VEL. The overall pooled rate of SVR12 based on intention-to-treat (ITT) and per-protocol (PP) analysis was 95.5% (462/484) and 95.4% (974/1021), respectively. The SVR12 was similar regardless of the use of RBV in GT3 compensated cirrhosis based on ITT (RR: 1.03, 95%CI: 0.99-1.07; $I^2$ = 0%) (Figure 1) and PP (RR: 1.03, 95%CI: 0.99-1.07; $I^2$ = 48%) analysis (Figure 2). The SVR12 remained comparable when subgroup analysis was performed based on study design, with less heterogeneity observed among RCTs (RR: 1.06, 95%CI: 1.00-1.13; $I^2$ = 0%) (Table 2).

### Treatment-related adverse events

The overall pooled rate of treatment-related adverse events was 7.2% (95%CI: 4.4-11.0)[16,24]. Treatment with SOF/VEL plus RBV increases the pooled risk of treatment-related adverse events compared to SOV/VEL without RBV (RR: 4.20, 95%CI: 1.29-13.68; $I^2$ = 0%) (Figure 3).

### Subgroup analysis

**Treatment-experienced:** The overall SVR12 among treatment-experienced GT3 compensated cirrhosis patients was 96.4%[16]. The use of RBV did not result in a higher SVR12 among treatment-experienced GT3 compensated cirrhosis patients (96% *vs* 96%).

**Baseline RAS mutation:** Baseline RAS testing was performed in 17.0% of subjects, from two studies[16,22]. Among those with baseline RAS mutation, the addition of RBV was associated with a higher SVR12 in patients treated with SOF/VEL (96% *vs* 87%, $P$ = 0.12).

### Validation of meta-analysis results

We performed sensitivity analysis to assess whether an individual study had a dominant effect on the overall pooled results. No individual study with a dominant effect was detected after serial exclusion of

**Table 1 Baseline characteristics of included studies**

| Ref. | Study design | Sample size (*n*) | Age | Subtypes (3A/3B), (%) | Co-infection with HIV (%) | Baseline NS5A RAS (%) | Prior treatment (%) | SVR12 ITT (%) | SVR12 PP (%) | Treatment-related adverse event (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Pianko *et al* [22], 2015 | RCT | 52 | I: 54.0 (44-65); C: 56 (45-68) | NR | 0 | NR | I: 100.0; C: 100.0 | I: 96.2; C: 88.5 | I: 96.2; C: 88.5 | NR |
| Esteban *et al* [16], 2018 | RCT | 204 | I: 51 ± 7.6; C: 51 ± 7.3 | I: 100.0/0.0; C: 99.0/1.0 | I: 15.5; C: 13.9 | I: 21.8; C: 19.4 | I: 27.2; C: 26.7 | I: 96.1; C: 91.1 | I: 96.1; C: 92.0 | I: 4.9; C: 1.0 |
| von Felden *et al*[23], 2018 | Cohort study | 65 | NR | NR | I: 2.9; C: 13.3 | I: 11.4; C: 0.0 | I: 37.1; C: 23.3 | I: 100.0; C: 96.7 | NR | NR |
| Drysdale *et al*[26], 2019 | Cohort study | 414 | NR | NR | NR | NR | NR | NR | I: 98.0; C: 91.7 | NR |
| Pasulo *et al* [27], 2019 | Cohort study | 130 | NR | NR | NR | NR | NR | NR | I: 93.9; C: 98.4 | NR |
| Hlaing *et al* [24], 2019 | Cohort study | 60 | NR | NR | NR | NR | NR | NR | I: 100.0; C: 96.0 | I: 31.4; C: 8.0 |
| Wong *et al* [30], 2020 | Cohort study | 163 | NR | NR | NR | NR | NR | I: 97.8; C: 97.5 | I: 97.8; C: 97.5 | NR |

HIV: Human immunodeficiency virus; NS5A: Non-structural protein 5A; RAS: Resistance-associated substitution; SVR12: Sustained virological response 12 wk post treatment; ITT: Intention-to-treat; PP: Per-protocol; RCT: Randomized controlled trial; I: Intervention (sofosbuvir/velpatasvir + ribavirin), C: Control (sofosbuvir/velpatasvir), NR: Not reported.

**Table 2 Subgroup analysis**

| Outcome | Subgroup | | No. of studies | Effect size (RR with 95%CI) | $I^2$ |
|---|---|---|---|---|---|
| SVR12 (ITT analysis) | Overall | | 4 | 1.03 (0.99-1.07) | 0 |
| | Study design | RCT | 2 | 1.06 (0.99-1.13) | 0 |
| | | Non-RCT | 2 | 1.01 (0.97-1.06) | 0 |
| | Effect estimates | Fixed model | 4 | 1.04 (1.00-1.08) | 0 |
| | | Odd's ratio | 4 | 2.32 (0.91-5.89) | 0 |
| SVR12 (PP analysis) | Overall | | 6 | 1.03 (0.99-1.07) | 48 |
| | Study design | RCT | 2 | 1.06 (1.00-1.13) | 0 |
| | | Non-RCT | 4 | 1.02 (0.97-1.07) | 65 |
| | Publication type | Full-text | 4 | 1.00 (0.96-1.04) | 0 |
| | | Abstract | 2 | 0.99 (0.88-1.10) | 86 |
| | Effect estimates | Fixed model | 6 | 1.04 (1.01-1.07) | 48 |
| | | Odd's ratio | 6 | 2.36 (1.07-5.19) | 14 |

SVR12: Sustained virological response 12 wk post treatment; ITT: Intention-to-treat; PP: Per-protocol; RCT: Randomized controlled trial.

individual studies. Our findings remained consistent when analysis was performed using a fixed-effect model and OR as the effect measure (Table 2). Based on $I^2$ analysis for heterogeneity, significant statistical heterogeneity was noted with the analysis for SVR12 for PP cohorts, which was reduced when only RCTs were considered. The funnel plot did not reveal significant publication bias for our primary outcome (Supplementary Figure 3).

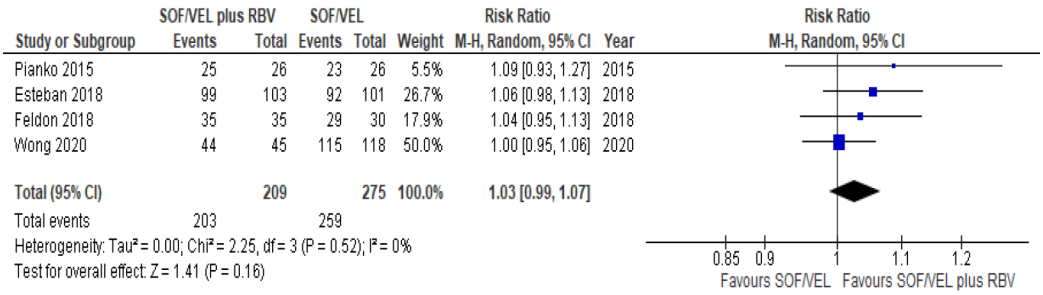


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**Figure 1 Sustained virological response by intention-to-treat analysis from sofosbuvir/velpatasvir with or without ribavirin.** SOF/VEL: Sofosbuvir/velpatasvir; RBV: Ribavirin.

