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EDITORIAL

Molecular mechanisms underlying SARS-CoV-2 hepatotropism and liver damage

Jorge Quarleri, M Victoria Delpino

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Abstract

In coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) primarily targets the respiratory system, but evidence suggests extrapulmonary organ involvement, notably in the liver. Viral RNA has been detected in hepatic tissues, and in situ hybridization revealed virions in blood vessels and endothelial cells. Electron microscopy confirmed viral particles in hepatocytes, emphasizing the need for understanding hepatotropism and direct cytopathic effects in COVID-19-related liver injury. Various factors contribute to liver injury, including direct cytotoxicity, vascular changes, inflammatory responses, immune reactions from COVID-19 and vaccinations, and drug-induced liver injury. Although a typical hepatitis presentation is not widely documented, elevated liver biochemical markers are common in hospitalized COVID-19 patients, primarily showing a hepatocellular pattern of elevation. Long-term studies suggest progressive cholestasis may affect 20% of patients with chronic liver disease post-SARS-CoV-2 infection. The molecular mechanisms underlying SARS-CoV-2 infection in the liver and the resulting liver damage are complex. This "Editorial" highlights the expression of the Angiotensin-converting enzyme-2 receptor in liver cells, the role of inflammatory responses, the impact of hypoxia, the involvement of the liver's vascular system, the infection of bile duct epithelial cells, the activation of hepatic stellate cells, and the contribution of monocyte-derived macrophages. It also mentions that pre-existing liver conditions can worsen the outcomes of COVID-19. Understanding the interaction of SARS-CoV-2 with the liver is still evolving, and further research is required.

Key Words: SARS-CoV-2; COVID-19; Hepatotropism; Angiotensin-converting enzyme-2

Core Tip: The hepatotropism of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a growing concern amid the coronavirus disease 2019 (COVID-19) pandemic. Despite its respiratory focus, the virus significantly affects various organs, notably the liver, leading to complications like inflammation, abnormal function tests, and, in severe cases, organ damage. This complex involvement worsens disease outcomes. Understanding the virus's interplay with the liver, mediated by the Angiotensin-converting enzyme-2 receptor, is crucial for tailored treatments. The liver's pivotal role in the immune response emphasizes the need to comprehend SARS-CoV-2 hepatotropism. Ongoing research is vital for uncovering mechanisms, clinical implications, and effective strategies in managing COVID-19 patients with liver involvement.

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INTRODUCTION

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), belonging to the *Betacoronavirus* genus within the *Coronaviridae* family, is a positive-sense, single-stranded RNA virus with an enveloped structure. It shares close genetic relatedness with severe acute respiratory syndrome coronavirus-1 (SARS-CoV-1) and Middle East respiratory syndrome CoV. The genome of SARS-CoV-2 is approximately 30000 base pairs long, encoding 16 nonstructural and 4 structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The spike protein assumes a critical role in the SARS-CoV-2 life cycle by governing viral attachment, fusion, entry, and transmission. This glycoprotein contains the S1 and S2 domains as functional components able to act as ligands for receptor binding and downstream membrane fusion, respectively. Notably, the receptor binding domain within the S1 unit exhibits significant genetic variability within the coronavirus genome[1,2].

When it comes to infecting the majority of host cells, the SARS-CoV-2 spike engages with its primary receptor, Angiotensin-converting enzyme-2 (ACE2). The process is further facilitated by host transmembrane proteases, such as serine 2 [transmembrane serine protease 2 (TMPRSS2)], which play a crucial role in priming the spike protein for receptor interaction and subsequent entry into the host cell. In the facilitation of viral entry may also act additional host co-factors, such as neuropilin-1, glycosaminoglycans, C-type lectins, and furin. Noteworthy is the spike protein's specific binding to ACE2 and TMPRSS2, which collectively support viral entry. The differential expression of ACE2 and TMPRSS2 in various tissues, including the airways, lungs, nasal/oral mucosa, and intestine, underscores the multifaceted nature of the viral entry process across different cellular environments. The affinity of the spike protein for the ACE2 receptor plays a critical role in determining the replication fitness and severity of SARS-CoV-2 infection[1,2].

In the context of Coronavirus Disease 2019 (COVID-19), produced by the infection with SARS-CoV-2, the most profound pathological modifications are predominantly evident within the respiratory system. Nevertheless, it is of utmost significance to acknowledge that this viral infection imposes deleterious consequences on various other bodily organs. Notably, evidence has been presented of the presence of SARS-CoV-2 viral RNA in extrapulmonary organs, including the liver[3-6]. Building upon the excellent review conducted by Roshanshad *et al*[6], this editorial seeks to provide supplementary insights into the molecular mechanisms underlying SARS-CoV-2 hepatotropism and liver damage. The specific cellular location of viral replication remains unclear because of the use of whole-tissue homogenization techniques for nucleic acid extraction. Subsequent examinations, employing in situ hybridization analysis, identified the presence of SARS-CoV-2 virions within the lumen of blood vessels and endothelial cells in the portal veins of liver tissues derived from COVID-19 patients[7,8]. Furthermore, electron microscopic assessments of liver specimens from two COVID-19 patients who succumbed to the disease and exhibited elevated liver enzyme levels revealed the presence of intact viral particles within the cytoplasm of hepatocytes[9].

Although the precise etiology of liver injury in the context of COVID-19 remains partially understood, various factors have been postulated to contribute to this phenomenon (Figure 1), including direct cytotoxic effects, vascular changes, immunological and inflammatory responses associated with COVID-19, immune responses triggered by COVID-19 vaccination, and drug-induced liver injury (DILI)[10-12].

The assessment of hepatotropism concerning SARS-CoV-2 and the possible manifestation of direct cytopathic effects are crucial for a comprehensive understanding of the mechanisms underlying liver injury in COVID-19. It is worth noting that a typical hepatitis presentation has not been extensively documented[7,9,13], despite recent albeit limited discoveries.

The prevalence of elevated liver biochemical markers in individuals with COVID-19 varies in different studies but, in hospitalized patients, these abnormalities can be observed in the vast majority of them. These are primarily characterized by a hepatocellular pattern of elevation. The extent of these elevations is typically mild, and the likelihood of encountering substantial increases in alanine aminotransferase or aspartate aminotransferase levels (> 20-fold upper normal limit), liver synthetic dysfunction, or elevated serum bilirubin levels remains relatively uncommon among COVID-19 patients[14-17]. Remarkably, recent extended follow-up investigations have unveiled that after SARS-CoV-2 infection, progressive cholestasis may impact as many as 20% of individuals with chronic liver disease (CLD), demonstrating a proclivity toward increased severity[18].

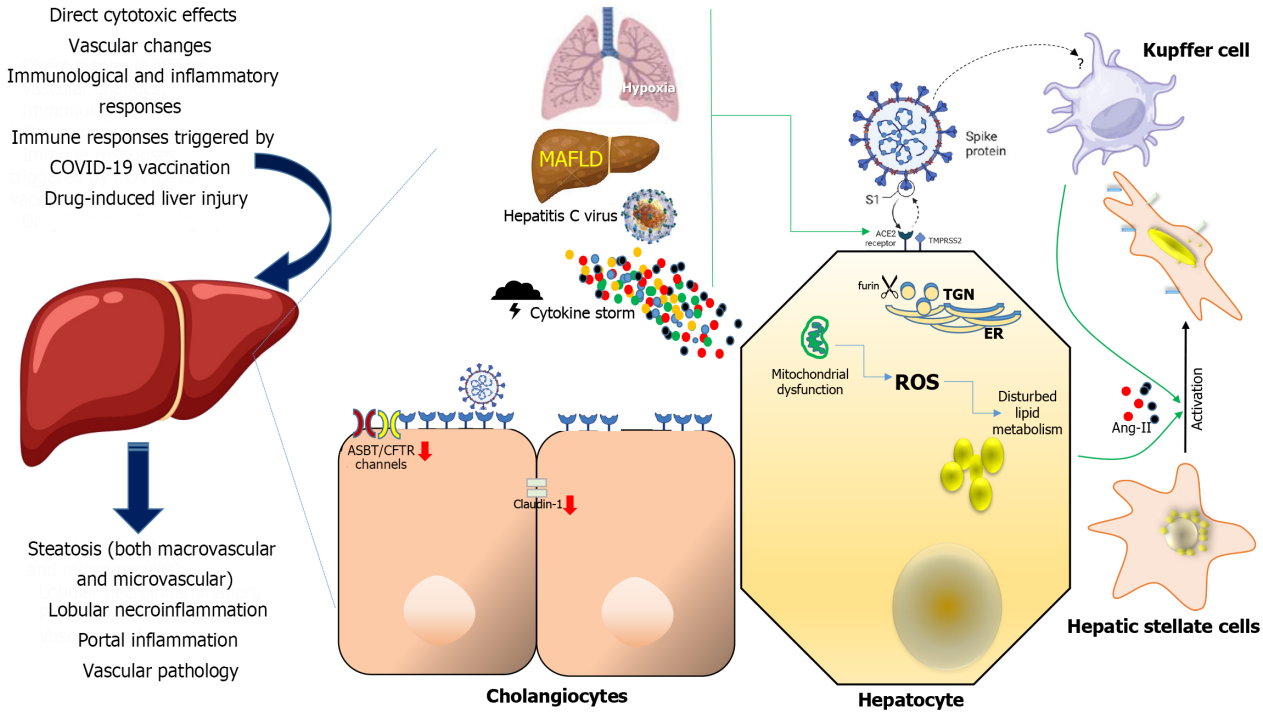

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Figure 1 Mechanisms of severe acute respiratory syndrome coronavirus-2 disease-induced liver injury and their consequences at organ level (left). Severe acute respiratory syndrome coronavirus-2 cellular targets involved in liver damage (center and right). Various factors have been postulated to contribute to liver injury in the context of coronavirus disease 2019 (COVID-19), including direct cytotoxic effects, vascular changes, immunological and inflammatory responses associated with COVID-19, immune responses triggered by COVID-19 vaccination, and drug-induced liver injury. In the context of liver injury associated with COVID-19, the histological patterns encompass features such as steatosis (both macrovascular and microvascular), lobular necroinflammation, portal inflammation, and vascular pathology. At the cellular level, hypoxia, metabolic dysfunction-associated fatty liver disease, and concomitant hepatitis C virus infection, and the cytokine storm may upregulate the Angiotensin-converting enzyme-2 (ACE2), transmembrane serine protease 2 and furin expression in hepatocytes. Mitochondrial dysfunction has been affected directly by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection of hepatocytes which in turn may be connected to pre-existing inflammation and the adverse impacts of excessive and dysfunctional adipose tissue. In cholangiocytes, SARS-CoV-2 Leads to a decrease in the mRNA expression of Claudin-1 and downregulates the expression of hepatobiliary transporters, such as ASBT and the chloride channel CFTR. The ACE-2 expression in Kupffer cells is still controversial. Hepatic stellate cells appear do not express ACE2 in any activation state. Their activation is a pivotal event in the progression of chronic liver disease, as these cells serve as the primary source of fibrosis, and it is induced by proinflammatory and profibrotic signals, including angiotensin II, which is generated by the catalytic action of ACE as part of the profibrotic branch of the renin-angiotensin system. Liver and Kupffer cell are created with BioRender.com. ROS: Reactive oxygen species; ACE2: Angiotensin-converting enzyme-2; TMPRSS2: Transmembrane serine protease 2; MAFLD: metabolic dysfunction-associated fatty liver disease; ER: Endoplasmic reticulum; TGN: Trans-Golgi network.

ACE2 AND VIRAL ENTRY CO-FACTORS EXPRESSION IN HEPATIC CELLS CONTRIBUTING TO SARS-COV-2 HEPATOTROPISM

A comprehensive understanding of tissue reservoirs supporting SARS-CoV-2 replication remains a critical research challenge. This is, in part, attributed to the inherent challenges associated with procuring biopsy samples from individuals presently infected with the virus, coupled with the requisite use of high-level laboratory containment facilities. The well-established understanding includes the interaction of the viral spike protein (S) with ACE2 for cellular entry, emphasizing the crucial roles of TMPRSS2 and furin enzymes in the infection process[1]. Consequently, examination of the expression of these receptors during the early stages of infection provided valuable insights into the potential permissiveness of hepatic cells. Notably, the liver exhibits minimal expression of ACE2 and TMPRSS2 proteins, whereas their highest expression is observed in the intestine and gall bladder. However, it is noteworthy that ACE2 expression appears to be absent in the lungs, where infection unequivocally occurs. Then, studies using single-cell RNA sequencing to analyze samples from healthy human livers revealed that although hepatic ACE2 expression is relatively low but still detectable. The expression level in cholangiocytes, the epithelium lining the bile duct, is similar to that found in lung alveolar cells[12,19]. Interestingly, sinusoidal endothelial cells appear to lack ACE2 expression, which aligns with earlier findings resembling SARS-CoV-1[20]. Recent observations concerning SARS-CoV-2-induced endothelitis in major intrahepatic arteries, coupled with the heightened presence of ACE2 in other endothelial cell types, such as those within the central and portal veins, which are similarly susceptible to infection by the virus, suggest the potential significance of this discovery[7]. TMPRSS2 and furin gene expression are broadly distributed across various liver cell types[21]. Notably, when three distinct single-cell RNA sequencing datasets from healthy liver tissue were collectively analyzed, it was observed that very few hepatocytes co-expressed both ACE2 and TMPRSS2[22].

To investigate the susceptibility of liver cell types to SARS-CoV-2 infection, experimental models involving cellular and organoid cultures have played a pivotal role. Hepatocellular carcinoma-derived cell lines such as Huh-7 and HepG2 have demonstrated the ability to support the entire viral life cycle[23]. A significant expression of ACE2 and TMPRSS2 in liver parenchymal cells was reported using bioinformatic analyses from a single-cell transcriptome database[21]. Permissiveness was demonstrated when pseudotyped lentiviral particles expressing the full-length spike protein of SARS-CoV-2 were inoculated to primary hepatocytes obtained from ACE2-humanized mice[24].

Importantly, research conducted in both murine and human subjects has revealed an increase in hepatic ACE2 expression within hepatocytes in the presence of liver fibrosis or cirrhosis, as already documented[25]. This finding holds significant relevance because pre-existing liver injury may exacerbate the susceptibility of hepatic tissues to the hepatitis C virus, SARS-CoV-2[26]. The impact of liver injury and pre-existing liver conditions on the propensity of SARS-CoV-2 to target the liver is still not well understood, and there is a notable absence of studies that have specifically investigated the histological alterations occurring in individuals with both COVID-19 and CLD. However, it is worth noting that previous investigations conducted before the emergence of COVID-19 have reported a significantly more than 30-fold elevation in ACE2 expression within the livers of patients suffering from cirrhosis related to the hepatitis C virus compared to individuals without underlying liver conditions[25,27] (Figure 1). These findings may be associated with the gene expression patterns observed in metabolic dysfunction-associated fatty liver disease (MAFLD), previously known as non-alcoholic fatty liver disease[28]. The presence of MAFLD within the broader context of metabolic syndrome may contribute to the exacerbation of COVID-19 severity. Molecular investigations have revealed elevated expression levels of crucial viral entry receptors, including ACE2, furin, and TMPRSS2, in individuals diagnosed with MAFLD. Furthermore, the liver mRNA expression of ACE2 and TMPRSS2 was found to be upregulated in individuals without active infection. Moreover, in obese patients with MAFLD, there was an observed upregulation of ACE2 in the liver as well as in subcutaneous and visceral adipose tissues compared with obese individuals lacking MAFLD[17,29,30] (Figure 1).

In addition, it has been established that hypoxia, a characteristic feature of severe cases of COVID-19, serves as a key regulatory factor in the upregulation of ACE2 expression in hepatocytes[17,25,31,32] (Figure 1). This phenomenon may explain the prevalence of extrapulmonary dissemination of SARS-CoV-2 in patients experiencing acute respiratory distress syndrome and other hypoxic conditions. Notably, in a manner analogous to findings in other organ systems, it is conceivable that inflammatory conditions and diseases affecting the liver, as reported[33,34], could elevate the expression of ACE2. Given the potential implication of DILI in the development of liver damage in COVID-19 patients[35,36], it is particularly interesting to investigate whether such conditions or specific pharmaceutical agents may induce excessive ACE2 expression within the hepatic environment. In contrast, while not yet substantiated in human subjects, Brevini *et al* [37] have recently delineated in a murine model the potential of ursodeoxycholic acid to inhibit ACE2, suggesting its potential as a promising therapeutic and prophylactic strategy against SARS-CoV-2.

In vitro experiments have demonstrated that the spike (S) protein of beta-coronaviruses exhibits a significant increase in its binding affinity for its receptor when it is pre-incubated with trypsin, a process involving proteolytic activation[1]. It's worth noting that liver epithelial cells express trypsin[38] and various other serine proteases, which are continuously involved in extracellular matrix remodeling and liver regeneration[39]. Considering this scenario, there is a plausible suggestion that the expression of ACE2, a pivotal factor for the precise targeting and recognition of SARS-CoV-2 within the liver, might be comparatively diminished in comparison to other tissues where extracellular proteolytic activity is less pronounced[40,41].