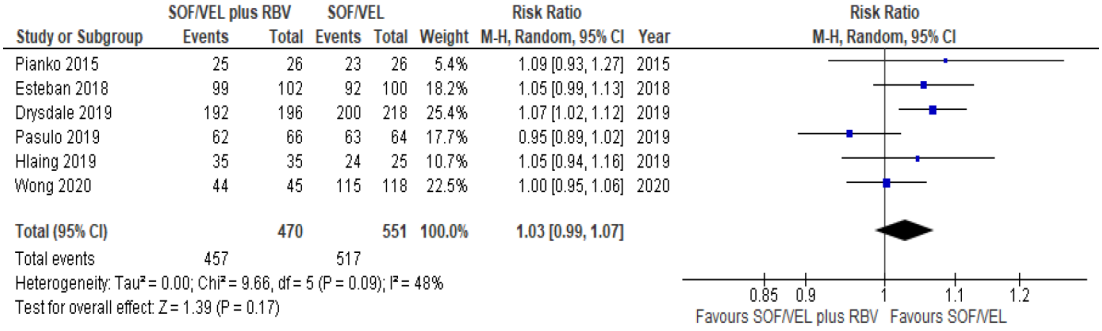


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**Figure 2 Sustained virological response by per-protocol analysis from sofosbuvir/velpatasvir with or without ribavirin.** SOF/VEL: Sofosbuvir/velpatasvir; RBV: Ribavirin.

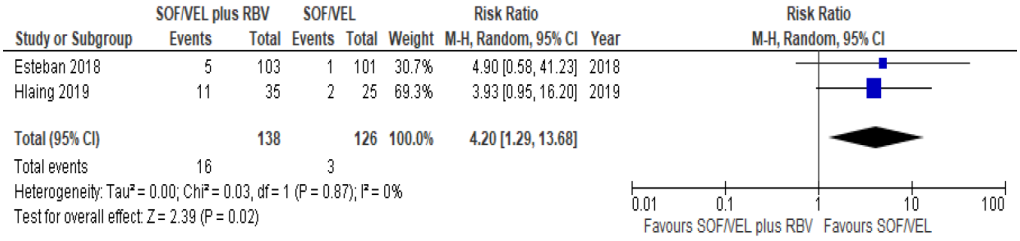


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**Figure 3 Severe adverse events from sofosbuvir/velpatasvir with or without ribavirin.** SOF/VEL: Sofosbuvir/velpatasvir; RBV: Ribavirin.

## DISCUSSION

GT3 HCV cirrhosis is considered the last frontier of HCV microelimination in the era of DAA use. Not only is GT3 the second most common genotype globally, affecting 45 million HCV patients worldwide [28], it has also been associated with significantly poorer outcomes, including higher risk of steatosis, faster progression to cirrhosis, and accelerated progression to hepatocellular carcinoma[29]. The benefit of RBV among GT3 compensated cirrhosis receiving SOF/VEL remains controversial. While the European Association for the Study of the Liver guidelines recommend routine RBV use, the AASLD guidelines recommend RBV only when baseline RAS mutation is present.

In this meta-analysis, we found that RBV has a limited role as a routine add-on therapy in GT3 compensated cirrhosis treated with SOF/VEL. The overall SVR12 was similar, regardless of the use of RBV. This finding remained robust when subgroup analysis was performed based on study design and prior treatment experience. In terms of safety, the addition of RBV increased the pooled risk of treatment-related adverse events, defined as symptomatic anemia requiring transfusion or a drop in hemoglobin > 2 g/dL. Five studies reported severe adverse events, defined as the need for hospitalization, intensive care unit, permanent disability, death and treatment cessation[16,22-25]. Overall, treatment-related severe adverse events were rare (0.8%) and were comparable regardless to the use of

RBV. The most common minor adverse event was asthenia, followed by headache[16,24].

Our findings suggest that the routine use of RBV in GT3 compensated cirrhosis patients treated with SOF/VEL should be reconsidered. Similar findings were observed in real-world studies demonstrating high SVR12 of around 95% in GT3 compensated cirrhosis patients, regardless the use of RBV[25,30]. Given the limited benefit and higher risk of treatment-related adverse events with RBV use, 12 wk of SOF/VEL among GT3 compensated cirrhosis patients provides a simplified approach to safely omit the need for routine genotype and resistance testing, thus allowing rapid treatment upscale[31]. Meanwhile, retreatment using the combination of SOF, VEL and voxilaprevir has also been shown to be an efficacious strategy, both in clinical trials and real-world settings[32,33].

There were several strengths in our meta-analysis. First, we conducted a comprehensive search of four electronic databases, including grey literature, with the help of a medical librarian. All relevant data were extracted independently using a predefined template to compare both the efficacy and safety of RBV and SOF/VEL in GT3 compensated cirrhosis patients. All corresponding authors were contacted for any missing data through emails. All included studies were homogeneous in terms of patient characteristics, intervention, and outcome measures. Finally, our findings remained robust under various permutations of sensitivity analysis. To our knowledge, this is the first meta-analysis evaluating the safety and efficacy of adding RBV to SOF/VEL, specifically among GT3 compensated cirrhosis patients.

We acknowledge that there were limitations to this study. First, the number of subjects with baseline RAS mutations tested were small and only derived from two studies[16,22]. Although SVR12 was higher in the RBV group, it did not achieve statistical significance. Moreover, few papers reported the specific side effects during the treatment period, thus it was not possible to investigate the dose-dependent effect of RBV. We were unable to exclude indication bias among the nonrandomized trials. Although the decision to initiate RBV may be confounded by indication bias, our findings were consistent between RCTs and non-RCTs. Finally, more studies are needed to investigate the treatment outcome among GT3b patients because GT3b are under-represented from the existing literature[34].

## CONCLUSION

Among GT3 compensated cirrhosis patients, adding RBV to 12 wk of SOF/VEL did not significantly increase SVR12. As RBV was associated with a higher risk of treatment-related adverse events, routine addition of RBV among GT3 compensated cirrhosis patients receiving SOF/VEL should be reconsidered.

## ARTICLE HIGHLIGHTS

### *Research background*

With direct-acting antiviral therapy that is safe, effective and simple to use, future research should address linkage of care of hepatitis C virus (HCV) to achieve elimination.

### *Research motivation*

As ribavirin (RBV) is associated with a higher risk of treatment-related adverse events, RBV as routine add-on therapy to sofosbuvir/velpatasvir (SOF/VEL) should be reconsidered among compensated genotype 3 (GT3) cirrhosis patients.

### *Research objectives*

RBV as routine add-on therapy was not associated with higher sustained virological response at 12 wk post-treatment (SVR12) in GT3 compensated cirrhosis patients receiving SOF/VEL.