In concordance with these findings, recent discoveries have brought attention to the existence of a furin-like proteolytic site within the S protein of SARS-CoV-2, a feature not found in other coronaviruses belonging to the same lineage[1]. It is interesting to note that furin expression is mostly observed in organs that are hypothesized to be susceptible to SARS-CoV-2 infection. These organs include the pancreas, kidney, liver, and salivary glands[21].

ACE2-INDEPENDENT SARS-COV-2 HEPATOTROPISM

While our understanding of the tissue-specific determinants governing SARS-CoV-2 infection remains limited, there is a growing recognition of the involvement of additional accessory receptors in viral entry. Notably, studies have suggested that the high-density lipoprotein scavenger receptor B type 1 (SR-B1) plays a facilitating role in ACE2-dependent coronavirus attachment in vitro, drawing parallels with hepatitis C virus infection. Likewise, therapeutic interventions targeting SR-B1 have shown efficacy in mitigating the lipoprotein-mediated enhancement of SARS-CoV-2 infection. It is important to note, however, that using immunohistochemistry analysis of liver tissue was confirmed only sporadic ACE2 expression within the hepatic tissue[42]. Besides, it is crucial to acknowledge that additional factors, such as ganglioside (GM1)[43], may influence the interaction between the spike (S) protein and ACE2. Consequently, there is an imperative need for more comprehensive research into the S protein-ACE2 interactome to gain a deeper understanding of the molecular mechanisms involved and explore potential therapeutic avenues.

Ou *et al*[44,45] used pseudovirions carrying the spike (S) protein of SARS-CoV-2 to assess their ability to infect various cell lines. When exposed to viral vectors expressing the SARS-CoV-2 S protein, HuH7 and Calu3 cells (a cell line originating from human lung cancer) were more susceptible to transfection than reference pseudovirus. Additionally, these investigations suggested that the PIKfyve-TCP2 endocytotic pathway, which is expressed at lung-like levels in the liver and gall bladder[15], could be important for the viral entry process[46].

EXPERIMENTAL MODELS FOR STUDYING SARS-COV-2 HEPATOTROPISM

HuH7 cells has been reported as a permissive model to develop a novel and effective functional viromics screening method to forecast the possibility of zoonotic occurrences with known lineage B betacoronaviruses. This model was employed to investigate the binding and recognition processes of both SARS-CoV-1 and SARS-CoV-2[47]. This approach further confirmed the affinity of SARS-CoV-2 for hepatocytes. It is important to note that in their study, HuH7 cells were identified as the third most permissive cell line, following pulmonary (Calu3) and intestinal ($CaCo_2$) cell models, which represent organs with histopathological evidence of SARS-CoV-2 infection[47]. However, it is important to recognize that a cell's ability to attach and internalize viral particles does not always indicate that the particular cell type is also supportive of efficient viral reproduction. In this regard, it has shown that HuH7 cells indeed facilitate SARS-CoV-2 viral multiplication[23,48]. It has been determined that hepatocyte cell lines are robust permissive cell types for infections with SARS-CoV-1 and SARS-CoV-2. Notably, HuH7 cells have recently been used in SARS-CoV-2 immunostaining assays as a positive control[49]. It is essential to underscore that the findings suggesting hepatocytes as potential hosts for SARS-CoV-2 primarily stem from studies conducted with cancer cell lines. To establish the clinical relevance of these observations, it is crucial to conduct a comparison of ACE2 protein expression in HuH7 cells with that observed in primary human hepatocytes.

Post-mortem autopsies have yielded evidence supporting the concept of direct infection of liver cells by SARS-CoV-2. Several studies have recorded the identification of SARS-CoV-2 in a notable portion of post-mortem liver biopsies, employing techniques such as PCR and *in situ* hybridization. However, the direct invasion of hepatocytes by the virus was not consistently confirmed. Nonetheless, certain researchers managed to demonstrate the presence of distinct coronavirus particles, including spike structures, within the cytoplasm of hepatocytes in individuals with COVID-19. These observations were accompanied by signs of mitochondrial swelling and apoptosis, suggesting a potential link between the virus and cellular damage in the liver[7,9,50]. The diverse spectrum of histological injury patterns observed in individuals infected with SARS-CoV-2, including features such as macrovascular and microvascular steatosis, lobular necroinflammation, portal inflammation, and vascular pathology (Figure 1), likely emphasizes the intricate and multifactorial nature underlying abnormal liver test results in the context of COVID-19-associated liver injury[14-16,51]. Perhaps the most compelling evidence of SARS-CoV-2's ability to infect liver tissue was recently presented by Wanner *et al*[52]. In their study, the authors presented multiple lines of evidence for SARS-CoV-2 liver tropism, including the direct identification of SARS-CoV-2 genomic material within hepatocytes using *in situ* hybridization. In our study and theirs, infectious SARS-CoV-2 was isolated from post-mortem liver tissue[53]. Furthermore, Wanner *et al*[52] delineated activity profiles through transcriptomic and proteomic analyses in hepatic samples, affirming the presence of established SARS-CoV-2 entry receptors and facilitators of infection, encompassing ACE2, TMPRSS2, procathepsin L, and the Ras-related protein Rab-7a. The analyses also unveiled pronounced upregulation in interferon responses, JAK-STAT signaling, and liver-specific metabolic modulation. These findings collectively suggest a viral activity profile bearing notable resemblances to other hepatotropic viral infections, notably hepatitis C virus infection[52]. Moreover, it is imperative to conduct further investigations aimed at unraveling the molecular alterations initiated in hepatocytes subsequent to SARS-CoV-2 infection.

Valuable insights into this matter can be derived from the research conducted by Yang *et al*[54]. Using organoids created from human hepatocytes generated from pluripotent stem cells and primary adult human hepatocytes, their work confirmed SARS-CoV-2 hepatotropism. Using these organoids, the S-expressing pseudovirus of SARS-CoV-2 demonstrated the ability to infect human hepatocytes, leading to substantial viral replication. Additionally, gene expression analyses indicated that primary hepatocytes infected with SARS-CoV-2 exhibited heightened expression of pro-inflammatory cytokines, coupled with the downregulation of essential metabolic functions, as evidenced by the inhibition of CYP7A1, CYP2A6, CYP1A2, and CYP2D6 expression[54]. Wang *et al*[9] made a noteworthy advancement when they used electron microscope imaging to examine liver tissues from two deceased COVID-19 patients. They found that the hepatocytes they studied had viral structures that resembled SARS-CoV-2 virions. This data suggests that, even in the absence of a traditional hepatitis pattern, the histological alterations seen in these individuals might be the result of SARS-CoV-2's direct cytopathic effects[55]. It is important, therefore, that more research utilizing more extensive biopsy or autopsy cohorts in conjunction with all-encompassing imaging methods, including immunological electron microscopy, could be necessary to validate these preliminary findings about the existence of SARS-CoV-2 in hepatocytes [56].

THE RELEVANCE OF CHOLANGIOCYTES AS SARS-COV-2 CELLULAR TARGET IN LIVER

Bile duct epithelial cells, also referred to as cholangiocytes, fulfill pivotal functions in both the generation and regulation of bile, while also contributing to immune responses[57]. Single-cell sequencing of long-term liver ductal organoid cultures derived from human tissues revealed the persistence of ACE2 and TMPRSS2 expression[58] (Figure 1). Cholangiocytes were infected with SARS-CoV-2, causing syncytia formation. Twenty-four hours after the infection, there was a notable rise in the amount of SARS-CoV-2 genomic RNA. When the virus was inoculated to adult human cholangiocyte organoids, similar outcomes were seen, thus showing that SARS-CoV-2 infection in vitro may occur in human liver ductal organoids[54], raising the possibility of viral replication within the bile duct epithelium in vivo. Despite the notably elevated expression of ACE2 in cholangiocytes compared to hepatocytes, there are no reports of direct proof of SARS-CoV-2 infection in cholangiocytes in COVID-19 patients. Since hepatocytes and cholangiocytes are the primary producers of bile and because biliary fluids and cholangiocytes' apical membrane interact directly and continuously, the presence of

SARS-CoV-2 viral RNA or proteins in bile may be an indirect indicator of cholangiocyte SARS-CoV-2 infection. Currently, there is just one case report that shows SARS-CoV-2 RNA exists in bile, while bile samples from two other small sample series tested negative. Such disparities could be attributed to the circumstance that the bile sample yielding a positive result was obtained during the surgical resolution of bile duct obstruction, whereas the bile sample yielding a negative result was obtained from post-mortem autopsies conducted 48 h after death[59,60].

Tight junctions are essential for cholangiocytes to act as a barrier that protects parenchymal liver cells from potentially hazardous components of bile. Notably, *in vitro* studies have shown that viral infection with SARS-CoV-2 Leads to a decrease in the mRNA expression of tight junction proteins such as claudin 1 in cholangiocytes, implying a compromised barrier function of these cells[58]. This disruption could result in liver injury, because it may allow toxic bile components to leak into the periductal space and adjacent liver parenchyma. Furthermore, SARS-CoV-2 infection downregulates the expression of hepatobiliary transporters, such as SLC10A2/ASBT and the chloride channel ABCC7/CFTR[58](Figure 1). This downregulation of hepatobiliary transporters could compromise the sensing and signaling of bile acids by cholangiocytes and the secretion of bicarbonate. Consequently, this could contribute to the identified biliary changes in individuals with COVID-19[61]. Additionally, inflammatory pathways were increased in SARS-CoV-2 infected cholangiocytes, indicating the establishment of a reactive phenotype[54]. Prospect investigation are needed to investigate if and how SARS-CoV-2 promoted cytokine production favoring inflammation and fibrosis, potentially playing a role in the development of the "reactive cholangiocyte phenotype". Such alterations have the potential to propagate inflammation and fibrosis[57].

ARE KUPFFER CELLS AND HEPATIC STELLATE CELLS SUSCEPTIBLE TO SARS-COV-2 INFECTION?

Alveolar macrophages and monocyte-derived macrophages (MDM) are known to express ACE2, and immunohistochemistry has revealed evidence of viral protein infection of alveolar macrophages caused by both SARS-CoV-1 and SARS-CoV-2[62-64]. Nonetheless, during a histopathological assessment of ACE2 tissue distribution, no staining for ACE2 was detected in Kupffer cells and other hepatic immune cells, despite the typical observation of Kupffer cell proliferation in the livers of individuals with COVID-19[9,65] (Figure 1).

Recent investigations in response to the COVID-19 pandemic have involved more comprehensive examinations of ACE2 expression patterns. These investigations included de novo single-cell RNA sequencing analyses and *in silico* evaluations of RNA sequencing databases. The results of these studies have consistently shown that Kupffer cells do not express ACE2. In contrast, a recent report that differentiates ACE2 expression in tissue macrophages demonstrated a high level of expression even among Kupffer cells[65]. However, it is crucial to emphasize that the evidence and findings reported thus far are based on samples from healthy human livers. Therefore, it may be necessary to quantify ACE2 expression in samples taken from individuals who had either an acute liver injury or underlying chronic liver illness to gain a more comprehensive understanding of different patterns of ACE2 expression in macrophages under such conditions[65,66].

It is worth noting that following liver injury or Kupffer cell depletion, MDM can infiltrate the liver and efficiently replenish the resident hepatic macrophage population[67-69]. While *in vitro* observations have indicated that MDM may not efficiently support the replication of SARS-CoV-1 (and likely SARS-CoV-2), infected MDM could serve as carriers of the pathogen, facilitating the infection of ACE2-expressing cells in the affected organ[70]. Additionally, Kupffer cell activation and proliferation are commonly observed due to systemic inflammation, and Kupffer cell activation has been reported in liver specimens from deceased COVID-19 patients. Through the propagation of inflammatory signals, monocytic cells may be important in SARS-CoV-2-mediated liver damage, even if ACE2 expression among Kupffer cells is a matter of debate[64].

Pre-existing chronic liver diseases seem to be independent risk factors associated with unfavorable outcomes in COVID-19, with the cirrhosis grade identified as a predictor of mortality in patients infected with SARS-CoV-2[71]. Since hepatic stellate cells are the main source of fibrosis, their activation is a crucial step in the development of chronic liver disease[72,73]. Activation is induced by proinflammatory and profibrotic signals, including angiotensin II, and arises fibrosis through the enzymatic activity of ACE within the profibrotic segment of the renin-angiotensin system[74] (Figure 1). Interestingly, ACE2 acts as an antagonist to ACE, generating the anti-inflammatory and anti-fibrotic peptide angiotensin-(1–7) and lowering the ratio of angiotensin II to angiotensin–(1–7) as a result[74]. Nevertheless, neither fibrogenic nor activated cells nor quiescent hepatic stellate cells have been shown to express ACE2[74-76]. These findings imply that these cells may not serve as highly permissive hosts for SARS-CoV-2. Nevertheless, the pro-inflammatory environment instigated by direct or indirect injury to hepatocytes and cholangiocytes in the context of COVID-19 may establish conditions conducive to the activation of hepatic stellate cells, thereby initiating the process of fibrosis (Figure 1). This scenario may be particularly pertinent for individuals who have already underlying chronic liver diseases, such as MAFLD as a condition characterized by steatosis in > 5% of the liver parenchyma. While available data indicate that liver injury caused by COVID-19 is typically mild and temporary, long-term surveillance studies are essential to fully assess the possibility of hepatic fibrosis developing as a long-term effect of COVID-19, especially in patients with pre-existing liver diseases. In the context of MAFLD, inflamed hepatocytes, along with other somatic cells, may manifest mitochondrial dysfunction[77,78]. Conversely, SARS-CoV-2 has been observed to directly impact mitochondrial function in hepatocytes[79] (Figure 1). Individuals with these conditions may undergo liver injury and exhibit elevated liver function tests due to direct viral cytotoxicity. Nevertheless, liver injury in these individuals may also be associated with pre-existing inflammation and the detrimental effects of excessive and dysfunctional adipose tissue. The interconnected influences of these factors may synergistically contribute to a more severe progression of both MAFLD and COVID-19.

Another pathogenic mechanism involves additional fat accumulation in hepatocytes triggered by SARS-CoV-2. COVID-19 induces dyslipidemia[80], and autopsy studies reveal a high prevalence of steatosis in COVID-19 patients[9,81,82]. As mentioned before, individuals with MAFLD exhibit elevated levels of ACE2 and various serine proteases in the liver[30], suggesting that preexisting steatosis may enhance susceptibility to COVID-19-induced damage. Reciprocally, COVID-19 may exacerbate existing steatosis. The quantitative significance of these dynamics remains uncertain and warrants further investigation in future research[83,84].

CONCLUSION

The understanding of the interaction of SARS-CoV-2 with the liver is still evolving, and more research is needed to fully elucidate the molecular mechanisms involved in liver tropism and damage in COVID-19. The complexity of these mechanisms underscores the importance of monitoring and managing liver function in patients with COVID-19, particularly those with underlying liver conditions.

FOOTNOTES

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EDITORIAL

Metabolomics in liver diseases: A novel alternative for liver biopsy?

Yasuo Tanaka

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Abstract

Hepatitis C virus (HCV) remains a significant public health problem as it can cause acute and chronic hepatitis. Chronic HCV infection is a major cause of liver fibrosis, and evaluation of liver fibrosis is essential because the prognosis of patients with chronic HCV infection is closely related to the stage of fibrosis. Liver fibrosis is traditionally evaluated based on pathological analysis of biopsy specimens, which is considered the gold standard. Nevertheless, liver biopsy is invasive and susceptible to sampling error and inter- and intraobserver variation in pathological interpretation; it is also costly. Therefore, noninvasive diagnostic investigations have been developed, including the use of fibrotic markers, scoring systems based on routine blood tests, and transient elastography with magnetic resonance imaging or ultrasonography. Recently, metabolomics, an emerging technology, has been used to detect the fibrosis stage. In this editorial, I comment on the article titled "Metabolomics in chronic hepatitis C: Decoding fibrosis grading and underlying pathways" by Ferrasi *et al* published in the recent issue of the *World Journal of Hepatology*. I discuss previous studies on the use of metabolome analysis for the diagnosis of HCV-related liver fibrosis and the potential development of biopsy-free diagnostic techniques.

Key Words: Metabolomics; Hepatitis C virus; Liver fibrosis; Liver cirrhosis; Serum biomarker

Core Tip: Metabolomics, a rapidly emerging technology, offers a non-invasive alternative to conventional blood tests and transient elastography with magnetic resonance imaging or ultrasonography for fibrosis staging. I consider the article titled "Metabolomics in chronic hepatitis C: Decoding fibrosis grading and underlying pathways" by Ferrasi *et al,* published in the latest issue of the *World J Hepatol*. I review prior studies concerning the role of metabolomics in diagnosing hepatitis C virus-related liver fibrosis and establishing a foundation for non-invasive diagnostic techniques.