### *Research methods*

Systematic review and meta-analysis.

### *Research results*

Our study aimed to evaluate the efficacy and safety of SOF/VEL, with or without RBV in GT3 compensated cirrhosis patients.

### *Research conclusions*

In routine clinical practice, the application of pretreatment resistance-associated substitution testing for patients with GT3 compensated cirrhosis is often limited by cost and availability. Moreover, such a strategy should be balanced with the need for closer monitoring for adverse events from RBV such as anemia. In order to address these gaps, we performed a systematic review and meta-analysis to compare the efficacy and safety of RBV in GT3 compensated cirrhosis patients treated with SOF/VEL.

### *Research perspectives*

SOF/VEL is an effective pan-genotypic direct-acting antiviral combination for the treatment of chronic HCV infection. While the addition of RBV to SOF/VEL improved SVR12 in GT3 decompensated cirrhosis patients, the benefits of RBV in GT3 compensated cirrhosis patients receiving SOF/VEL remains unclear.

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## FOOTNOTES

**Author contributions:** Loo JH and Xu WXF contributed equally as the first-author; Wong YJ contributed to study concept and design; Loo JH, Tay WX, Low JT, Ang LS, Xu WXF contributed to systematic review of literature; Loo JH, Xu WXF contributed to drafting of manuscript; all authors did critical review of manuscript.

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**Country/Territory of origin:** Singapore

**ORCID number:** Jing Hong Loo 0000-0002-2781-4245; Wen Xin Flora Xu 0000-0003-4252-3689; Jun Teck Low 0000-0002-0213-4008; Wei Xuan Tay 0000-0001-5716-803X; Le Shaun Ang 0000-0003-3422-630X; Yew Chong Tam 0000-0002-3201-2634; Prem Harichander Thurairajah 0000-0002-9411-1523; Rahul Kumar 0000-0002-5092-4821; Yu Jun Wong 0000-0002-0727-1183.

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META-ANALYSIS

# Spontaneous bacterial empyema in cirrhosis: A systematic review and meta-analysis

William Reiche, Smit Deliwala, Saurabh Chandan, Babu P Mohan, Banreet Dhindsa, Daryl Ramai, Abhilash Perisetti, Rajani Rangray, Sandeep Mukherjee

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**William Reiche,** Department of Internal Medicine, CHI Creighton University Medical Center, Omaha, NE 68124, United States

**Smit Deliwala,** Department of Internal Medicine, Michigan State University at Hurley Medical Center, Flint, MI 48503, United States

**Saurabh Chandan, Rajani Rangray, Sandeep Mukherjee,** Division of Gastroenterology and Hepatology, CHI Creighton University Medical Center, Omaha, NE 68124, United States

**Babu P Mohan, Daryl Ramai,** Department of Gastroenterology, University of Utah, Salt Lake City, UT 84132, United States

**Banreet Dhindsa,** Division of Gastroenterology and Hepatology, University of Nebraska Medical Center, Omaha, NE 68198, United States

**Abhilash Perisetti,** Division of Gastroenterology, Parkview Health, Fort Wayne, IN 46845, United States

**Corresponding author:** William Reiche, DO, Doctor, Department of Internal Medicine, CHI Creighton University Medical Center, 7500 Mercy Road, Omaha, NE 68124, United States. reichewilliam@gmail.com

## Abstract

### *BACKGROUND*

Spontaneous bacterial empyema (SBE) occurs when a hepatic hydrothorax becomes infected and runs a course similar to spontaneous bacterial peritonitis (SBP). It remains underdiagnosed as patients with cirrhosis do not routinely undergo diagnostic thoracentesis. Current understanding is limited by small cohorts, while studies reporting its association with ascites/SBP are conflicting.

### *AIM*

To explore the incidence of SBE, to determine its association with ascites, and to summarize what is known regarding treatment and outcomes for patients with SBE.

### *METHODS*

Major databases were searched until June 2021. Outcomes include the incidence of SBE in pleural effusions, SBP in peritoneal fluid, and SBE in patients without

ascites within our cohort of patients with cirrhosis. We performed a meta-analysis using a random-effects model with pooled proportions and 95% confidence intervals (CI). We assessed heterogeneity using $I^2$ and classic fail-safe to determine bias.

***RESULTS***

Eight studies with 8899 cirrhosis patients were included. The median age ranged between 41.2 to 69.7 years. The majority of the patients were Child-Pugh B and C. Mean MELD score was 18.6 ± 8.09. A total of 1334 patients had pleural effusions and the pooled incidence of SBE was 15.6% (CI 12.6-19; $I^2$ 50). Amongst patients diagnosed with SBE, the most common locations included right (202), left (64), and bilateral (8). Amongst our cohort, a total of 2636 patients had ascites with a pooled incidence of SBP of 22.2% (CI 9.9-42.7; $I^2$ 97.8). The pooled incidence of SBE in patients with cirrhosis but without concomitant ascites was 9.5% (CI 3.6-22.8; $I^2$ 82.5).

***CONCLUSION***

SBE frequently occurs with concurrent ascites/SBP; our results suggest high incidence rates of SBE even in the absence of ascites. The pleura can be an unrecognized nidus and our findings support the use of diagnostic thoracentesis in patients with decompensated cirrhosis after exclusion of other causes of pleural effusion. Thoracentesis should be considered particularly in patients without ascites and when there is a high suspicion of infection. The need for diagnostic thoracentesis will continue to be important as rates of multi-drug resistant bacterial infections increase and antibiotic susceptibility information is required for adequate treatment.

**Key Words:** Spontaneous bacterial peritonitis; Spontaneous bacterial peritonitis; Postparacentesis circulatory dysfunction; Refractory ascites; Hepatic hydrothorax

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**Core Tip:** Identification of risk factors for developing spontaneous bacterial empyema and characterization of spontaneous bacterial empyema are lacking. This is a systematic review and meta-analysis describing spontaneous bacterial empyema and the relationship to ascites in patients with cirrhosis. We investigated the incidence of spontaneous bacterial empyema, the incidence of spontaneous bacterial peritonitis, and the incidence of spontaneous bacterial empyema without ascites in a meta-analysis including eight studies.

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## INTRODUCTION

Hepatic hydrothorax (HH) is one of the pulmonary complications observed in cirrhotic patients and attributed to portal hypertension which leads to a transudative effusion. The true prevalence of HH in cirrhotic patients is unclear but estimated to be at 10%. Infection of the HH (pleura and pleural fluid) is termed spontaneous bacterial empyema (SBE) and represents a distinct and underdiagnosed infectious etiology in patients with decompensated cirrhosis. This entity's existence has frequently been debated; the true nature is uncertain[1-4]. Knowledge regarding this complication has been limited due to a lack of clinical studies. At the same time, several studies suggest SBE is not spontaneous but occurs due to ruptured pleuroperitoneal blebs, which cause diaphragm defects. The negative pressure created in the pleural cavity creates unidirectional flow into the pleural space where low protein ascitic fluid can accumulate and propagate infection[5]. Conversely, other studies have found that SBE can develop without spontaneous bacterial peritonitis (SBP) and can even be diagnosed in patients without ascitic fluid, representing an overlooked infection nidus[5-8]. More robust data is needed within SBE to guide efficient clinical decision-making due to the significant burden on patients and healthcare resources[9, 10]. Patients inflicted by pleural pathology can have prolonged stay with increased mortality[7,11,12].

The exudative nature of SBE is well established; however, risk factors for developing this condition are less clearly elucidated. Several studies have found that patients who develop HH or SBE are more likely to have lower levels of pleural fluid protein and a higher Child-Pugh or MELD score to support the diagnosis[13,14]. Additionally, the concurrence of hydrothorax and ascites remains unknown. We

aim to fill the current understanding by performing a systematic review and incidence meta-analysis exploring the incidence of SBE and its association with ascites.

## MATERIALS AND METHODS

### Protocol

This review has been in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement (PRISMA) and Meta-analyses of Observational Studies in Epidemiology reporting standards (Supplementary Tables 1 and 2)[15].

### Eligibility criteria, literature search, and search strategy

An expert librarian conducted a systematic literature search using a priori protocol to identify studies reporting the incidence, associations, and outcomes of SBE in patients with cirrhosis. The search strategies included "spontaneous bacterial empyema," "SBE," and "SBEM." The search was run in June 2021 across multiple databases, including Ovid EBM Reviews, Ovid Embase (1974+), Ovid Medline (1946+ including epub ahead of print, in-process, and other non-indexed citations), Scopus (1970+), Web of Science (1975+), and PubMed. The search was restricted to articles in English and identified searches were exported to a reference manager (EndNote). We cross-checked reference lists of identified sources for additional relevant studies. Any discrepancy was resolved by a third reviewer (SC). Detailed search strategy presented as Supplementary material.

### Study selection

This meta-analysis included studies that evaluated patients with SBE. SBE was defined as positive pleural fluid culture and polymorphonuclear leukocytes (PMN) count > 250 cells/mm$^3$ or negative pleural fluid culture and PMN count > 500 cells/mm$^3$, without evidence of pneumonia/parapneumonic effusion on imaging[5-7]. Studies reporting performance in pediatric age groups (< 18 years), conference abstracts, case reports, and non-English studies were excluded. Studies were restricted to full-text. Two authors decided on the final selection (WR, SC). Details presented in PRISMA flow diagram, Figure 1.

### Data extraction and quality assessment

Two reviewers (WR, SD) independently extracted eligible information into an a priori designed Google excel spreadsheet. The Qumseya scale for quality assessment of cohort studies for systematic reviews and meta-analyses consisted of nine questions (Supplementary Figure 1). We assessed each study for its design, measurements, outcomes, and patient characteristics. Each risk of bias had a maximum score of 10. Studies with less than six were considered low, 6-7 were moderate, and > 8 were deemed to be high quality[16].

### Outcomes assessed

(1) Incidence of SBE in patients with cirrhosis; (2) Incidence of SBP in patients with cirrhosis; (3) Incidence of SBE in patients without concomitant ascites.

### Statistical analysis

Statistical analysis was performed using Comprehensive Meta-Analysis (CMA 3.0) software (Biostat, Englewood, NJ). Pooled estimates and corresponding 95% confidence intervals (CI) for dichotomous variables were calculated using the random-effects inverse variance method[17]. Heterogeneity was measured by Cochrane Q and $I^2$ statistics, with values of < 30%, 31%-60%, 61%-75%, and > 75% suggesting low, moderate, substantial, and considerable heterogeneity, respectively[18,19]. A funnel plot combined with Egger's tests was performed to assess publication bias. A p-value of 0.05 or less combined with asymmetry in the funnel plots was used to measure significant publication bias. If < 0.05, the trim-and-fill computation was used to evaluate the effect of publication bias on the interpretation of the results. Three levels of impact were reported based on the concordance between the reported results and the actual estimate if there was no bias. The impact was reported as minimal if both versions were estimated to be the same, modest if the effect size changed substantially. Still, the final finding would remain the same and severe if the bias threatens the conclusion of the analysis[20]. To evaluate an individual study's effect on the collective outcome, sensitivity analysis was completed.

## RESULTS

### Study characteristics

An initial search identified 155 publications after removing duplicates. After screening full-text articles, eight studies were eligible for qualitative and quantitative synthesis, as shown in Supplemen-

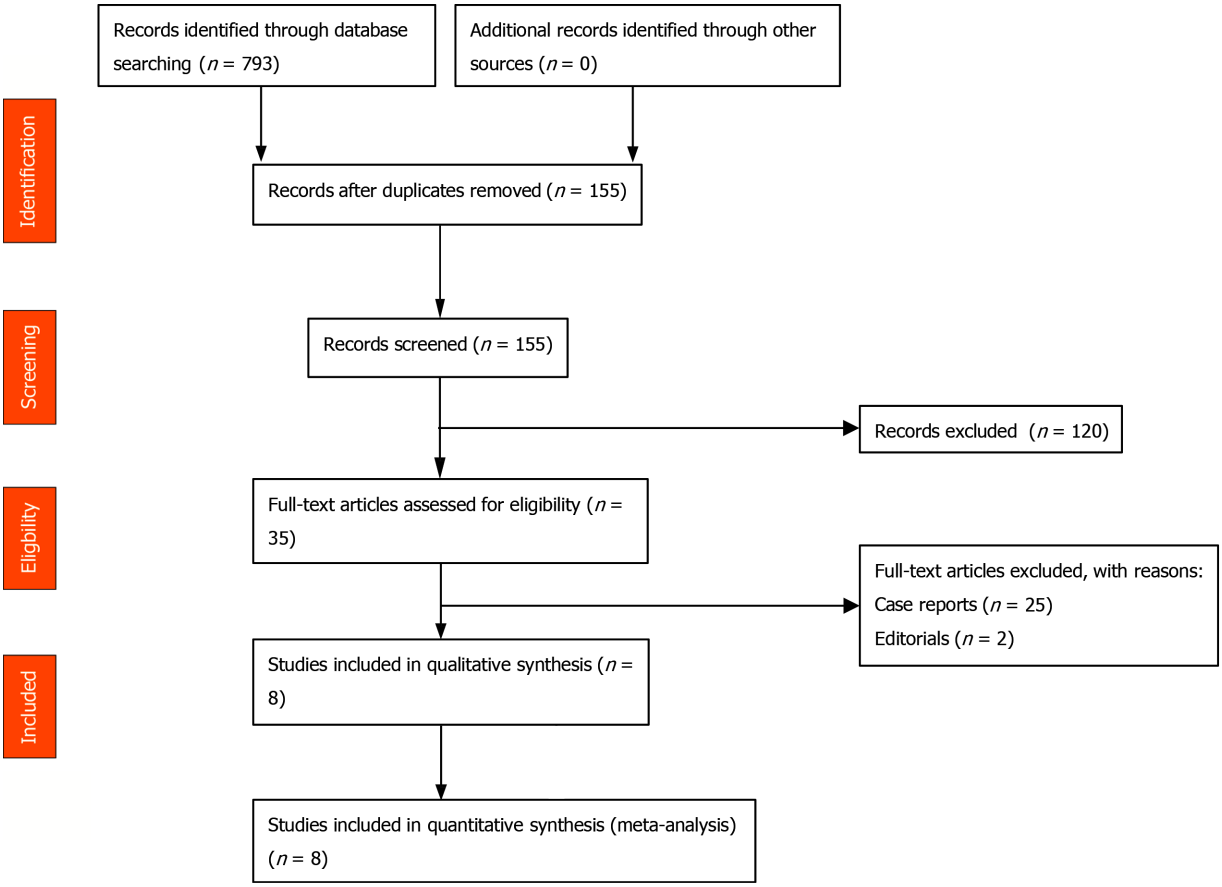


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**Figure 1 Preferred reporting items for systematic reviews and meta-analyses statement flow diagram.**