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INTRODUCTION

Hepatitis C virus (HCV) remains a significant public health concern as it can lead to acute and chronic hepatitis. The development of direct-acting antiviral therapy has substantially improved the rate of sustained virologic response and has generated interest in the goal of HCV elimination. In 2016, the World Health Organization called for the elimination of HCV infection by the year 2030[1].

Chronic HCV infection is a major cause of liver fibrosis, which is characterized by the formation of fibrous scar tissue resulting from the accumulation of extracellular matrix proteins, primarily cross-linked collagens. This tissue replaces injured liver tissue[2] and can lead to liver cirrhosis, defined as the histological development of regenerative nodules surrounded by fibrous bands. In turn, liver cirrhosis can lead to portal hypertension and end-stage liver disease[3].

Assessing the stage of liver fibrosis is essential because the prognosis of patients with liver fibrosis is closely linked to the stage of fibrosis, with those having advanced fibrosis being at higher risk for experiencing liver disease-related clinical events, such as hepatic failure and hepatocellular carcinoma[4]. Physicians require accurate methods to evaluate the progression of liver fibrosis to guide patient management and predict long-term outcomes.

Liver biopsy has traditionally been considered the gold-standard investigation for evaluating such disease. Nevertheless, it has several limitations. It is an invasive procedure that is associated with potential sampling error, inter- and intraobserver variability in pathological interpretation, and high cost[5]. To address these limitations, noninvasive diagnostic investigations have been developed.

Direct fibrotic markers, such as hyaluronic acid[6] and tissue inhibitor of metalloproteinase-1[7], and scoring systems based on routine blood tests, such as the Fibrosis-4 Index based on four factors[8] and the Aspartate Transaminase to Platelet Ratio Index[9], are cost-effective and easily accessible alternatives to liver biopsy.

Transient elastography using magnetic resonance imaging[10] or ultrasonography[11] is another option. However, their availability is limited due to the high cost of equipment.

Recently, novel diagnostic investigations based on emerging technologies, such as metabolomics, have been developed. Metabolomics involves comprehensive profiling and comparison of metabolites in biological samples, including plasma, serum, urine, and cell and tissue extracts[12]. The collected samples undergo pretreatment, and metabolites are measured using nuclear magnetic resonance or mass spectrometry (MS) combined with liquid chromatography (LC-MS), gas chromatography (GC-MS), or electrospray ionization (ESI-MS). Metabolomics offers a unique advantage because it represents the current physiological "state" of an individual, allowing exploration of factors that influence the human phenotype. The data obtained from these analyses are analyzed to determine the signatures of cellular biochemical activity. This approach is relatively novel; therefore, few studies have evaluated the associations between the metabolome and HCV-related liver disease and even fewer related to HCV-related liver fibrosis (Table 1).

Fitian *et al*[13] performed a comprehensive analysis of the global serum metabolomes of 30 patients with hepatocellular carcinoma, 27 patients with HCV-related cirrhosis, and 30 healthy controls using GC-MS and ultrahigh-performance LC-MS-MS. They found a strong association between elevated levels of bile acids (such as taurochenodeoxycholate and taurocholate) and dicarboxylic acids (such as azelate, undecanedioate, and sebacate) and cirrhosis.

Sarfaraz *et al*[14] evaluated noninvasive biomarkers for liver fibrosis, steatosis, and inflammation in patients with chronic HCV, and found that the upregulated metabolites in severe fibrosis included 1,7 dimethylxanthine, caffeine, methylsuccinate tyrosine, histidine, 2-hydroxyisovalerate, propionate, methionine, methylguanidine, 2-oxoisocaproate, and formate. Conversely, the downregulated metabolites included N-acetylaspartate, creatinine, urea, threonine, glycine, methylhistidine, adenosine, N-acetylglycine, glutamine, and asparagine.

Cano *et al*[15] examined serum metabolomics and fibrosis progression in HCV patients 1 year after transplantation. Patients at fibrosis stages F0–F1 were categorized as slow "fibrosers," whereas those at stages F2–F4 were categorized as rapid fibrosers. The investigators found that the levels of glycocholic acid, taurochenodeoxycholic acid, and sphingomyelins (SMs) (d18:0/18:0) were increased in rapid fibrosers. Conversely, the ratio of branched-chain amino acids to aromatic amino acids was reduced in rapid fibrosers. Furthermore, they developed a model to discriminate between rapid and slow fibrosers using an algorithm consisting of four lipid metabolites: two SMs [SM (d18:2/16:0) and SM (38:1)] and two phosphatidylcholines (PCs) [PC (16:0/16:0) and PC (16:0/18:0)]. This model accurately classifies rapid and slow fibrosers after transplantation.

Gaggini *et al*[16] analyzed the sera collected at baseline from 75 HCV patients using GC-MS and LC-MS, and revealed that low ceramide (18:1/22:0), ceramide (18:1/24:0), and diacylglycerol (42:6) levels and a high phosphocholine (40:6)

Table 1 Metabolites as the fibrotic biomarkers of hepatitis C

Ref.	Analyzed cases	Analytical method	Increased metabolites in fibrosis progression	Decreased metabolites in fibrosis progression
Fitian *et al*[13], 2014	Cirrhosis *vs* healthy non-diabetic controls	GC/MS, UPLC/MS-MS	Bile acids (taurochenodeoxycholate, taurocholate, *etc.*), dicarboxylic acids (azelate, undecanedioate, sebacate, *etc.*)	
Sarfaraz *et al*[14], 2016	F3- 4 *vs* F0- 2 (Metavir)	^{1}H-NMR	1,7 dimethylxanthine, caffeine, methylsuccinate, tyrosine, histidine, 2-hydroxyisovalerate, propionate, methionine, methylguanidine, 2-oxoisocaproate, formate	N-acetylaspartate, creatinine, urea, threonine, glycine, methylhistidine, adenosine, N-acetylglycine, glutamine, asparagine
Cano *et al*[15], 2017	F2- 4 *vs* F0- 1 (Metavir)	UPLC/MS	Glycocholic acid, taurochenodeoxycholic acid, sphingomyelins (d18:0/18:0)	BCAA/ArAA
Gaggini *et al*[16], 2019	F5- 6 *vs* F3- 4 *vs* F1- 2 (Ishak score)	UPLC/QTOF-MS	Phosphocholine (40:6)	Ceramides (18:1/22:0), (18:1/24:0), diacylglycerol (42:6)
Shanmuganathan *et al*[17], 2021	F2- 4 *vs* F0- 1 (Metavir)	MSI-CE-MS, ^{1}H-NMR	Choline, histidine	
Khalil *et al*[18], 2022	Cirrhosis *vs* non-cirrhosis *vs* healthy controls	UPLC/MS	Taurocholic acid, glycholic acid, glycoursodeoxy-cholic acid, taurochenodeoxycholic acid, glycochen-odeoxycholic acid	
Ferrasi *et al*[19], 2023	F1 *vs* F2 *vs* F3 *vs* F4 (Metavir)	ESI/MS		

^{1}H-NMR: Proton nuclear magnetic resonance. GC: Gas chromatography; MS: Mass spectrometry; UPLC: Ultrahigh-performance liquid chromatography; QTOF: Quadrupole time-of-flight; MSI-CE: Multisegment injection-capillary electrophoresis; ESI: Electrospray ionization; BCAA/ArAA: The ratio of branched-chain amino acids (BCAA) to aromatic amino acids (ArAA).

level were associated with greater fibrosis.

Shanmuganathan *et al*[17] demonstrated that serum levels of choline and histidine were consistently higher in HCV patients with late-stage (F2–F4) liver fibrosis compared to early-stage (F0–F1) fibrosis.

Khalil *et al*[18] found that changes in serum levels of several bile acids exhibit a linear trend across hepatocellular carcinoma, cirrhosis, non-cirrhosis, and healthy controls, potentially reflecting disease progression. Furthermore, receiver operating characteristic (ROC) curve analysis identified five conjugated acids (taurocholic acid, glycocholic acid, glycoursodeoxycholic acid, taurochenodeoxycholic acid, and glycochenodeoxycholic acid) that effectively distinguished hepatocellular carcinoma (HCC) from patients with non-cirrhotic livers.

Ferrasi *et al*[19] provided new insights into the pathogenesis and progression of liver fibrosis in HCV infection through metabolite analyses. They analyzed sera from 46 HCV patients and 50 healthy controls using ESI-MS. ESI is a soft-ionization technique that limits ion excitation, resulting in minimal or no analyte fragmentation[20]. This ionization technique has revolutionized the analysis of large biomolecules, such as the detection of coenzyme A in the present study. Statistical analysis was performed using partial least squares discriminant analysis and the variable importance score. The six most important ions were selected for each group, encompassing various metabolites categorized as sterols, lipids (glycerolipids, eicosanoids, sphingolipids, prenol lipid, and glycerophospholipids), coenzyme A, polypeptide, methyl-adenosine, amino acid derivatives, and acylcarnitines. The investigators performed ROC curve analysis to determine the diagnostic accuracy of metabolites associated with each grade of fibrosis. The metabolites demonstrated high sensitivity and specificity for each fibrosis grade except for F2. Consistent with the findings by Cano *et al*[15], detection of sterols, such as 18:0 and 20:5 cholesteryl esters, among patients with F1 fibrosis revealed downregulation of cholesteryl esters in rapid "fibrosers." Furthermore, the detection of diacylglycerols among patients with F1 fibrosis supported previous results that diacylglycerols were downregulated in patients with severe fibrosis[16]. Conversely, the significant upregulation of acylcarnitines among patients with F4 fibrosis mirrored the hyper-carcinogenic state observed in HCC patients [13]. These studies have provided useful information regarding detection of the fibrosis grade and underlying pathways in HCV infection.

However, the aforementioned results raise concerns about whether these metabolites are specific to HCV-related liver fibrosis or if they may also be caused by other etiologies, such as hepatitis B virus infection, alcohol consumption, and nonalcoholic steatohepatitis.

Given the absence of overlap between each fibrosis stage, the changes in metabolites with fibrosis progression remain unclear. In particular, it remains to be explored whether the metabolite levels exhibit a linear relationship with fibrosis stage. Furthermore, the biological significance of each metabolite is not yet known. Further studies with larger sample sizes are needed to verify these results.

CONCLUSION

Metabolomics is a newly developed technology that has several limitations due to the influence of several factors, including sampling time, collection protocol, and measurement methods. Furthermore, it is more time-consuming and expensive compared to other methods. However, this novel approach offers valuable information for diagnosis, prognosis, and treatment of liver disease. The role of metabolomics in HCV requires further investigation. In the future, metabolomics may enable the diagnosis of liver diseases without the need for biopsy.

FOOTNOTES

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MINIREVIEWS

Role of fecal microbiota transplant in management of hepatic encephalopathy: Current trends and future directions

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Abstract

Fecal microbiota transplantation (FMT) offers a potential treatment avenue for hepatic encephalopathy (HE) by leveraging beneficial bacterial displacement to restore a balanced gut microbiome. The prevalence of HE varies with liver disease severity and comorbidities. HE pathogenesis involves ammonia toxicity, gut-brain communication disruption, and inflammation. FMT aims to restore gut microbiota balance, addressing these factors. FMT's efficacy has been explored in various conditions, including HE. Studies suggest that FMT can modulate gut microbiota, reduce ammonia levels, and alleviate inflammation. FMT has shown promise in alcohol-associated, hepatitis B and C-associated, and non-alcoholic fatty liver disease. Benefits include improved liver function, cognitive function, and the slowing of disease progression. However, larger, controlled studies are needed to validate its effectiveness in these contexts. Studies have shown cognitive improvements through FMT, with potential benefits in cirrhotic patients. Notably, trials have demonstrated reduced serious adverse events and cognitive enhancements in FMT arms compared to the standard of care. Although evidence is promising, challenges remain: Limited patient numbers, varied dosages, administration routes, and donor profiles. Further large-scale, controlled trials are essential to establish standardized guidelines and ensure FMT's clinical applications and efficacy. While FMT holds potential for HE management, ongoing research is needed to address these challenges, optimize protocols, and expand its availability as a therapeutic option for diverse hepatic conditions.

Key Words: Hepatic encephalopathy; Fecal microbiota transplant; Cognitive impairment; Liver cirrhosis; Chronic liver disease

Core Tip: Hepatic encephalopathy (HE) is a reversible neurocognitive dysfunction and a frequent complication in patients with chronic liver disease. HE results from synergistic interaction between various mechanisms like increased ammonia production, systemic inflammation, disruption of the blood-brain barrier, and impairment of neurotransmission, leading to altered gut-brain-liver axis. Lactulose and rifaximin are the current mainstays of management of HE as they are known to decrease ammonia production. Fecal microbiota transplant is being studied as a potential microbiome targeted therapy that can improve the symptoms of HE by decreasing ammonia production, decreasing systemic inflammation, and improving intestinal barrier function.

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INTRODUCTION

Hepatic encephalopathy (HE) is a neurological condition that manifests in advanced liver disease, resulting in significant morbidity and mortality[1]. In the United States, 7–11 million people are affected by HE, with approximately 150000 new diagnoses reported annually. Among recently diagnosed cases, approximately 20% are associated with cirrhosis[2]. The prevalence of HE can vary depending on the severity of liver disease and the specific patient population under investigation. Table 1 provides a general overview of prevalence rates for common hepatic pathologies leading to HE[3,4]. It is important to note that these prevalence figures may differ depending on the study population, the diagnostic criteria employed, and other influencing factors. The occurrence of HE can also be influenced by comorbidities such as alcohol consumption, infections, and other complications associated with liver disease.

Clinical intervention holds promise for reversing HE, particularly in acute cases. Contributing factors leading to HE include ammonia toxicity, disrupted gut-brain communication, and inflammation. Increased ammonia levels resulting from liver disease exert neurotoxic effects. Altered gut microbiota and increased gut permeability facilitate the entry of toxins into the bloodstream, affecting brain function through neurotransmitter imbalances. Inflammatory processes in the body and brain further exacerbate the condition. Addressing these underlying factors is critical in the management of HE [5-7]. Effective management improves symptoms and quality of life, thereby significantly improving the well-being of patients, encompassing the treatment of underlying liver disease and the reduction of ammonia levels. Medications such as lactulose or rifaximin are approved by the Food and Drug Administration for the treatment of HE. These medications exert their therapeutic effects by modulating gut microbiota composition and decreasing gut ammonia levels[8]. Recently, fecal microbiota transplantation (FMT) has emerged as an alternative approach for modulating gut microbiota and ameliorating symptoms of HE. Kao *et al*[9] published a pioneering case report that documented the initial utilization of FMT as a therapeutic approach for the treatment of HE. While FMT is currently primarily used for Clostridium difficile infection, its application in HE is still evolving[10].

Table 1 Overview of the prevalence of hepatic encephalopathy in different chronic hepatic conditions

Condition	Total, *n* = 166192	Did not develop HE, *n* = 117433	Developed HE, *n* = 48759
Alcoholic cirrhosis	54194 (33)	30011 (26)	24183 (50)
Hepatitis C cirrhosis	49599(30)	31247(27)	18352(38)
Nonalcoholic cirrhosis	78111 (47)	62433 (53)	15678 (32)

Data are shown in *n* (%). HE: Hepatic encephalopathy

This review article aims to provide a comprehensive and scientifically rigorous overview of FMT. It will elucidate the pathogenesis of gut dysbiosis leading to HE, and discuss the efficacy, safety, limitations, and future prospects for the implementation of FMT therapy in managing patients with HE.

TYPES AND STAGING OF HE

The stages of HE can be assessed using the clinical grading system called West Haven Criteria (WHC) as recommended by the American Association for Study of Liver Diseases. HE can be clinically classified into four grades based on the symptoms at presentation, as shown in Table 2[11]. Grade I includes subtle personality changes. Grade II involves gross disorientation, inappropriate behavior, and lethargy. Grade III includes stupor and disorientation, while Grade IV represents a comatose state with or without decorticate or decerebrate posturing[11,12]. Other etiologies that can lead to changes in mentation should be evaluated and ruled out[8]. Based on the etiology, HE can be broadly classified into three types. Type A is HE secondary to acute liver failure, type B occurs in patients with a portosystemic shunt, and type C in patients with cirrhosis[11]. HE can be categorized as episodic if there is one episode over a 6-mo period, recurrent if there are multiple episodes in 6 mo, or persistent if the patient does not return to baseline[11].