tary Figure 1. Study locations included Spain, Taiwan, Egypt, and Pakistan between 1988-2017. Among eight studies, 8899 patients (270 males and 110 females; not all studies reported sex); were included, with the median age between 41.2 to 69.7 years. Most of the patients were Child-Turcott Pugh B and C, while the average MELD score was 18.6 ± 8.09. 202 cases were seen in the right pleural space, while 64 cases were seen in the left pleural space, eight cases of bilateral pleural effusions were reported. Study and baseline clinical characteristics have been summarized in Tables 1 and 2.

### *Quality assessment*

Scores for methodological quality assessment are shown in Supplementary Figure 2. Amongst eight studies, one was prospective,[8] five retrospective,[6,7,21-23] and two were cross-sectional[5,24]. All studies were performed in single-centers.

### *Meta-analysis outcomes*

**Incidence of SBE in patients with cirrhosis:** All eight studies reported the incidence of SBE in patients with cirrhosis[5-8,21-24]. A total of 1334 patients had pleural effusions, and the pooled incidence of SBE was 15.6% (CI, 12.6-19; $P < 0.001$, $I^2$ 50%). The true effect size in 95% of all comparable populations falls in the interval 0.12-0.21 (Figure 2).

**Incidence of SBP in patients with cirrhosis:** Seven studies reported ascites and incidence of SBP[5-7,21-24]. After pooling the results of 2636 patients, the incidence of SBP was 22.2% (CI, 9.9-42.7; $P < 0.001$, $I^2$ 97.8%). The true effect size in 95% of all comparable populations falls in the interval 0.01-0.90 (Figure 3).

**Incidence of SBE in patients without concomitant ascites:** Six studies reported SBE without concomitant ascites[5-8,21,22]. The pooled incidence of SBE in patients without concomitant ascites was 9.5% (CI, 3.6-22.8; $P < 0.001$, $I^2$ 82.5 %). The true effect size in 95% of all comparable populations falls in the interval 0-0.76 (Figure 4).

### *Validation of meta-analysis results*

**Sensitivity analysis**: We completed a one-study removal sensitivity analysis to assess if one study had a dominant effect on the meta-analysis. Statistical significance and direction of findings for all outcomes remained unchanged.

**Table 1 Study details**

| Ref. | Year, country, study type | Total patients | Patients w/ PE | Patients w/ SBE | Patients w/ ascites | Patients with SBP | SBE w/o ascites | Age(mean) | Sex(m/f) | R PE | L PE | B/l PE | Treated patients | MELD score | CP score | Mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Xiol *et al*[8], 1996 | 1996, Spain, Prospective | 120 | 120/120 | 16/120 | 95/120 | 14/18 | 6/24/ | | | | | | 19/24 | | 10.67 (1.20) | |
| Chen *et al*[21], 2003 | 2003, Taiwan, Prospective | 862 | 132/862 | 17/132 | 451/862 | 104/451 | 2/411 | 53.7 (13.2) [17n] | 13/4[17n] | 17/17 | | | | | 11.5 (1.6) [17n] | |
| Chen *et al*[7], 2011 | 2011, Taiwan, Retrospective | 3390 | 508/3390 | 81/508 | 1729/3390 | 44/1729 | 14/81 | 60.0 (12.8) [81n] | 55/26 [81n] | 60/81 | 21/81 | | 58/81 | 20.5 (8.0) | 9.7 (2.1) | 31/81 |
| Makhlouf *et al* [5], 2013 | 2012, Egypt, Prospective | 901 | 61/901 | 16/61 | 45/901 | 9/45' | 4/16' | 51.1 (11.00) [16n] | 15/1 [16n] | 53/61 | 5/61 | 3/61 | | | 0 [CP A], 1 [CP B], 15 [CP C]//11.8 (1.3) | 4/16 |
| Mansour *et al* [22], 2013 | 2013, Egypt, Prospective | 98 | 98/98 | 14/98 | 94/98 | 16/94 | 1/14' | 69.7 (16.5) [14n] | 8/6 [14n] | 12/14 | 1/14 | 1/14 | | 27.2 (5.7) | | |
| Emam *et al* [24], 2015 | 2015, Egypt, Prospective | 322 | 322/322 | 46/322 | | 108/322 | 0/46 | 56.76 (6.23) [46n] | 30/16 [46n] | 42/46 | 2/46 | 2/46 | | | 0 [CP A], 4 [CP B], 42 {CP C] | |
| Abbasi *et al* [6], 2016 | 2016, Pakistan, Prospective | 206 | 23/206 | 7/23' | 152/206 | | 5/23' | 41.25 (13.593) [206n] | 149/57 [206n] | 18/23 | 3/23 | 2/23 | | | 62 [CP A], 61 [CP B], 83 [CP C] | |
| Mohamed *et al* [23], 2017 | 2017, Egypt, Prospective | 3000 | 70/3000 | 5/70' | 70/3000 | 17/70 | | | | | | | | | | |

PE: Pleural effusion; SBE: Spontaneous bacterial empyema; SBP: Spontaneous bacterial peritonitis; R: Right; L: Left; B/l: Bilateral; Treated patients: Successfully treated patients; MELD: Model for end-stage liver disease; CP: Child-pugh.

**Heterogeneity**: The $I^2$ was consistently between 50%-75% across most outcomes suggesting considerable heterogeneity of our sample.

**Publication bias**: A publication bias analysis and estimated symmetry could not be completed because fewer than ten studies were included.