IMPACT OF HE ON PATIENT'S QUALITY OF LIFE AND PROGNOSIS

HE greatly impacts patients' quality of life and prognosis. The severity can range from mild cognitive impairment to severe neurological dysfunction, affecting memory, cognition, and daily functioning[3]. Challenges in activities, social interactions, and employment are common. Frustration, anxiety, and depression are prevalent for patients and caregivers. Patients with HE need comprehensive psychological and social support and management strategies due to its significant negative impact on quality of life[3]. Apart from cognitive dysfunction, HE can also present with physical manifestations, including tremors, muscle stiffness, coordination difficulties, and asterixis. These physical symptoms can restrict a patient's mobility and hinder their performance of tasks that require precise motor skills[11,13]. Recurrence and progression of HE can further impair cognitive function and quality of life. Patients must receive proper medical treatment and follow-up to manage their liver disease and reduce the risk of recurrent HE. The prognosis of patients with HE varies based on underlying liver disease, severity, treatment response, and overall health. Severe HE, acute liver failure, and advanced chronic liver disease increase the mortality risk. Complications like hepatocellular carcinoma or liver failure worsen prognosis[14].

OVERVIEW OF FMT

Historical background and rationale

The microbiome targeted therapies that have been proposed as a therapeutic option in the management of patients with hepatic cirrhosis include prebiotics, probiotics, FMT, antibiotics, and synbiotics[15]. Probiotics are live microbial supplements of human origin which have shown to benefit the host by improving intestinal microbial balance when consumed adequately[15,16]. Prebiotics are nondigestible food ingredients that can selectively stimulate the growth of beneficial bacteria in the human gut and thereby improving the host's health[16]. Synbiotic is the synergistic combination of prebiotics and probiotics[16]. A meta-analysis of 9 randomized control trials showed that prebiotics and probiotics were associated with significantly reduced relative risk of no improvement in minimal HE without any significant adverse events[16]. There are no studies in the literature that have compared the direct outcomes and adverse events of prebiotics, probiotics, synbiotics, and FMT.

FMT refers to the transfer of stool from healthy donors to patients with a dysbiotic gut environment in order to restore eubiosis[17]. Since the fourth century in China, human fecal material has been used in the form of a yellow soup to manage conditions such as diarrhea, constipation, and abdominal pain[18]. In 2013, the first human randomized controlled trial (RCT) was conducted to test the efficacy of FMT in patients with recurrent Clostridium difficile infection (CDI)[18]. The first successful use of FMT in non-infectious conditions like ulcerative colitis (UC) was reported in 1989

Table 2 Classification of hepatic encephalopathy based on American Association for Study of Liver Diseases[11]

Grade[1]		Explanation[2]	Suggested operational criteria[3]
Covert	Minimal	Tests measuring psychomotor speed, executive function, or neurophysiological abilities may change psychometrically or neuropsychological without showing any signs of a mental shift	A non-phenomenological abnormality on recognized psychometric or neuropsychological tests
	Grade 1	Trivial lack of awareness; Euphoria or anxiety; Shortened addition or subtraction	Despite being spatially and temporally oriented, this individual appears to have some cognitive/behavioral issues. decay concerning his clinical assessment that meets her standards, or to the carers
Overt	Grade 2	Lethargy or apathy; Gross disorientation; Obvious personality change; Inappropriate behavior	Disorientation with regard to time (at least three of the following are incorrect: day of the week, month, season, and year) plus/minus the other symptoms stated)
	Grade 3	Marked confusion; Somnolence to semi-stupor; Responsive to stimuli; Bizarre behavior	Disoriented also in terms of space (at least three of the incorrectly reported terms: nation, state or area, cities, location, plus/minus the other indicators)
	Grade 4	Comatose state; Unresponsive to pain; Decorticate or decerebrate posturing	Never react, not even with painful stimuli

[1]Unimpaired.
[2]Absolutely no encephalopathy, no previous hepatic encephalopathy (HE) diagnosis.
[3]Examined and found to be normal.

[19]. Over the past decade, the range of applications for FMT has significantly expanded[18]. The efficacy of FMT has been tested in various gastrointestinal infectious and noninfectious etiologies including CDI, UC, irritable bowel syndrome (IBS), primary sclerosing cholangitis, metabolic syndromes, HE, and D-lactic acidosis[20].

Mechanisms of action of gut dysbiosis and FMT in HE

The human gut microbiome consists of a diverse range of bacteria, fungi, viruses, and protozoa, all of which can have proinflammatory or anti-inflammatory effects, thereby influencing the inflammatory environment[21]. Patients with HE are particularly susceptible to disturbances in gut microbiota due to frequent antibiotic use[22]. Disruption of the gut-liver-brain axis is the primary cause of neurocognitive dysfunction in individuals with HE[6]. In recent years, culture-independent studies have revealed a link between alterations in the gut microbiome, cognitive function, and systemic inflammation. Changes in the microbiota in both minimal HE and overt HE have been associated with impaired cognition, endotoxemia, and inflammation[23].

A potential mechanism explaining the association between gut dysbiosis, the severity of cirrhosis, and cognitive function involves reduced production of bile acids in cirrhosis patients which can alter the indigenous gut microbiota [23]. Healthy gut microbiota such as *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales Cluster XIV* contribute to the production of short-chain fatty acids and maintenance of the gut barrier integrity[23]. In individuals with liver dysfunction, the liver's reduced detoxification ability or the bypassing of bacterial products (including endotoxins, ammonia, and bacterial DNA) through portosystemic shunts can lead to systemic inflammation and cognitive decline [24]. Disruptions in the inflammatory environment and the presence of toxins promote neuroinflammation, resulting in elevated levels of intra-astrocytic ammonia. This, in turn, leads to increased concentrations of osmotically active glutamate or glutamine, along with decreased levels of myoinositol and choline[25]. The possible mechanism of HE in cirrhotic patients with gut dysbiosis is displayed in Figure 1 (created with BioRender.com). A study conducted on a rat model demonstrated that FMT can alleviate intestinal edema, mucosal damage, and inflammatory infiltration induced by HE[26]. FMT was also associated with reduced ammonia levels and systemic inflammation, as evidenced by decreased levels of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α[26]. Another study in rats with D-galactosamine-induced liver injury and FMT with *B. adolescentis* revealed significant alterations in the gut microbial community, including a decrease in pathogenic taxon *Proteus* and an enrichment of taxa responsible for lipid and amino acid metabolism, such as *Coriobacteriaceae*, *Bacteroidales*, and *Allobaculum*[27].

A study revealed a positive correlation between the severity of cirrhosis, as measured by the Child-Turcotte-Pugh Score (CTP), and the presence of the taxon *Enterobacteriaceae*, while a negative correlation was observed with *Ruminococcaceae*[28]. In a larger study involving 219 patients with liver cirrhosis, progressive changes in the gut microbiome were found to be associated with decompensated cirrhosis. The cirrhosis dysbiosis ratio represents the ratio of autochthonous bacteria (such as *Ruminococcaceae*, *Lachnospiraceae*, and *Clostridales cluster XIV*) to non-autochthonous bacteria (including *Enterobacteriaceae* and *Bacteroidaceae*), was correlated with the Model for End-Stage Liver Disease (MELD) score and endotoxin levels[29].

An overabundance of *Streptococcus salivarius*, which has been implicated in increased ammonia production due to its urease activity, was associated with elevated ammonia levels and cognitive impairment in patients with minimal HE. No significant difference was observed in the stool microbiome between minimal HE and overt HE, however, significant differences were observed in the colonic mucosal microbiome of these patients[30]. Autochthonous genera (such as *Lachnospiraceae Roseburia*, *Lachnospiraceae Dorea*, and *Ruminococcaceae Faecalibacterium*) were associated with better cognitive function compared to non-autochthonous genera (including *Burkholderiaceae Other*, *Veillonellaceae Megasphaera*, *Rikenellaceae Alistipes*, *Streptococcaceae Streptococcus*, *Alcaligenaceae Sutterella*, and *Porphyromonadaceae Parabacteroides*), which

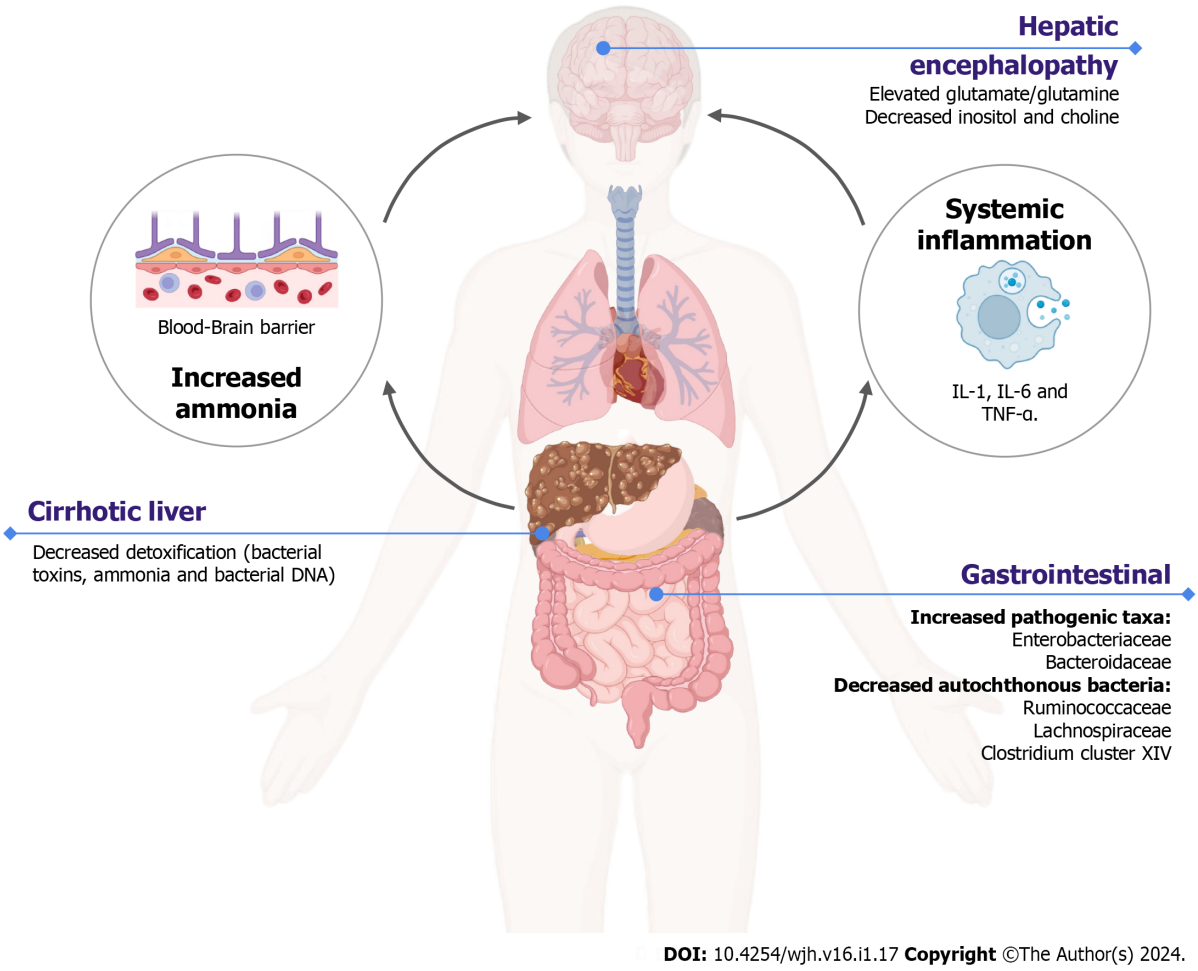

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Figure 1 Pathogenesis of hepatic encephalopathy in cirrhotic patients with gut dysbiosis.

were linked to poorer cognitive function in patients with both overt and non-overt HE[23]. Currently, Lactulose and rifaximin are commonly used as prophylaxis to reduce ammonia-producing bacteria and prevent recurrent episodes of HE[31,32]. However, long-term use of rifaximin can lead to drug resistance. FMT has shown outstanding clinical efficacy in the management of various conditions like CDI, inflammatory bowel disease, IBS, *etc.* It has also been explored in the management of HE and has shown promising results[33].

FMT delivery methods

Several techniques have been developed and tested for FMT, including colonoscopy, enema, nasogastric or nasojejunal tubes, and capsules. The outcomes of FMT have been associated with various factors, such as donor selection, sample selection, and delivery techniques[34]. Although there is limited data directly comparing related *vs* non-related donors, current evidence does not show a significant difference in outcomes[34]. The establishment of stool banks has increased the use of unrelated donors for FMT, providing easier access and availability[35]. Autologous stool FMT is a newer concept with limited evidence, involving the transplant of stool from patients themselves when their disease is in remission[21].

For upper gastrointestinal delivery, standard methods include esophagogastroduodenoscopy, nasogastric or nasojejunal tube, and oral capsules. Colonoscopy and enemas are commonly used for lower gastrointestinal delivery[34]. Oral capsules are the most recent development in stool delivery and are widely used due to their minimal invasiveness, convenience, and acceptance compared to other procedures[36]. The advantages and disadvantages of different delivery methods of FMT are discussed in Table 3[34,37,38].

Different methods used for fecal preparation include fresh fecal matter, frozen fecal matter, and lyophilized fecal matter (freeze-dried stool)[39]. Fresh FMT can readily be immediately transferred from a donor and has higher microbial load and diversity but it is logistically challenging to find a donor and transfer stool immediately[40]. On the other hand, frozen FMT can be conveniently stored and transported but it can lose efficacy if appropriate preservation and storage techniques are not maintained[15,40]. Lyophilized stool is the easiest to store and administer as it does not warrant invasive procedures for administration[40]. Multiple studies in patients with CDI have shown an overall efficacy of frozen fecal matter ranging from 81% to 100%. However, there are no significant differences in outcomes between fresh and frozen fecal preparations[34]. The efficacy of lyophilized stool also ranges from 78%-100%. The efficacy of lyophilized stool (78%) was significantly lower compared to fresh fecal preparation (100%), but equally effective compared to frozen stool (83%) based on a RCT[41]. No significant data is available comparing the use of different forms and delivery

Table 3 Comparison of different modes of fecal microbiota transplant delivery

Mode of delivery		Advantage	Disadvantage
Upper gastrointestinal tract	Nasogastric	Faster; Comparatively less expensive; Better tolerability	Risk of aspiration; Discomfort; Increased risk of small intestinal bacterial overgrowth
	Nasojejunal	Faster; Comparatively less expensive; Better tolerability	Risk of aspiration; Risk of bowel perforation; Increased risk of small intestinal bacterial overgrowth
	Oral capsule	Least invasive; Cost-effective; Easy to store	Risk of aspiration; Vomiting; Sometimes failure to reach intestinal target
Lower gastrointestinal tract	Colonoscopy	Direct visualization of GI tract; Standard risks of sedation and procedural intervention	Risk of bowel perforation; Higher cost of performing procedure
	Retention enema	Useful in patients with severe colitis or colon distention to avoid perforation; Less invasive as compared to colonoscopy	Difficulty to retain transplanted stool; Need for repeated small volume infusion; Possible retention in patients with poor sphincter tone

methods of FMT in the management of HE. The method of preparation of FMT can also impact the outcomes. Several studies in patients with UC have shown improved outcomes with anaerobically processed FMT as compared to aerobically processed FMT as many probiotics like *Faecalibacterium prausnitzii* are lost with aerobic stool processing[42]. Similarly, patients with HE might also benefit from anaerobically processed FMT[42]. The advantages and disadvantages of different delivery methods of FMT are discussed in Table 4[40]. FMT can be a robust technique for treating various conditions. However, there is no consensus on the specific route, dose, and preparation to be used for a particular condition.

HEPATIC CONDITIONS ASSOCIATED WITH HE

Chronic hepatitis B

Hepatitis B is a viral infection caused by the hepatitis B virus (HBV) and is typically transmitted through sexual, parenteral, or vertical routes. In 2019, it was estimated that there were 316 million infected individuals worldwide[43]. A study using a mouse model demonstrated that FMT can modulate the immune response and affect the host's susceptibility to HBV infection[44]. This finding highlights the crucial role of the gut microbiota in HBV infection. Furthermore, the composition of the gut microbiota varies across the different stages of chronic hepatitis B and these variations may be closely related to liver fibrosis[45]. Moreover, patients with liver cirrhosis experience an imbalance between beneficial and pathogenic bacteria, with a decrease in the abundance of beneficial bacteria such as Dialiste and Alistipes, and an increase in the abundance of pathogenic species within Actinobacteria[45]. This finding suggests that the gut microbiota may be involved in the pathogenesis of chronic hepatitis B progression. Therefore, the modulation of the gut microbiota through FMT could potentially influence the clinical course of HBV infection. Evidence suggests that the gut microbiota plays a critical role in the immune clearance of HBV.

Ren *et al*[46] conducted a case-control pilot study with an open-label design to assess the effectiveness of FMT in achieving hepatitis B e antigen (HBeAg) clearance. The study included a total of 18 patients, with 5 individuals assigned to the FMT arm and 13 to the control group. The results revealed that among the 5 patients in the FMT arm, 4 successfully achieved HBeAg clearance after undergoing 1-7 rounds of FMT treatment. Similarly, Chauhan *et al*[47] conducted a non-randomized pilot study where two out of twelve patients achieved HBeAg clearance. These findings indicate a positive association between FMT and the clearance of HBeAg. Guo *et al*[48] evaluated the efficacy of FMT in 35 patients with different stages of HBV-related chronic liver disease. The results showed that continuous FMT treatment led to improvements in liver function, controlled HBV-DNA replication, enhanced intestinal mucosal barrier function, and delayed the progression of HBV. Additionally, FMT demonstrated the ability to convert HBeAg-positive patients to HBeAg-negative status in 36.4% of cases, and it achieved negative conversion of HBV-DNA in 53.3% of patients[48]. The majority of the studies examined involve a limited number of participants, emphasizing the necessity for larger clinical studies to elucidate the findings pertaining to FMT as a treatment for HBV. Furthermore, there aren't studies evaluating the effects of FMT on HE resulting from HBV. Hence, further research is required to comprehensively understand the efficacy of FMT in this topic.

Chronic hepatitis C

Chronic infection with hepatitis C virus (HCV) leads to long-term liver inflammation, potentially resulting in liver fibrosis and cirrhosis, hepatic decompensation and chronic liver failure[49]. Studies have shown that the prevalence of liver cirrhosis 20 years after presumed HCV infection ranges from 7% to 18%[50]. The intestinal microbiota plays a significant role in influencing the onset and progression of HCV infection. Heidrich *et al*[51], reported a decrease in alpha diversity (observed richness or evenness of a specific taxa in an average sample within the habitat) measured by number of phylotypes and the Shannon Diversity Index associated with HCV infection, which further diminishes in cirrhotic patients. They also identified distinct microbial communities in the intestines of HCV patients, with non-cirrhotic individuals demonstrating a relatively higher abundance of *Veillonella spp.*, *Lactobacillus spp.*, *Streptococcus spp.*, and

Table 4 Comparison of different fecal microbiota transplant preparation methods

FMT preparation method	Efficacy range (%)	Preservation of microbial diversity	Advantages	Disadvantages
Fresh[40]	85-100	High	Contains diverse microbial population	Requires immediate availability of the patient
Frozen[40]	83-95	Moderate	Allows for long term storage	Loss of some microbial diversity during freezing; Comprise on efficacy if not stored properly and use of incorrect thawing techniques
Frozen lyophilized[40]	78-84	Moderate	Longer shelf life; Can be easily incorporated into a capsule	Loss of some microbial diversity during encapsulation

FMT: Fecal microbiota transplantation.

Alloprevotella spp., while the highest abundance is observed in cirrhotic patients. This positive association of increased abundance in liver fibrosis progression suggests a connection between these genera and liver fibrosis progression in the liver. Conversely, *Bilophila spp.*, *Clostridium IV spp.*, *Clostridium XlVb spp.*, *Mitsuokella spp.*, and *Vampirovibrio spp.* appear to be negatively associated with fibrosis progression, showing decreased abundance from healthy controls to non-cirrhotic and cirrhotic patients[51]. Furthermore, several studies have investigated the impact of HCV eradication on gut dysbiosis [52-55]. Wellhöner *et al*[52] analyzed changes in the gut microbiome following direct-acting antivirals treatment and achieved sustained virological response (SVR). The study observed an increase in alpha diversity in non-cirrhotic patients, but not in those with cirrhosis. Bajaj *et al*[53] also found that systemic inflammation and gut dysbiosis are present in HCV cirrhosis patients irrespective of achieving SVR. Taken together, these studies highlight the complex relationship between the intestinal microbiota, HCV infection, fibrosis progression, and treatment outcomes.

Despite the growing interest in FMT as a potential therapeutic intervention for HE in liver cirrhosis, there is a notable lack of articles specifically assessing its efficacy in patients with liver cirrhosis due to chronic HCV infection. This knowledge gap underscores the need for further research in this area to better understand the effectiveness, safety, and long-term outcomes of FMT in this specific patient population. Such studies would provide valuable insights into the potential benefits and limitations of FMT as a treatment modality for HE in liver cirrhosis associated with chronic HCV infection and help guide future clinical practice and therapeutic decision-making.

Alcoholic liver disease

Alcohol is a major risk factor for liver cirrhosis with risk increasing exponentially[56]. The prevalence of cirrhosis and heavy alcohol use varies by country, being higher in Europe (16%-78%) and the United States (17%-52%) compared to Asia (0%-41%)[57]. Chronic heavy alcohol consumption, even in the absence of liver disease, affects gut bacterial composition. The composition of the gut microbial community in patients with alcoholic liver cirrhosis (ALC) is characterized by a decrease in the commensal taxa *Clostridia*, *Bacteroidetes*, and *Ruminococcaceae* but an increase in *Lactobacillus*, *Bifidobacterium* and oral microbiota[58]. This dysbiosis leads to acetaldehyde-caused increased intestinal permeability, the increased blood concentration of endotoxins, and activation of inflammatory markers (TNF-α, IL-1β, IL-6, IL-8, and IL-10) likely inducing liver damage[59]. In patients with HE and ALC, *Escherichia/Shigella*, *Burkholderiales*, and *Lactobacillales* taxa predominated and led to an enhanced catabolism of arginine through ammonia-producing pathways[60]. This causes further systemic/neuroinflammation, hyperammonemia, endotoxemia, and microglial activation, increasing the risk of development of HE[23,61,62].

A potential role for FMT to improve the altered gut-brain axis in alcoholic liver disease and HE has been described. In a study involving 8 male patients with severe alcohol-associated hepatitis (SAH), FMT demonstrated significant improvements in liver disease severity within 1 wk, which were sustained over a median follow-up of almost a year[63]. HE was resolved in 71.4% of the FMT group[63]. Another similar study aimed to assess the longer-term outcomes (> 1 year) of FMT in SAH and found promising clinical benefits, including significantly lower incidences of HE[64]. The only article evaluating chronic conditions and FMT was a phase 1 randomized control trial with 20 patients with ALC and active drinking, participants were assigned either a placebo or an FMT enema from a donor with an enriched abundance in *Lachnospiraceae* and *Ruminococcaceae*[65]. Following a 2-wk period, FMT patients exhibited significant cognitive improvement, as assessed by both the Psychometric HE Score (PHES) and the EncephalApp (mobile application designed to evaluate cognitive function in HE) captured improvements in both off-time (periods of impaired cognitive performance) and on-time (periods of normal cognitive function) among the FMT-treated individuals. In addition, alcohol craving/consumption, quality of life, and diversity of microbiota also improved. These findings highlight the potential of FMT as a promising treatment option for patients with SAH, warranting further investigation through larger controlled studies.

Metabolic Dysfunction Associated Steatotic Liver Disease and nonalcoholic steatohepatitis

Metabolic Dysfunction Associated Steatotic Liver Disease (MASLD) is a liver disease characterized by the accumulation of fat in the liver. Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent liver diseases worldwide, with a global prevalence of 38.2%[66]. Emerging research has linked the gut microbiome dysbiosis and the pathogenesis of

MASLD through the dysregulation of the gut-liver axis[67,68]. In MASLD, there is a disruption in the integrity of the intestinal barrier, resulting in an increase in intestinal permeability. This increased permeability has been associated with the severity of hepatic steatosis[69]. The compromised intestinal barrier function leads to endotoxemia and inflammation, which can further contribute to alterations in bile acid profiles and metabolite levels produced by the gut microbiota[68]. Moreover, dysregulation of bile acids has been closely linked to the progression of MASLD[70]. At the phylum level, patients with MASLD have variations in the gut microbiota composition, characterized by an increase in *Proteobacteria* and *Firmicutes* and a decrease in *Bacteroidetes* abundance[68]. Although obesity is closely associated with NAFLD, the role of dysbiosis caused by obesity in influencing MASLD development remains a subject of debate due to conflicting evidence[68]. Nonetheless, considering the significant role of the gut microbiota in MASLD, it is necessary to explore therapeutic strategies aimed at modulating the gut microbiota such as probiotics, prebiotics, antibiotics, and FMT[71]. A study conducted on a mouse model, in which steatohepatitis was induced through a high-fat diet, demonstrated that FMT effectively attenuated steatohepatitis and restored gut microbiota balance in mice after an 8-wk intervention[72]. This intervention resulted in reduced serum levels of alanine aminotransferase and aspartate aminotransferase, increased expression of zonula occludens-1, associated with improved tight junction integrity, restoration of high-fat diet-induced mucosal damage, and a decrease in serum endotoxin levels[72]. Xue *et al*[73] conducted a RCT involving 47 patients assigned to the FMT group, wherein the patients received FMT *via* colonoscopy using donor stool. Remarkably, the results demonstrated that FMT led to a significant increase in the *Bacteroidetes/Firmicutes* ratio, thereby indicating a positive modulation of the gut microbiota. Furthermore, the FMT group exhibited noteworthy clinical improvements as evidenced by a reduction in hepatic fat attenuation, which was evaluated using FibroScan. Subsequently, Kootte *et al*[74] examined the effects of FMT on insulin sensitivity in patients with metabolic syndrome. Their findings revealed a significant improvement in insulin sensitivity at 6 wk after allogeneic FMT. However, a clinical trial conducted by Craven *et al*[75] did not find an improvement in insulin sensitivity but demonstrated that allogeneic FMT improved intestinal permeability after 6 wk. Furthermore, patients with MASLD resulting from dysbiosis often experience disruptions in the gut-brain axis, leading to cognitive deterioration[76]. As MASLD progresses, it can lead to elevated levels of ammonia and exacerbate cognitive impairment in conjunction with a pro-inflammatory environment[76]. FMT has demonstrated efficacy in ameliorating HE among cirrhotic patients[77]. Therefore, FMT holds significant potential as a safe and promising therapeutic approach to enhance cognition in individuals with MASLD.

Table 5 compiles and highlights existing research, showcasing FMT's capacity to rebalance gut equilibrium and mitigate cognitive impairments associated with HE[9,22,42,63,66,77-81]. There are a few studies that have assessed the efficacy of HE based on the severity. Of these studies, the study by Metha *et al*[81] on 10 patients treated with FMT for recurrent over HE (≥ 2 episodes of WHC criteria II-IV HE in 6 mo as previously described in Table 2) showed that 6 patients had sustained clinical response as well as significant improvement in ammonia levels, CTP and MELD score. On the other hand there were two readmissions for spontaneous bacterial peritonitis and three patients with overt HE[79]. The case report by Kao *et al*[9] showed improvement in ammonia levels, Inhibitory Control Test (ICT), and Stroop test after FMT enema in a patient with Grade I-II HE which later worsened after stopping the treatment.

To sum up, significant progress has been achieved in the potential application of FMT as a therapeutic option for HE among cirrhotic patients and careful selection of the donor can lead to improved outcomes in patients with HE. Prospective studies are required to compare the efficacy of FMT in patients with different stages of HE.

SAFETY OF FMT IN HE

FMT is grounded in the concept of bacterial displacement, where beneficial bacteria from a healthy donor are introduced to replace harmful pathogens in the recipient's gut. This process leverages competitive exclusion[82]. By restoring a balanced microbiome, FMT aims to alleviate disease and its progression. Analysis of cross-sectional stool genomics data has revealed significant dysregulation in the expression levels of specific genomics species between decompensated and compensated hepatic conditions[83]. This provides valuable scientific insights into the molecular alterations associated with disease progression and highlights potential targets for further investigation and therapeutic interventions[9]. Cognitive assessment using the ICT and the Stroop test demonstrated progressive improvement in cognition with consecutive FMT sessions, reaching the plateau after the 4th wk following three FMTs. Notably, cognition regressed to baseline 14 wk after discontinuing FMT which intriguingly was associated with a marked reduction in the levels of lachnospiraceae, a bacterial family associated with improved cognitive function. The promising outcomes of this study paved the pathway for the initiation of the first RCT.

In a notable study by Bajaj *et al*[77] in 2017, an open-label randomized trial was conducted involving a cohort of 20 cirrhotic patients experiencing recurring episodes of HE. The intervention arm involved the administration of lachnospiraceae and ruminococcaceae enema in addition to standard of care (SOC). The results of the study demonstrated a significant reduction in the occurrence of serious adverse events (SAEs) in the FMT arm (P = 0.02) compared to the SOC arm. In the FMT arm, 2 patients were hospitalized within 5 mo, but their conditions (acute kidney injury and chest pain) were deemed not related to FMT. In contrast, the SOC arm experienced 11 SAEs, including liver-related complications such as mental status changes, pneumonia, chest pain, portal vein thrombosis, anemia, gastroenteritis, and variceal bleeding. The FMT arm also showed significant cognitive improvement, indicated by improved PHES and encephal app Stroop scores, while the SOC did not exhibit similar improvements. The 12 mo follow-up of the patients revealed that FMT appeared to be safe and held potential for long-term efficacy[80].

In 2019, a subsequent study was conducted by Bajaj *et al*[22] comparing FMT using capsules enriched with lachnospiraceae and ruminococcaceae (administered in a dose of 15 capsules at a time) with a placebo group. The study focused

Table 5 Overview of important studies highlighting the efficacy and adverse effects of fecal microbiota transplant in the management of conditions associated with hepatic encephalopathy

Ref.	Type	Population	Intervention	Comparison	Outcomes					Adverse effects
					Cognitive impairment	Microbiota	Liver function	Scores	Not specified	
Bajaj *et al*[77], 2017	RCT	20 cirrhotic patients experiencing recurrent HE while on lactulose/rifaximin treatment	FMT enema involving donor material enriched in *Lachnospiraceae* and *Ruminococcaceae*	SOC (Lactulose and rifaximin)	A significant improvement in both the PHES total score and EncephalApp Stroop was observed within the FMT group but not in the SOC group	Following antibiotic treatment, there was a decline in beneficial taxa and microbial diversity, coinciding with an expansion of *Proteobacteria*. However, FMT led to an increase in both diversity and beneficial taxa (*Lactobacillaceae* and *Bifidobacteriaceae*)	No alterations were observed in AST, ALT, or albumin levels in either study arm	In the SOC arm, MELD scores remained stable. However, in the FMT arm, antibiotics initially worsened the MELD scores, but subsequent FMT intervention successfully restored them to baseline levels	In the SOC arm, the urine metabolic profile remained stable over time. Conversely, the FMT group exhibited altered metabolites due to antibiotics, which were subsequently restored post-FMT	FMT arm: Tolerated treatment with no mental status hospitalizations; two unrelated hospitalizations occurred; SOC arm: Eleven SAEs, with higher incidences of HE and liver-related complications
Bajaj *et al*[65], 2021	RCT, phase 1	10 patients with cirrhosis and alcohol use disorder, with an AUDIT-10 score of ≥ 8 during screening (FMT arm MELD score: 9.3 ± 2.6), and an equivalent of 10 patients in the placebo arm (9.5 ± 2.8)	FMT enema involving donor material enriched in *Lachnospiraceae* and *Ruminococcaceae*	Placebo	Cognitively, post-FMT patients exhibited improvements in both PHES and EncephalApp OffTime + OnTime	Post-FMT, an increase in diversity was observed, alongside elevated levels of *Odoribacter, Bilophila, Alistipes*, and *Roseburia*; Conversely, no changes were noted in the pre-placebo microbiota	There were no changes in AST, ALT, or albumin levels within the FMT group	The MELD score within the FMT group was similar at the study's conclusion (score at the end of the study: 8.6 ± 2.8)	In the FMT group, a noteworthy decrease in craving was evident among 90% of participants, whereas this reduction was observed in just 30% of the placebo group	A significant decrease in SAEs was observed in the FMT group compared to the placebo group (1 *vs* 7). The sole SAE in the FMT group was alcohol use disorder related, while 2 placebo-assigned patients required short-term antibiotics
Bloom *et al* [42], 2022	RCT, phase 2	A group of 10 cirrhotic patients, each having previously suffered at least one episode of overt HE and currently experiencing ongoing neurocognitive dysfunction	Healthy donors with normal BMI administered 15 oral FMT capsules on days 1, 2, 7, 14, and 21; Antibiotic pretreatment was not employed	None	PHES demonstrated improvement after three doses of FMT (+ 2.1), after five doses of FMT (+ 2.9), and at the 4-wk mark following the fifth dose of FMT (+ 3.1)	Baseline *Bifidobacterium* abundance was higher in FMT responders compared to nonresponders	Not reported	Not reported	Two taxa, namely *Bifidobacterium adolescentis* and *B. angulatum*, displayed a positive correlation with PHES scores. On the contrary, *Enterobacter asburiae* and *B. breve* showed a negative correlation with PHES scores	Four minor adverse effects were noted: nausea, bloating, fatigue, and constipation; One SAE involved the transmission of extended-spectrum beta-lactamase-producing *Escherichia coli* bacteremia through FMT
Li *et al* [78], 2022	Case series	2 patients diagnosed with liver cirrhosis resulting from hepatitis B, who faced recurring Grade 2-3 HE following TIPS intervention	Fecal microbiota transplant conducted three times using 50 g of fresh fecal intestinal flora suspension	None	Subsequent hospitalizations due to HE were not reported among the patients	Notable increases in *Ruminococcus, Akkermansia,* and *Oscillospiraceae* were observed, alongside decreased abundance of *Veillonella* and *Megasphaera*. These changes were	Liver function demonstrated improvement in Case 1, while Case 2 exhibited a nonsignificant enhancement	In Case 1, Child Pugh Score decreased from 10 to 5; In Case 2, it decreased from 11 to 7	There were no clinical manifestations, and the blood ammonia level decreased significantly	No FMT-related adverse events or infection complications occurred in Case 1. Temporary constipation persisted for 7 d in Case 2 following FMT