## DISCUSSION

To our knowledge, this is the first systematic review and meta-analysis exploring the incidence of SBE in patients with cirrhosis. The pleural space is a potential pocket for infection and often can be overlooked in cases of septic decompensation. SBE is recommended to be managed without a chest tube and requires the delivery of appropriate antibiotics and exclusion of pneumonia, placing importance on timely diagnostic thoracentesis. Our study includes one prospective, five retrospective, and two cross-sectional studies amongst four countries with 8899 patients and 1334 cases of pleural effusions. The

**Table 2 Spontaneous bacterial empyema diagnostic criteria**

| Ref. | Xiol *et al*[8], 1996 | Chen *et al* [21], 2003 | Chen *et al* [7], 2011 | Makhlouf *et al*[5], 2013 | Mansour *et al*[22], 2013 | Emam *et al* [24], 2015 | Abbasi *et al* [6], 2016 | Mohamed *et al*[23], 2017 |
|---|---|---|---|---|---|---|---|---|
| Year, country, study type | Spain, Prospective | Taiwan, Prospective | Taiwan, Retrospective | Egypt, Prospective | Egypt, Prospective | Egypt, Prospective | Pakistan, Prospective | Egypt, Prospective |
| SBE-diagnostic criteria | Positive PF culture and a PMN cell count > 250 cells/mm$^3$ or negative PF culture, compatible clinical course, and a PF PMN > 500 cells/mm$^3$; Exclusion of parapneumonic infections: no image of pneumonia on CXR or CT and evidence of pleural effusion before the infectious episode or PF transudate characteristics during infection | Positive PF culture and a PMN cell count > 250 cells/mm$^3$ or PMN cell count > 500 cells/mm$^3$; no pneumonia on CXR or CT; PF transudate characteristics during infection or evidence of pleural effusion before the infected episode | Positive PF culture and a PMN cell count > 250 cells/mm$^3$ or, negative PF culture, PMN cell count > 500 cells/mm$^3$; no evidence of pneumonia on CXR or CT and evidence of pleural effusion before the infectious episode or PF transudate characteristics during infection | Positive PF culture and a PMN count of 250 cells/mm$^3$ or, if a negative culture, a PF PMN count of > 500 cells/mm$^3$ and the absence of pneumonia or a contiguous infection process on CXR | Positive PF culture or, if negative, a PF PMN count > 500 cells/μL without radiographic evidence of pneumonia | Positive PF culture or, if negative, a PF PMN count > 500 cells/mm$^3$ without radiographic evidence of pneumonia or a contiguous infection process on CXR | PF with PMN cell count > 500 cells/mm or positive culture with PMN cell count > 250 cells/mm$^3$ with exclusion of a parapneumonic effusion | Positive PF culture and PMN count > 250 cells/mm$^3$ or negative PF culture and PMN count > 500 cells/mm$^3$; no evidence of pneumonia/parapneumonic effusion was observed on CXR or CT |

SBE: Spontaneous bacterial empyema; SBP: Spontaneous bacterial peritonitis; PF: Pleural fluid; PMN: Polymorphonuclear leukocyte; CXR: Chest x ray; CT: Computerized tomography.

criteria for diagnosis of SBE were consistent throughout most of the studies and the parameters for cell count and culture results were identical to widely accepted definitions of SBE[25]. Our results support the current understanding that SBE most commonly occurs in patients with ascites or concomitant SBP. Studies have been conflicting on its association with ascites/SBP. Our results uncovered SBE at 9.5%, which was previously unknown and demonstrates the high incidence. In our cohort, roughly 22% had ascites and SBP, suggesting that the high SBE rates near SBP incidence-indicating that the pleural space is a potential space for infection and should be considered to complete a thorough evaluation.

Just as the peritoneal fluid is susceptible to translocation and infection leading to SBP, the development of HH in the pleura is a risk factor for SBE. SBE without HH occurs at less than 3%, but it increases up to 30% with underlying HH. Although HH prevalence is 10%, this is likely underestimated, as patients with HH do not routinely undergo thoracentesis[8,26]. Indications for thoracentesis include patients with HH who develop fever, pleuritic pain, encephalopathy, or a sharp drop in renal function [5]. Pleural fluid characteristics to diagnose HH include a total cell count of PMN < 250/uL, total protein < 2.5 g/dL, albumin gradient > 1.1g/dL, protein quotient < 0.5, or LDH gradient < 0.6. A PMN count > 250/uL with a positive pathogen detected or > 500/uL and a negative pathogen confirm SBE. Computed tomography (CT) can often be helpful in the setting of SBE to detect pleural abscesses that may require more immediate drainage. SBE development often occurs spontaneously or due to the flow of infected ascites from the peritoneal to pleural space. Infected ascites develops from a variety of mechanisms predominantly related to portal hypertension including (but not limited to) bacterial translocation from increased gastrointestinal permeability and bacterial overgrowth from intestinal dysmotility. SBE must be suspected in every patient with HH, as its symptomatology varies greatly. In our study, cohorts from Egypt and Spain mainly exhibited fever and dyspnea, while the remaining cohorts had cough, dyspnea, pleuritic pain, or tachypnea[5,8,22-24].

Amongst the included studies, sterile effusions were most common, while positive cultures commonly reported enteric organisms-*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *enterococcus* on pleural studies. The distinction between SBE and empyema secondary to pneumonia is important as treatment differs greatly. 3rd generation cephalosporins such as cefotaxime and ceftriaxone were most used, followed by cefazolin, ampicillin/sulbactam, fluoroquinolones, and meropenem. Carbapenems should be used for possible extended-spectrum beta-lactamase-producing strains in high-risk patients. Aspiration and pigtail catheters were used in a minority of studies, often in cases of frank pus and were not associated[5,24]. Repeat thoracentesis is not routinely performed and is