						accompanied by an overall increase in microbiota diversity				
Bajaj *et al*[22], 2019	RCT, phase 1	20 cirrhotic patients experiencing recurrent HE and undergoing lactulose and rifaximin treatment. Out of these, ten were assigned to the FMT arm (MELD score of 9.5 ± 2.6) and ten were placed in the placebo arm (MELD score of 10.9 ± 4.2)	Administration of 15 FMT capsules from a single donor enriched in *Lachnospiraceae* and *Ruminococcaceae*	Placebo	A noteworthy improvement in OffTime + OnTime was evident within the FMT group compared to baseline. Conversely, significant PHES improvement was not observed in the FMT group, and placebo exhibited no significant changes	After FMT, duodenal mucosal diversity rose, featuring higher *Ruminococcaceae* and *Bifidobacteriaceae*, and reduced *Streptococcaceae* and *Veillonellaceae*. Similar reductions in *Veillonellaceae* were seen post-FMT in sigmoid and stool samples	Not reported	The MELD score within the FMT group was similar at the study's conclusion (score at the end of the study: 8.7 ± 2.9)	Following FMT, Duodenal E-cadherin and Defensin A5 increased, while IL-6 and serum LBP reduced	In the placebo group, 6 patients experienced SAEs: Five HE episodes, two infections, and one renal insufficiency case. In addition, 1 patient was transferred to hospice and deceased. In contrast, the FMT group had only one HE episode, with no reported deaths
Mehta *et al* [79], 2018	Case series	10 patients, previously treated with FMT for recurrent HE (defined as ≥ 2 episodes of West Haven grade II–IV HE in the last 6 mo)	FMT was introduced *via* colonoscopy into the right colon 7–10 d after the episode of HE	None	Not reported	Not reported	Not reported	A reduction in both CTP and MELD scores was observed from baseline to post-treatment week 20	The arterial ammonia concentration showed a considerable decrease at post-treatment week 20	1 patient died due to bronchopneumonia complicated by sepsis 2 mo after FMT. Additionally, 2 patients were readmitted due to spontaneous bacterial peritonitis
Kao *et al*[9], 2016	Case report	A 57-yr-old male with grade 1-2 HE, with liver cirrhosis (MELD score of 10), attributed to alcohol and hepatitis C	Weekly FMT was administered, with the first application performed *via* colonoscopy and the subsequent sessions through retention enema	None	Mental status was assessed through the ICT and Stroop test. At 4 wk after the third FMT, the ICT score changed from 17 (baseline) to 5, and the Stroop test score changed from 250.9 to 183.5. However, by the 14-wk mark, these values reverted to baseline levels	Following FMT, there was a reduction in the relative abundance of *Lachnospiraceae*	Not reported	Not reported	Not applicable	No adverse events or infectious complications linked to FMT occurred
Bajaj *et al*[80], 2019	RCT, long term outcomes (> 12 mo) of a 2017 study	20 patients with cirrhosis experiencing recurring episodes of HE	FMT enema involving donor material enriched in *Lachnospiraceae* and *Ruminococcaceae*	SOC (Lactulose and rifaximin)	The FMT group experienced fewer HE episodes during long-term follow-up compared to SOC. Additionally, cognitive function, evaluated using the PHES total score and EncephalApp Stroop, significantly favored the FMT group	During long-term follow-up, FMT displayed increased Burkholderiaceae and decreased *Acidaminococcaceae*. However, *Lachnospiraceae* and *Ruminococcaceae* remained relatively stable. Microbiota composition remained similar post-FMT, regardless of short or long-term follow-up, when compared to the pre-FMT state	Not reported	Changes in MELD scores exhibited similarity between the two groups	The FMT group experienced significantly fewer hospitalizations compared to the SOC group during the long-term follow-up	The intervention was well-tolerated in the FMT group, demonstrating a favorable long-term safety profile

Philips *et al* [63], 2017	Pilot study	8 patients diagnosed with steroid-ineligible severe alcohol-asSOCiated hepatitis (MELD score: 31 ± 5.6) and 18 control subjects (MELD score: 27 ± 5.2)	Thirty grams of donor stool samples infused daily for 7 d through a nasoduodenal tube	SOC (specifics not provided)	HE resolved in 6 out of 8 patients after FMT (71.4%).	1 yr post-FMT, there was an increase in Firmicutes and a reduction in *Proteobacteria* and *Actinobacteria*. Noteworthy species changes included decreased *Klebsiella* pneumoniae and increased *Enterococcus villorum*, *Bifidobacterium longum*, and *Megasphaera elsdenii*	The mean bilirubin levels significantly decreased from 20.5 ± 7.6 mg/dL to 2.86 ± 0.69 mg/dL after treatment	Child-Turcotte-Pugh, MELD, and MELD Sodium scores showed significant reductions post-treatment in comparison to baseline	Survival was notably better in the FMT group when compared to healthy controls. Additionally, post-FMT improvements were observed in bile, carotenoid, and pantothenate pathways	Excessive flatulence was reported as a complaint by 50% of FMT patients
Philips *et al* [81], 2022	Retrospective analysis	47 patients diagnosed with severe alcohol-asSOCiated hepatitis (MELD score: 28.1 ± 4.7) and 25 control subjects (MELD score: 28.2 ± 6.3)	The FMT group received 100 mL of freshly processed stool samples daily *via* a nasoduodenal tube for 7 d	Pentoxifylline (400 mg thrice daily for 28 d)	During follow-up, the FMT group exhibited significantly lower HE incidences compared to the SOC group	In the FMT group, there was a decrease in *Proteobacteria* and an increase in *Actinobacteria* and *Bacteroides*. Genus-level analysis revealed higher *Bifidobacterium* and lower *Acinetobacter*. Within the SOC group, higher levels of *Erwinia* and *Porphyromonas* were noted, along with lower beneficial *Bifidobacterium* at 1-2 yr. Beyond the 2-yr mark, FMT led to higher beneficial *Bifidobacterium* levels	Not reported	Not reported	During follow-up, the FMT group exhibited lower instances of ascites, infections, hospitalizations, and alcohol relapse in comparison to the SOC group. A longer time to relapse was noted, along with a trend towards improved survival at 3 yr	Acute variceal bleeding was the most common cause of death in the FMT group, whereas infection predominated in the SOC group

ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: Basal metabolic index; FMT: Fecal microbiota transplantation; HE: Hepatic encephalopathy; ICT: Inhibitory Control Test; MELD: Model for End-Stage Liver Disease; PHES: Psychometric Hepatic Encephalopathy Score; RCT: Randomized controlled trial; SAE: Serious adverse event; SOC: Standard of care.

on patients with cirrhosis experiencing recurrent episodes of HE. The placebo group had a higher number of SAEs compared to the FMT group. The FMT group (n = 10) had one reported SAE, whereas the placebo group (n = 10) reported 11 SAE (P < 0.05). Most SAEs in the placebo group were related to liver disease progression and resulted in hospitalizations and emergency room (commonly referred to as ER) visits. Six placebo patients experienced SAEs, with one patient having multiple events including episodes of HE and renal insufficiency without HE. The remaining five patients had one SAE each, including infections (pneumonia and cellulitis), HE and electrolyte abnormalities. Four placebo patients did not have any SAEs. In the FMT group, only one patient had an episode of HE as an SAE, while the remaining nine patients did not experience any SAEs during the follow up period. One placebo patient required ER visits/hospitalization for altered electrolytes, which resolved within 24 h. Unfortunately, one placebo patient with multiple admissions was transferred to hospice and passed away 4 mo after enrollment. None of the FMT assigned patients died during the follow up period. The results also revealed improvements in the microbiota composition, inflammatory markers, and cognitive scores, indicating the potential of FMT. In the FMT group, duodenal microbiota diversity was enhanced and was also marked by a decrease in the levels of serum lipopolysaccharide binding protein and interleukin-6.

Another study conducted by Bloom *et al*[42] demonstrated improved cognitive function in patients with a history of cirrhosis and overt HE after 5 doses of oral FMT capsules were given over 3 wk. FMT donors were healthy adults with normal body mass index, carefully chosen through a rigorous screening process (Figure 2: Created with biorender.com)

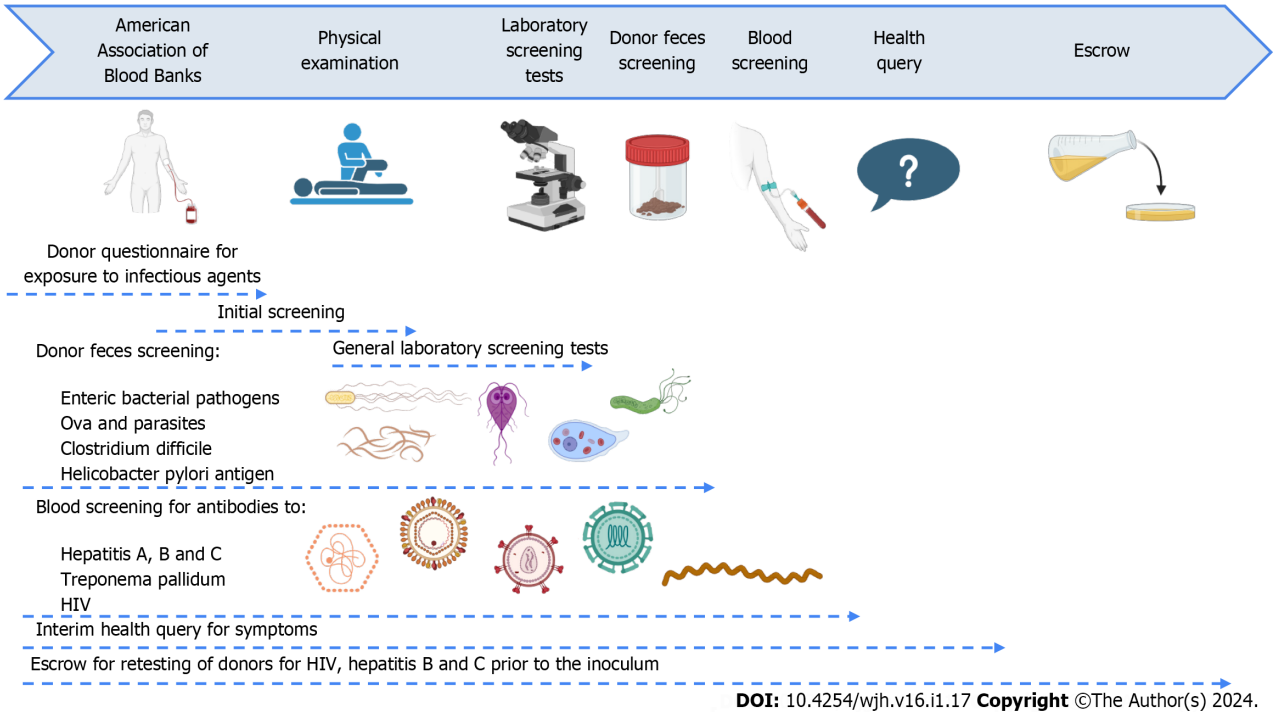

Figure 2 Illustrative portrayal of a screening protocol employed for the selection of prospective healthy fecal microbiota transplant donors. HIV: Human immunodeficiency virus.

outlined in a previously published study[36]. Following the completion of the FMT treatment, there was a notable average improvement of 3.1 points in the PHES after 4 wk. FMT resulted in mild and transient gastrointestinal side effects in a few patients. However, it is important to note that one patient experienced SAE in the form of esbl-producing *E. coli* bacteremia following FMT. In spite of the reported cases of FMT-transmitted infections, a recent systematic review encompassing 4241 patients concluded that FMT is generally safe, demonstrating a notably low incidence of SAE related to microbiota[84]. Additionally, there are more promising studies establishing the process of FMT for the treatment of HE in cirrhotic patients[85]. Even though alterations in the efficacy of FMT have been noticed, immunocompromised patients, including those with cirrhosis, may require multiple FMT treatments to achieve a cure. A study by Shogbesan *et al*[86] found that the success rate of FMT increased from 88% to 93% when multiple FMTs were administered. However, in decompensated cirrhotic patients, the efficacy of FMT may be diminished due to worsened immune deficiencies resulting in a lower success rate compared to other immunocompromised patients or the general population[87].

LIMITATIONS AND CHALLENGES

FMT has several limitations and challenges that need to be addressed. Firstly, the standard microbiota composition for FMT in the donor and the receptor sets a basis for which patients will respond, and the optimal treatment duration remains largely unknown. Large-scale, randomized, and controlled clinical trials are necessary to validate and standardize the clinical application of FMT in HE cases[88].

Several studies have been conducted, including randomized and controlled trials, to evaluate the efficacy and safety of FMT in HE. However, the number of patients enrolled in these studies is relatively small, limiting the generalizability of the findings. Additionally, factors such as the optimal dose, duration and administration route of FMT, long-term effects of FMT, mortality, the need for prior antibiotic use to facilitate engraftment, and donor selection based on their microbiota profile still need further investigation[22,42,77,89].

Furthermore, while some studies have reported decreased hospitalizations and severe adverse events in the FMT group compared to the placebo, these outcomes were not the primary endpoints in those trials[85,90]. Some other evidence of FMT in patients with decompensated cirrhosis has shown a marginally higher rate of death and SAE compared to the average immunocompetent population[87]. FMT as a one-time infusion, has been found to be less effective than expected[87]. Therefore, a true meta-analysis to get a better conclusion by combining the available literature is currently not feasible, because of the scarcity of large research trials and limited published evidence. This also limits the widespread use of FMT, making it primarily available in academic centers. Detailed information regarding the health status of donors and sourcing of donor material is often lacking in the included studies, which could potentially introduce confounding factors[87,88].

In conclusion, although FMT has shown therapeutic efficacy in treating HE in cirrhotic patients, there are limitations and challenges that need to be addressed. Further research with larger cohorts and robust study protocols is necessary to fully understand the role of FMT in cirrhotic patients and establish standardized guidelines for its clinical application[85].

CONCLUSION

FMT is currently being studied as a treatment option for HE. The evidence is limited due to the quality and the number of studies performed. This review offers a summary of current studies in various clinical conditions, delivery and preparation methods, safety, limitations and future aspects in the field of FMT for management of HE. The review also highlights the important aspects of hepatic conditions associated with HE, and their pivotal role in the pathogenesis and understanding how the microbiome is affected in each pathology, and how the FMT could help in these clinical scenarios. Significant efforts need to be directed towards addressing the doses, delivery methods, and safety of FMT, as well as larger studies performed in humans to better understand and assess the quality and benefit of the intervention. Patients with hepatic conditions that cause HE will greatly benefit from more advances in the medical research of FMT that remains as a promising therapy for HE in different contexts.

FOOTNOTES

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Abstract

Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most common liver disease worldwide, with an estimated prevalence of 31% in Latin America. The presence of metabolic comorbidities coexisting with liver disease varies substantially among populations. It is acknowledged that obesity is boosting the type 2 diabetes mellitus "epidemic," and both conditions are significant contributors to the increasing number of patients with MASLD. Non-alcoholic steatohepatitis represents a condition of chronic liver inflammation and is considered the most severe form of MASLD. MASLD diagnosis is based on the presence of steatosis, noninvasive scores and altered liver tests. Noninvasive scores of liver fibrosis, such as serum biomarkers, which should be used in primary care to rule out advanced fibrosis, are simple, inexpensive, and widely available. Currently, guidelines from international hepatology societies recommend using noninvasive strategies to simplify case finding and management of high-risk patients with MASLD in clinical practice. Unfortunately, there is no definite pharmacological treatment for the condition. Creating public health policies to treat patients with risk factors for MASLD prevention is essential.

Key Words: Nonalcoholic fatty liver disease; Primary care; Metabolic risk; Liver; Metabolism

Core Tip: Metabolic dysfunction-associated steatotic liver disease must be prevented in primary care by focusing on risk factors for metabolic syndrome and noninvasive fibrosis scores so that early detection is possible. To avoid a late diagnosis, primary care physicians need to reinforce in their routine examinations the need for lifestyle changes through healthy diet and exercise and implement pharmacological treatment when disease progression with the presence of fibrosis is identified. The treatment must be individualized, and in many cases several pharmacological options may be used to avoid disease progression, resulting in multisystemic involvement.

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INTRODUCTION

Metabolic syndrome (MetS) represents a multifaceted disorder distinguished by cardiovascular risk factors associated with central fat deposition and insulin resistance. These metabolic alterations have ramifications for various organs, with particular emphasis on the liver, a pivotal organ responsible for metabolizing diverse substances[1,2]. Epidemiological evidence indicates that the global diagnosis of MetS exceeds one billion individuals, primarily attributable to lifestyle factors[3]. Within the Brazilian population, the prevalence of MetS stands at 33%, surpassing the international prevalence range of 20%-25%[4].

CONTRIBUTING FACTORS TO METABOLIC DYSFUNCTION-ASSOCIATED STEATOTIC LIVER DISEASE

Type 2 diabetes

Type 2 diabetes stands as one of the most pivotal risk factors contributing to the onset of non-alcoholic steatohepatitis (NASH), advanced fibrosis/cirrhosis, hepatocellular carcinoma, and mortality[5]. Emerging evidence underscores the bidirectional relationship between these ailments, wherein the presence of metabolic dysfunction-associated steatotic liver disease (MASLD) heightens the susceptibility to type 2 diabetes.

The etiology of MASLD remains incompletely elucidated. Among the various identified origins, insulin resistance accompanied by subclinical inflammation emerges as a prominent contributor. Within this proinflammatory milieu, an augmented influx of free fatty acids (FFAs) into the liver precipitates hepatic infiltration of lipids, subsequently instigating liver injury through processes such as lipid peroxidation and mitochondrial dysfunction[6].

Dyslipidemia

In MASLD, the accrual of hepatic fat ensues through several pathways: Deficient uptake of circulating lipids; heightened hepatic de novo lipogenesis; inadequate compensatory augmentation in fatty acid oxidation; and alterations in lipid export, particularly as constituents of very low-density lipoprotein (VLDL). The increased uptake of lipids and accelerated rates of de novo lipogenesis within MASLD contribute to augmented hepatic triglyceride accumulation. This process is accompanied by the excessive production and release of voluminous, triglyceride-enriched VLDL particles, facilitating the mobilization and transportation of fat from the liver to peripheral tissues[7].

The overproduction of VLDL particles in the context of MASLD triggers a cascade of plasma lipoprotein irregularities, manifesting as atherogenic dyslipidemia characterized by elevated serum triglyceride levels and diminished high-density lipoprotein (HDL) cholesterol levels. Similarly, an atherogenic lipoprotein phenotype, featuring a preponderance of low-density lipoprotein particles, accumulation of triglyceride-rich lipoproteins and their remnants, and intermediate-density lipoprotein, is evident. These apolipoprotein-B-containing lipoproteins are fundamentally implicated in the progression of atherosclerosis[7].

Obesity

In MASLD, the accumulation of hepatic fat arises through distinct pathways. Obesity, recognized as a pervasive and epidemic-level chronic ailment on a global scale, has seen a marked escalation in prevalence worldwide. According to findings from a population-based cohort study, individuals classified as overweight or obese, devoid of additional metabolic abnormalities such as diabetes, hypertension, or dyslipidemia, exhibited a two-fold heightened risk of developing MASLD compared to their eutrophic counterparts[8]. Failure to control obesity during the steatosis stages

triggers an intrahepatic inflammatory process.

During this phase, immune cells release cytokines that amplify the inflammatory response contributing to the fibrotic process, which becomes evident with prolonged inflammation. Following liver injury, the customary counter-regulatory mechanism typically facilitates the replacement of deceased or apoptotic hepatocytes. However, in instances where this mechanism falters, as observed in sustained obesity, fibrosis ensues, possibly representing an unsuccessful attempt to counteract liver injury and promote tissue regeneration. The cumulative outcome of these ongoing processes manifests as scarring, encompassing cirrhosis and neoplasia[9].

Hypertension and cardiovascular disease

The prevalence of hypertension in MASLD patients spans a range of 40%-70%, and recent evidence underscores a robust association with an elevated risk of incident prehypertension and hypertension[10].

The precise nature of the relationship between MASLD and hypertension remains incompletely elucidated. There are indications that the systemic inflammation accompanying MASLD may trigger the sympathetic nervous system, potentially contributing to hypertension. Additionally, insulin resistance could play a role in promoting hypertension by fostering increased concentrations of FFAs, leading to perivascular fatty deposits in proximity to vessels and the renal sinus. Elevated levels of homocysteine in MASLD coupled with intestinal dysbiosis may incite heightened oxidative stress, further substantiating a link to hypertension[10]. Another study proposed that MASLD and hypertension may share a multifactorial association involving biochemical, genetic, nutritional, and lifestyle factors[11].

In patients with MASLD, cardiovascular disease stands as the predominant cause of mortality. Risk factors for cardiovascular disease, including hypertension, dyslipidemia, insulin resistance, smoking, and central obesity, are intricately connected to MetS and risk factors for MASLD. Screening for these conditions bears significant clinical implications for disease mitigation and the prevention of cardiovascular events[12].

EPIDEMIOLOGY OF METABOLIC FATTY LIVER DISEASE

The escalation in the prevalence of MASLD worldwide is intricately linked to sedentary lifestyle choices and the consumption of processed foods[13]. It is noteworthy that MASLD stands as the primary cause of liver-related morbidity and mortality[14]. Recent investigations have honed in on the metabolic facet of fatty liver disease, pinpointing liver fat storage as the unifying factor. A consensus panel of international experts has proposed the substitution of the term non-alcoholic fatty liver disease (NAFLD) with MASLD, aligning it with metabolic comorbidities. This novel nomenclature, grounded in the classification of causative factors, holds promise for refining phenotypic characterization and facilitating the identification of new biomarkers and therapeutic modalities[15,16].

These criteria possess the potential to surmount challenges associated with defining alcohol consumption, catalyzing advancements in our understanding of pathophysiology and streamlining the execution of clinical trials. The diagnosis of MASLD will encompass individuals exhibiting fatty liver and dysmetabolism, irrespective of reported alcohol consumption[15,16]. This diagnosis considers the presence of hepatic steatosis (confirmed through imaging, biomarkers, or histology) alongside at least one of the following features: Overweight/obesity; type 2 diabetes; and metabolic dysregulation. The latter criterion is satisfied when a minimum of two features are present including increased waist circumference, hypertension, hypertriglyceridemia, low HDL cholesterol, prediabetes, insulin resistance, and subclinical inflammation. The criteria for assessing MASLD in lean individuals with fatty liver hinge on the identification of at least two metabolic risk abnormalities[15,16]. While the terminology for MASLD remains subject to ongoing discussions, alternative terms such as metabolic associated liver disease and alcoholic liver disease have been proposed, as noted during the European Association for the Study of the Liver (EASL) Congress in 2023[17].

MASLD, formerly NAFLD

In Brazil, a survey conducted among individuals in the middle-aged and elderly demographic revealed a prevalence of 35.2% for MASLD[18]. In a meta-analysis encompassing 35599 patients, the prevalence of NAFLD in those with type 2 diabetes was reported at 59.67%, with results ranging from 29.60% to 87.10%[19]. Recently, an extensive meta-analysis, incorporating data from over 24 million individuals, identified an elevated risk of severe liver disease in this cohort. Conversely, a reduced risk of severe liver disease was observed in individuals with a body mass index (BMI) exceeding 30 kg/m^2. Nonetheless, the study suggested a less favorable prognosis in the presence of central adiposity, particularly among females[20].

A global prevalence assessment of MASLD, drawing upon data from 205307 subjects across 14 countries, indicated a prevalence of 9.7% in lean patients. Moreover, MASLD was found to be more prevalent among middle-aged individuals (45-59 years) and those of Asian descent[21].

Studies categorically delineate MASLD into two classifications: Simple steatosis, which infrequently progresses to cirrhosis; and steatohepatitis, or NASH, a process with the potential to culminate in the development of cirrhosis and hepatocellular carcinoma[22]. Approximately 30% of MASLD patients exhibit steatohepatitis[23], a progressive condition resulting in severe liver dysfunction, including cirrhosis in 20%-25% of cases[17,24]. This progression is marked by the presence of macrovesicular steatosis, lobular inflammation, hepatocyte degeneration, and fibrosis.

Clinical Practice Guidelines for MASLD Management, collaboratively proposed by the EASL, European Association for the Study of Obesity, and European Association for the Study of Diabetes, advocate for a 7% to 10% reduction in body weight for overweight/obese patients with NAFLD[17,25]. A congruent weight reduction target is endorsed by the American Association for the Study of Liver Diseases[26].

Steatosis

Hepatic steatosis is characterized by the presence of more than 5% lipid content in hepatocytes, a diagnosis established through imaging or histological examinations[27]. The three principal sources of FFAs in the liver include non-esterified fatty acids from adipose tissue (60%), de novo lipogenesis in the liver (25%), and FFAs from the diet in the form of chylomicrons (15%). The liver metabolizes fat primarily through the beta-oxidation of FFAs, a process predominantly occurring in mitochondria, peroxisomes, and cytochrome P-450, in situations of energy surplus or through the export of FFAs as VLDLs[27].

Plasma non-esterified fatty acid levels escalate when adipocytes are overloaded, leading to an augmented process of lipolysis. Adipose tissue responds to hormones such as glucagon, epinephrine, and adrenocorticotropic acid by releasing non-esterified fatty acids. Postprandial lipolysis in adipose tissue is inhibited by insulin after meals. In instances of insulin resistance within adipocytes, inadequate postprandial lipolysis transpires. Steatosis induced by impaired beta-oxidation of fatty acids can also result from mitochondrial dysfunction, as observed in conditions like alcoholic steatosis, NASH, and acute fatty liver of pregnancy and through the use of medications such as valproic acid[27].

Steatosis renders the liver parenchyma vulnerable to aggression, manifested through the release of FFAs and oxidative stress, both conducive to cellular injury and the development of steatohepatitis. Genetic polymorphisms, environmental factors, and dietary influences can induce inflammation, fibrosis, and progression to cirrhosis[27].

In summary, the accumulation of fat in the liver can be elucidated by insulin resistance, leading to heightened peripheral lipolysis, increased hepatic lipid uptake, and elevated triglyceride biosynthesis.

NASH

NASH denotes a chronic state of liver inflammation, representing an inflammatory subtype within the spectrum of NAFLD. In NASH, steatosis serves as evidence of hepatocyte damage characterized by ballooning and inflammation, with or without concurrent fibrosis[28]. The prevailing hypothesis posits that NASH evolves from NAFLD, precipitated by the so-called "second hit." The precise manifestation of this second hit remains inconclusive, although prevalent theories implicate oxidative stress, specific cytokines, and lipopolysaccharides. FFAs and hyperinsulinemia synergistically potentiate lipid peroxidation and the release of free radicals, directly inflicting injury upon hepatocytes by recruiting neuroinflammatory mediators. Prolonged liver injury eventually activates stellate cells, laying the groundwork for the development of liver fibrosis.

Given the histological dynamism of NASH, it is imperative to establish a consensus on parameters signifying disease progression, irrespective of its advancement or regression. From a regulatory standpoint, a one-point expansion in fibrosis stage is indicative of deterioration. The extent of scarring does not increase linearly, with a notable surge observed in the progression of fibrosis from stage 2 to stage 3. This bears noteworthy implications for the validation of biomarkers targeting fibrosis measurement rather than its distribution[28]. Although often clinically asymptomatic, steatohepatitis has the potential to evolve into cirrhosis or end-stage liver disease or necessitate liver transplantation[28].

NASH constitutes a multifaceted condition with metabolic complications, rendering its treatment intricate. The ideal therapeutic approach would effectively reverse liver damage and fibrosis, ameliorate additional metabolic parameters, address cardiovascular comorbidities, or at the very least exhibit no deleterious effects. Despite the wealth of information accumulated on the pathogenesis of NASH over the past decade, no approved therapy has yet emerged[28].

DIAGNOSIS OF MASLD

Various diagnostic and monitoring modalities have been employed in the assessment of MASLD, including ultrasonography (US), computed tomography, magnetic resonance imaging (MRI), and more recently the controlled attenuation parameter utilized in conjunction with transient elastography and MRI elastography. While US is widely accessible, its interobserver reproducibility is not notably high, and it lacks sensitivity for detecting mild steatosis. Similarly, computed tomography exhibits limited sensitivity in identifying mild steatosis and entails patient exposure to radiation. Although MRI accessibility is constrained, it boasts high reproducibility when employing multi-echo fat quantification techniques and proton spectroscopy. Approximately 25% of individuals with isolated steatosis progress to NASH, with a positively correlated escalation in the degree of steatosis heightening the risk of disease progression. Among those diagnosed with NASH, around 25% advance to chronic hepatopathy, characterized by fibrosis, cirrhosis, and an elevated risk of complications, including portal hypertension and hepatocellular carcinoma[29] (depicted in Figure 1).

An extensive comprehension of the outcomes of clinical trials and the significance of reported treatment effects necessitates a reflection on diverse approaches to diagnose MASLD. Although liver biopsy stands as the gold standard technique for a thorough MASLD diagnosis, enabling the identification of inflammation and the classification of fibrosis stages (F0-4), its invasive nature and associated risks constrain widespread utilization. Consequently, most preceding clinical studies have resorted to US, liver enzymes, or various indices for MASLD diagnosis. It is imperative to develop dependable noninvasive methods for evaluating liver fibrosis and is crucial for estimating disease progression and guiding therapy[30].

The suspicion of MASLD is grounded in the identification of steatosis *via* US or abnormal liver test results in patients harboring risk factors (obesity, type 2 diabetes, and/or MetS). The selection of noninvasive tools should follow a sequential approach, guided by local availability and the context of primary healthcare utilization. Simple, economical, and widely accessible serum biomarkers, such as fibrosis-4 (FIB-4) or NAFLD fibrosis score (NFS), exhibiting a high negative predictive value (88%-95%) for excluding advanced fibrosis, should constitute the first-line assessment. Individuals at low risk (FIB-4 < 1.3 or NFS fibrosis score < -1.455) require no further evaluation and are advised to adopt

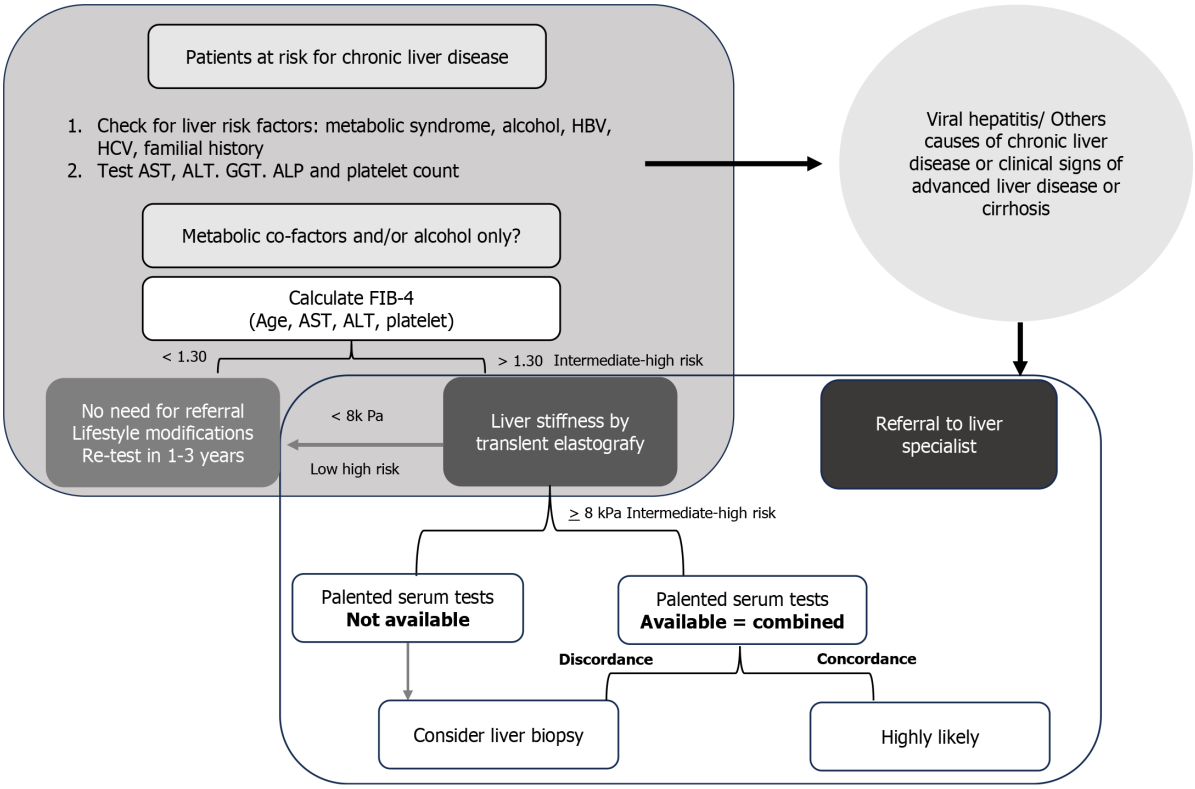

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Figure 1 Algorithm proposed by the European Association for the Study of Liver targets patients at high risk of metabolic dysfunction-associated steatotic liver disease seen in primary health care. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FIB-4: Fibrosis-4; GGT: Gamma-glutamyl transferase; HBV: hepatitis B virus; HCV: Hepatitis C virus.

lifestyle modifications and engage in regular exercise. Those at intermediate risk (FIB-4 between 1.30 and 2.67 or NFS -1.455 to 0.672; accounting for 30% of cases) and high risk (FIB-4 > 2.67 or NFS > 0.672; comprising 12%-15% of cases, with a positive predictive value of 75%-90%) of advanced fibrosis should be referred to a specialized center for expert evaluation.