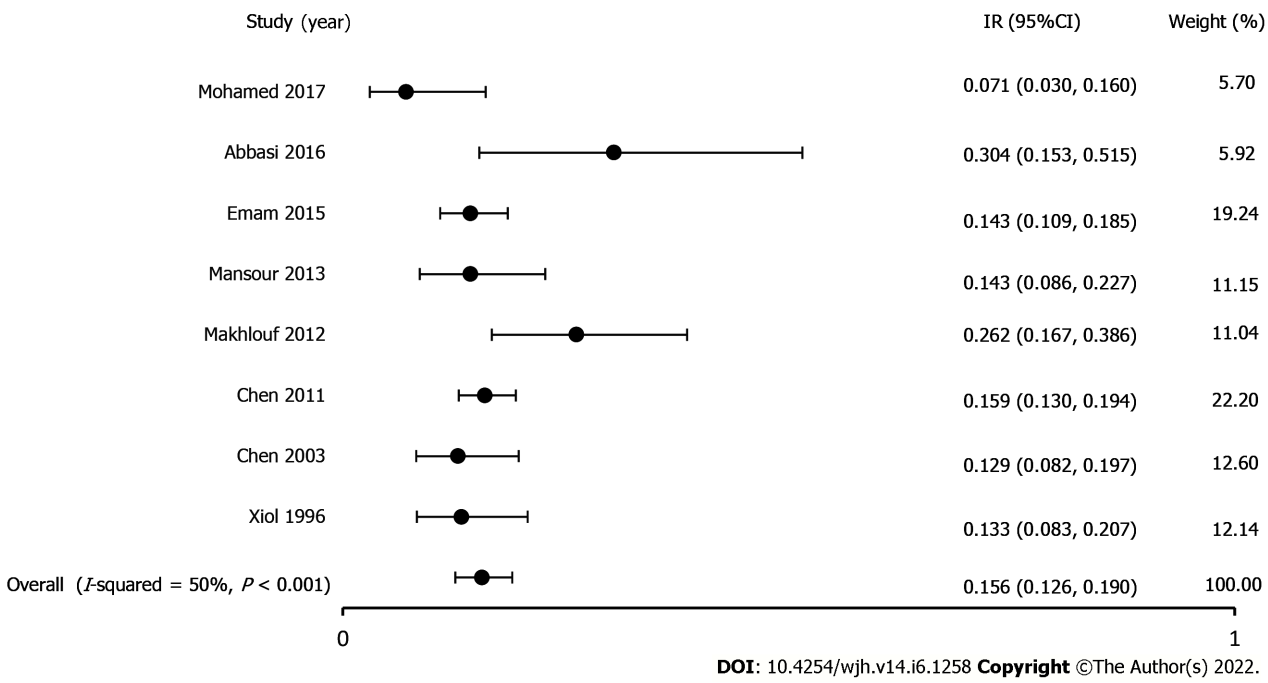


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**Figure 2 Incidence of spontaneous bacterial empyema in patients with cirrhosis.**

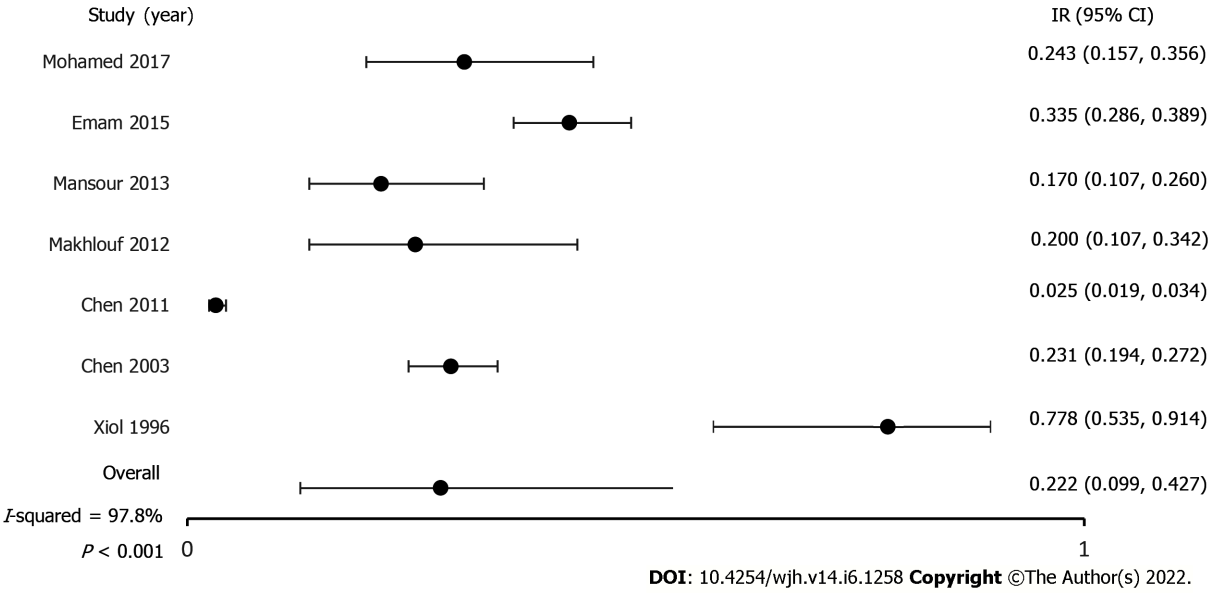


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**Figure 3 Incidence of spontaneous bacterial peritonitis in patients with cirrhosis.**

only undertaken in non-responding cases. Albumin infusion at 1.5g/kg on day 1 and 1g/kg on day 3 has shown benefit in SBP and has been used in SBE; none in our included studies. Antibiotic duration based on SBP experience has been recommended; however, the evidence was based on a few cohorts and case-control studies[27]. In our meta-analysis, two studies reported a duration of seven to ten days followed by a control thoracentesis[7,8]. Antibiotic response varied; one included study found SBE resolved in 72% of patients; however, the need for aspiration and second-line antibiotic therapy is frequent. This same study found 43% of patients died before second line therapy could be initiated[7]. A chest tube was only used in one of the patients and this patient had biochemical analysis suggestive of empyema[5,7].

Just as hepatic hydrothorax is known to decrease survival, SBE is known to impact mortality negatively[28]. In our meta-analysis we found the mortality rate of SBE in patients receiving treatment ranged from 20%-38%[5,21]. Compared to patients without SBE, patient with SBE have been shown to have a higher likelihood of death or liver transplantation at one year[26]. First-line treatment failure, odds ratio (OR) 7.56 followed by ICU admission (OR 5.53), and concomitant bacteremia (OR 4.32),

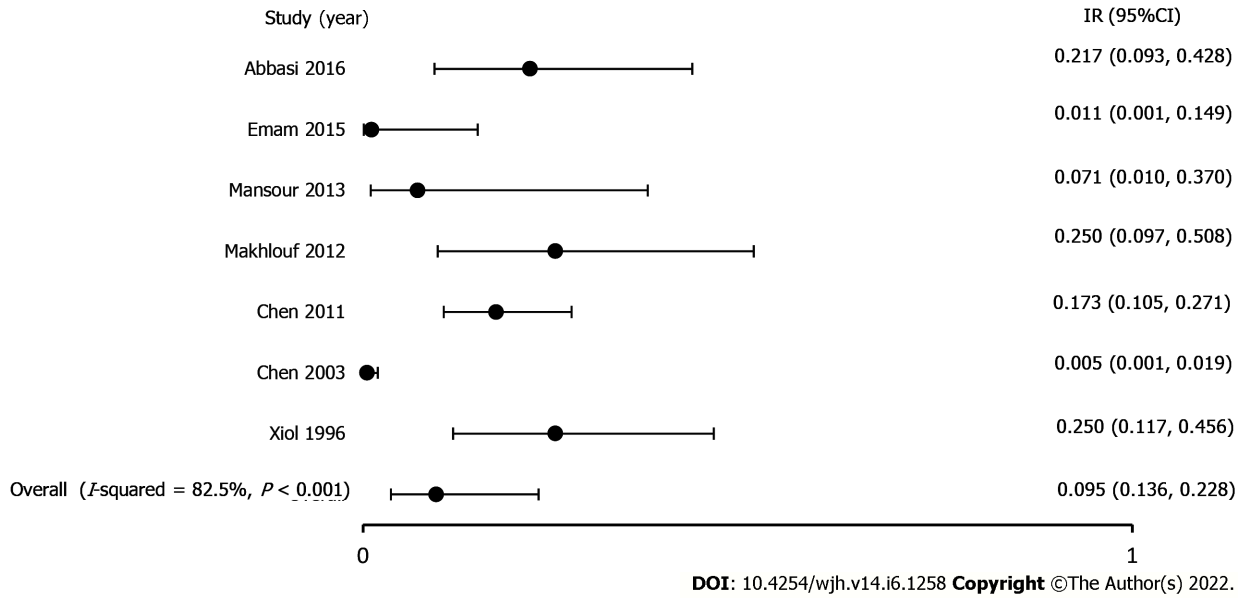


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**Figure 4 Incidence of spontaneous bacterial empyema in patients without concomitant ascites.**

concomitant SBP (OR 2.51), CPS (OR 1.59), and MELD-Na (OR 1.21) correlated to increased mortality [7]. MELD-Na has been shown to most accurately predict SBE associated hospital mortality with an area under the curve of 0.793, followed by serum sodium-0.778 and CPS-0.744. INR, pleural total protein, sex, creatinine, followed by diabetes mellitus, MELD-Na, MELD, bilirubin have been identified as predictors of dual SBP and SBE infection[23]. Five patients underwent an orthotopic liver transplant (OLT) a few months after SBE and all were alive at follow-up five years after OLT[8]. HH management is based on therapeutic principles of treating ascites-diuretics, sodium restriction, and fluid removal in symptomatic cases. Transjugular intrahepatic portosystemic shunt (TIPS) has been beneficial in cases of recurrent HH by reducing portal hypertension pressures. Indwelling pleural catheters (IPCs) may be an option for patients who are not TIPS candidates. IPCs have been associated with fewer complications compared to chest tubes.[29] Chest tubes have been associated with increased mortality unless pus has been demonstrated in the pleural space[26,27]. The development of SBE is significant for patients in the peri-transplantation period, as a few studies have suggested that independent SBE be considered an indication for liver transplantation evaluation and MELD exception points due to its impact on outcomes[8,26,27].

A limitation for determining the incidence of SBE was the lack of studies and a small number of included patients[8,22]. Despite this, we used a newer quality assessment scale to elicit the performance characteristics of the included studies. Follow-up data, including mortality, antibiotic duration, and the number of successfully treated patients, were only reported in two studies[7,8]. Majority of included patients were Child-Pugh class B or C, while a majority lacked a MELD score. MELD and Child-Pugh scores were reported in two studies[22,23]. There was considerable heterogeneity in the included studies attributed to study location, patient selection, and characteristics. To illustrate the range of true effects, we additionally provided prediction intervals to our outcomes[30]. The lack of long-term results in our studies translates to our current limited understanding of this disease process and its impact on respiratory mechanisms and overall mortality. A publication bias was not provided due to fewer than ten studies.

## CONCLUSION

This study highlights the importance of considering SBE and HH in the differential for patients with cirrhosis who have pleural effusion. HH in the setting of cirrhosis is not routinely evaluated. The pleura can be an unrecognized nidus and our findings support the use of diagnostic thoracentesis in patients with decompensated cirrhosis after exclusion of other causes of pleural effusion. Thoracentesis should be considered particularly in patients without ascites and when there is a high suspicion of infection. It helps rule out empyema due to pneumonia and allows for targeted antibiotic therapy against enteric organisms. Additionally, as rates of multi-drug resistant (MDR) organisms increase globally, the need for organism identification for targeted treatment will become even more crucial, making timely thoracentesis of key importance[31]. Future observational and long-term studies will help elucidate further the mortality rates, optimal treatment route and duration, and risk factors for SBE.

## ARTICLE HIGHLIGHTS

### *Research background*

Spontaneous bacterial empyema (SBE) is analogous to spontaneous bacterial peritonitis (SBP); however, much less is understood regarding its incidence rate, treatment strategies, and management.

### *Research motivation*

The current understanding of SBE is limited by small sample size and results regarding its association with ascites are conflicting. Previous studies have noted patients who have cirrhosis and SBE may have poorer outcomes therefore more information regarding its association with ascites/SBP, incidence, treatment, and effect on outcomes are needed.

### *Research objectives*

To identify the incidence of SBE in patients with cirrhosis, the incidence of SBP in patients with cirrhosis, and the incidence of SBE in patients without concomitant ascites. Additionally, we performed a systematic review of the treatment and outcomes of SBE.

### *Research methods*

We performed a meta-analysis using a random-effects model with pooled proportions and 95% confidence intervals (CI). We assessed heterogeneity using $I^2$ and classic fail-safe to determine bias.

### *Research results*

A total of 1334 patients had pleural effusions and the pooled incidence of SBE was 15.6% (CI 12.6-19; $I^2$ 50). Amongst patients diagnosed with SBE, the most common locations included right (202), left (64), and bilateral (8). Amongst our cohort, a total of 2636 patients had ascites with a pooled incidence of SBP of 22.2% (CI 9.9-42.7; $I^2$ 97.8). The pooled incidence of SBE in patients with cirrhosis but without concomitant ascites was 9.5% (CI 3.6-22.8; $I^2$ 82.5).

### *Research conclusions*

SBE frequently occurs with concurrent ascites/SBP; our results suggest high incidence rates of SBE even in the absence of ascites. The pleura can be an unrecognized nidus and our findings support the use of diagnostic thoracentesis in patients with decompensated cirrhosis after exclusion of other causes of pleural effusion. Thoracentesis should be considered particularly in patients without ascites and when there is a high suspicion of infection. The need for diagnostic thoracentesis will continue to be important as rates of multi-drug resistant bacterial infections increase and antibiotic susceptibility information is required for adequate treatment.

### *Research perspectives*

This study suggests the baseline incidence of SBE is high in patients with cirrhosis and diagnostic thoracentesis should be considered after underlying pulmonary and cardiac causes have been ruled out, especially when there is high concern for infection. High index of suspicion for SBE must be maintained especially in cirrhosis patients with pleural effusions and without underlying ascites. Timely treatment is warranted given high associated mortality of SBE. Future prospective studies are needed, as it remains unclear if long term prophylaxis against SBE is warranted in patients with decompensated cirrhosis.

## FOOTNOTES

**Author contributions:** Reiche W acquisition of data, drafting the article, final approval; Deliwala S acquisition of data, analysis, interpretation of data, drafting the article; Chandan S conceptualization, study search, critical revision, final approval; Mohan B statistical analysis; Dhindsa B, Ramai D, Perisetti A data collection and study search; Rangray R and Mukherjee S critical revision, final approval

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**Country/Territory of origin:** United States

**ORCID number:** William Reiche 0000-0001-8797-5764; Smit Deliwala 0000-0001-5239-9533; Saurabh Chandan 0000-0002-2661-6693; Babu P Mohan 0000-0002-9512-8693; Banreet Dhindsa 0000-0002-9858-0941; Daryl Ramai 0000-0002-2460-7806; Abhilash Perisetti 0000-0003-4074-6395; Rajani Rangray 0000-0002-8590-9277; Sandeep Mukherjee 0000-0002-0538-3253.

**Corresponding Author's Membership in Professional Societies:** American College of Gastroenterology, 58606; American Association for the Study of Liver Diseases, 259789.

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