Another noninvasive approach involves estimating liver fibrosis through elasticity assessment. A recent development in this realm is a shear elasticity probe based on one-dimensional transient elastography utilizing ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves, initially termed FibroScan. While initially employed for patients with chronic hepatitis C, ongoing studies are investigating hepatic elastography in MASLD, exploring diverse cutoff values for patients with NASH[31].

Unfortunately, existing noninvasive or minimally invasive biomarkers remain limited[32]. Endeavors have been made to formulate clinical parameters capable of reliably identifying fibrosis in MASLD patient cohorts. Various scores have been devised to ascertain the presence of fibrosis using clinical data and laboratory outcomes, aiming to integrate routine parameters of liver injury (*e.g.*, transaminase activity) and risk characteristics (*e.g.*, diabetes)[33]. The most commonly utilized scores are FIB-4 and the NFS. FIB-4 comprises four straightforward parameters: age; platelets; and the serum transaminases aspartate aminotransferase and alanine aminotransferase. NFS encompasses seven parameters: age; BMI; glycated hemoglobin; serum transaminases; platelets; and albumin. In primary care settings, where the prevalence of advanced fibrosis is low (5%), FIB-4 emerges as the preferred option due to its simplicity, and the fact that serum transaminases and platelet count are routinely requested by physicians in standard examinations. In large populations, a FIB-4 threshold of up to 1.30 effectively excludes the risk of advanced liver fibrosis with a substantial degree of accuracy (60%-80%). Individuals with a FIB-4 between 1.30 and 2.67 are deemed at intermediate risk for advanced fibrosis, warranting further investigations such as elastography, which can be performed before or after referral to a medical specialist. Those with a FIB-4 > 2.67 are classified as high risk and should be directed to specialized services for additional investigations, potentially including liver biopsy. It is noteworthy that guidelines from international hepatology societies advocate for the use of noninvasive strategies, which can simplify case finding and management of high-risk MASLD patients in clinical practice[34].

TREATMENT

As MASLD or NAFLD manifests as a multifactorial ailment, various integrated treatment strategies are employed with primary objectives of retarding the progression to severe forms, such as fibrosis (NASH), and managing associated risk

factors including obesity, diabetes, dyslipidemia, and hypertension[34].

Initiating therapeutic measures entails lifestyle interventions involving dietary modifications and exercise. Medication may be introduced as a secondary measure, particularly as fibrosis advances, and bariatric surgery becomes a viable option in the tertiary phase. It is noteworthy to underscore the significance of assembling an interdisciplinary team comprising a nutritionist, endocrinologist, physical educator, psychologist/psychiatrist, cardiologist, and hepatologist [36]. The percentage of weight loss is directly correlated with NASH progression, irrespective of the chosen method[17, 25].

Consideration for pharmacological intervention arises if diet and exercise prove ineffective in disease control. Following the EASL recommendations (2023)[17], the presence of steatohepatitis, diagnosed through noninvasive methods or liver biopsy, accompanied by macro and microvesicular steatosis, mixed inflammatory infiltrate, hepatocellular ballooning in centrilobular vein areas (Zone III), Mallory's corpuscles, and fibrosis, warrants pharmacological intervention. This holds true for less severe cases but with a high risk of progression. Given the limited scope of drugs and surgical treatments for NASH, lifestyle changes, including dietary adjustments, increased physical activity, and exercise, remain the cornerstone of its management.

Physical activity and exercise

Regular physical activity serves as a pivotal adjunct to metabolic regulation. A favorable correlation exists between sedentary behavior and susceptibility to MASLD. Individuals adhering to a health-conscious lifestyle exhibit diminished likelihood of developing pivotal factors contributing to the onset of the disease, including insulin resistance, diabetes, and glucose intolerance. Additionally, engaging in physical activity facilitates a reduction in visceral and hepatic adipose tissue, along with a decrease in circulating FFAs in the plasma[28].

Calorie restriction

Dietary considerations, with a particular focus on calorie intake, especially derived from carbohydrates, the primary energy source for the human body, play a crucial role in regulating the body's glycemic levels. Manipulating dietary carbohydrate intake, either through restriction or substitution with complex carbohydrates, exerts an influence on enhancing serum glucose and triglyceride levels. It contributes to the elevation of HDL levels and exerts an impact on pancreatic β cells involved in insulin elimination[27]. A calorie-restricted diet, meticulously assessed and calculated by a professional considering basal metabolism and accounting for individual physical and behavioral variations, is recommended as a contributing factor to the regression of NAFLD.

Pharmacological treatment

Pharmacological intervention is warranted when the disease manifests a moderate degree of fibrosis, denoted as F2 > 2, as determined through transient hepatic elastography or liver biopsy. Based on this data, the initial and economically feasible treatment may involve the use of vitamin E at a dosage of 800 IU and pioglitazone at 30 mg. If applicable, anti-GLP1 medications such as liraglutide or semaglutide are recommended for overweight and obese patients to achieve a reduction of 7%-10% in body weight. Emerging pharmacological options discussed at EASL 2023, such as retatrutide, are being considered. Bariatric surgery stands as a viable treatment option with favorable outcomes, particularly for patients with a BMI > 35[17,25,26].

Pharmacotherapy aims to mitigate the progression from the early stages of NASH to advanced fibrosis. The correlation between fibrosis and overall mortality, cardiovascular risk, and the transition from cirrhosis to hepatocellular carcinoma is significant, particularly in individuals aged over 50 with concurrent diabetes, elevated alanine aminotransferase, MetS, and NASH, where greater inflammatory activity is observed.

CONCLUSION

MAFLD necessitates preventive measures within the domain of primary healthcare, centering attention on the identification of risk factors associated with MetS and the utilization of noninvasive fibrosis scoring systems to facilitate early detection. Moreover, in instances characterized by moderate to severe fibrosis, it is imperative to advocate for referral to a specialized hepatology department for comprehensive evaluation and subsequent monitoring. To preclude delayed diagnoses, primary care practitioners should consistently underscore, in their routine counselling sessions, the imperative for lifestyle modifications encompassing a wholesome diet and regular exercise. Furthermore, the introduction of pharmacological interventions becomes imperative upon the identification of disease progression, concomitant with the presence of fibrosis. Such therapeutic interventions must be tailored to individual patient profiles, often necessitating the utilization of diverse pharmacological options to forestall disease progression and foster a comprehensive multisystemic approach.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Direct-acting antivirals failed to reduce the incidence of hepatocellular carcinoma occurrence in hepatitis C virus associated cirrhosis: A real-world study

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Abstract

BACKGROUND

Direct-acting antivirals (DAAs) revolutionized the treatment of chronic hepatitis C virus (HCV)-associated disease achieving high rates of sustained virological response (SVR). However, whether DAAs can reduce the occurrence of hepatocellular carcinoma (HCC) in patients with HCV-associated cirrhosis who are at high risk have not been concluded.

AIM

To investigate the effect of DAAs on the occurrence of HCC in patients with HCV-associated cirrhosis after achieving SVR.

METHODS

Of 427 inpatients with HCV-associated cirrhosis were enrolled in Tianjin Second People's Hospital from January 2014 to April 2020. 118 patients weren't received antiviral treatment with any reasons named non-antiviral treatment group, and 236 patients obtained from the 309 DAAs treatment patients according to the propensity score matching named DAAs treatment group. Demographic information and laboratory data were collected from baseline and the following up. Kaplan-Meier curve and Log-Rank test were used to compare the incidence and cumulative incidence of HCC between the two groups. Cox proportional risk regression was used to re-evaluate the risk factors for HCC.

RESULTS
HCC incidence was 4.68/100PY (95%CI, 3.09-6.81) in the DAAs treatment group, while it was 3.00/100PY (95%CI, 1.50-5.37) in the non-antiviral treatment group, and the relative risk was 1.82 (95%CI, 0.93-3.53, $P > 0.05$). The incidence of HCC at 12, 24, 36 and 48 months was 3.39%, 6.36%, 8.47% and 10.17% in the DAAs treatment group, and it was 0%, 0%, 3.39% and 9.32% in the non-antiviral treatment group, respectively. Age > 58 [hazard ratio (HR) = 1.089; 95%CI, 1.033-1.147; $P = 0.002$] and liver stiffness measurement > 27.85 kPa (HR = 1.043; 95%CI, 1.022-1.065; $P = 0.000$) were risk factors for HCC in all patients ($n = 427$), and DAAs treatment didn't show protective efficacy.

CONCLUSION
DAAs treatment seems failed to reduce the incidence of HCC occurrence in HCV-associated cirrhosis in 48 months, and even increased the incidence of HCC in 36 months.

Key Words: Direct-acting antivirals; Sustained viral response; Cirrhosis; Hepatocellular carcinoma; Risk factor

Core Tip: We evaluated the effect of direct-acting antivirals (DAAs) on the development of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV)-associated cirrhosis during long-term follow-up. We performed propensity score matching, Kaplan-Meier curve and Log-Rank test, the incidence and cumulative incidence of HCC in DAAs treatment group ($n = 236$) and non-antiviral treatment group ($n = 118$) were retrospectively evaluated, and the risk factors for HCC were evaluated by Cox regression. We found that DAAs treatment of HCV-associated cirrhosis failed to reduce the incidence of HCC over 48 mo. Age and liver stiffness measurement were risk factors for developing HCC in all patients ($n = 427$), and DAAs treatment showed no protective effect.

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INTRODUCTION

According to an estimate by the World Health Organization[1], more than 185 million people worldwide have been infected with hepatitis C virus (HCV), of which 350000 people died from HCV infection each year. China is a region with high incidence of HCV infection. It has been estimated[2] that the prevalence of HCV in China is between 0.4% and 2.0%, with over 14 million infected persons. HCV infection has been identified as an independent risk factor for hepatocellular carcinoma (HCC) development, especially in patients with cirrhosis[3]. In patients with HCV-associated cirrhosis, the risk of developing HCC is estimated at 3% to 8% per year[4]. Multiple studies have shown[5,6] all-cause mortality and the risk of HCC were reduced among chronic hepatitis C (CHC) patients who achieved sustained virological response (SVR) with IFN-based antiviral therapy. A large number of studies[7,8], however, have indicated that IFN therapy can only bring a low SVR rate, in addition, the therapeutic indications for IFN are limited, which effectiveness varies with the degree of fibrosis, stage of liver disease, viral genotype, and presence of comorbidities[9]. New regimens with direct-acting antiviral agents (DAAs) have not only changed the scope and spectrum of treatment, but also had high efficacy, sufficient safety and few contraindications to be used in patients with advanced liver disease who are not recommended to be treated with interferon[4]. Moreover, through different combined treatment solutions, more than 95% of SVR can be achieved, regardless of HCV genotype or degree of fibrosis[10,11]. Kanwal *et al*[12], through a large retrospective cohort study, found that DAAs treatment can reduce the occurrence of HCC in patients with HCV, and Calvaruso *et al*[10] discovered that DAAs treatment can reduce the occurrence of HCC in patients with HCV-associated cirrhosis. However, some studies[13,14] have shown that DAAs can increase the occurrence of HCC[15]. Hence, in this study, we evaluated the efficacy of DAA on prevention HCC in HCV-associated cirrhosis patients who were at high risk after achieving SVR, in the real-world. The Kaplan-Meier curve and log-rank test were used to compare the incidence of HCC development in our hospital with or without DAAs treatment. Cox regression was used to retrospectively study the risk factors of HCC in HCV-associated cirrhosis patients after achieving SVR with DAAs.

MATERIALS AND METHODS

Study design

This was an institutional review board-approved retrospective clinical cohort study conducted at a large hepatology hospital in China. Patients with HCV-related cirrhosis were enrolled from January 2014 through April 2020. DAA or

liver-protective therapy was prescribed by experienced specialists according to the patient's condition. Informed consent was obtained from all enrolled patients. Enrolled patients were followed and reviewed by specialists during follow-up. Prior to implementation, education was provided to all hepatologists who would manage or verify orders for HCV patients. Physician leadership and health care teams also participated in education about the protocol and its implementation.

Included patients

A total of 427 consecutive inpatients with HCV-associated cirrhosis were enrolled in Tianjin Second People's Hospital from January 2014 to April 2020. Of these, 309 patients received DAA treatment and the remaining 118 patients did not receive antiviral therapy.

Inclusion criteria: (1) Age ≥ 18, no gender limitation; (2) the serum anti-HCV and HCV RNA of all patients were positive, and the diagnosis was in line with the diagnostic criteria of hepatitis C associated cirrhosis in China's Hepatitis C Prevention and Treatment Guidelines 2019 Edition[16]; and (3) obtaining informed consent from all patients. Exclusion criteria: (1) Patients successfully treated with interferon combined with ribavirin; (2) patients who received direct antiviral therapy but did not achieve SVR; (3) For patients clinically diagnosed with HCC or previously diagnosed with HCC, the diagnostic criteria for HCC should be based on the 2018 European Association for the Study of the Liver (EASL) guidelines[4] and American Association for the Study of Liver Diseases guidance of HCC[17] and evaluated by imaging or pathological examination; (4) Patients with previous history of extrahepatic tumor; (5) patients who have received or are awaiting liver transplantation; (6) patients combined with HIV, HAV, HBV, HEV infection; (7) patients with alcoholic liver disease, autoimmune hepatitis, drug-induced liver injury, genetic metabolic liver disease and other liver diseases; and (8) patients with no follow-up records or incomplete follow-up data. A total of 836 inpatients with chronic HCV infection in our hospital were evaluated, and 409 patients were excluded from this study, as shown in Figure 1.

The Medical Ethics Committee of Tianjin Second People's Hospital approved the study protocol, which conformed to the ethical guidelines of the Declaration of Helsinki amended in 2008.

Therapy

Antivirus solution: According to the guidelines[16,18], the 309 patients were treated by experienced clinicians at or above the attending level with the following regimen: (1) Sofosbuvir 400 mg/d + daclatasvir 60 mg/d + ribavirin 1000 mg/d (12 wk) regimen (95 cases); (2) sofosbuvir 400 mg/d+ Velpatasvir 100 mg/d (12 wk) (60 cases); (3) sofosbuvir 400 mg/d+ ribavirin 1000 mg/d (12 wk) (57 cases); (4) Ombitasvir 300 mg/d+ dasabuvir 500 mg/d (12 wk) (44 cases); (5) sofosbuvir 400 mg/d+ ledipasvir 90 mg/d+ ribavirin 1000 mg/d (12 wk) regimen (22 cases); (6) Elbasvir and Grazoprevir 50 mg/d (12 wk) (17 cases); and (7) Dasabuvir 60 mg+ Asunaprevir 100 mg (24 wk) regimen (14 cases).

Non-antiviral treatment: Inclusion reasons: (1) Because some patients could not choose pegylated interferon (peg-IFN) + ribavirin due to decompensation of cirrhosis, and DAA drugs were not released in China before 2017; and (2) Some patients cannot choose the treatment due to the economic burden.

Patients without antiviral treatment were received the corresponding hepatoprotective treatment and symptomatic treatment.

Demographics and laboratory parameters

We recorded baseline data including gender, age, weight, height, body mass index, compensatory/decompensated cirrhosis, Child-Pugh score, nonspecific liver nodules, hypertension, diabetes, fatty liver, HCV genotype, HCV RNA (viral load was quantified by direct-PCR, Roche Diagnostics, 1080 US Highway 202 South, Branchburg, NJ 08876, BMI), Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II), carcinoembryonic antigen (Cobas E601 electrochemiluminescence analyzer, Basel, Switzerland), alpha fetoprotein (AFP) (Cobas E601 electrochemiluminescence analyzer, Basel, Switzerland); serum biochemical indicators: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-alanine transferase (γ-GT), total bilirubin (TBIL), total protein (TP), albumin (ALB), renal function, creatinine, uric acid, glomerular filtration rate, blood glucose, triglyceride, total cholesterol, low density lipoprotein, high density lipoprotein, all above assays were carried out in Hitachi 7180, automatic biochemical analyzer, Japan. coagulation function: prothrombin time (PT), international standardized ratio of prothrombin time (INR); blood routine: red blood cell, hemoglobin, white blood cell (WBC), platelet (PLT); liver stiffness measurement [liver stiffness measurement (LSM), in kPa] and controlled attenuation parameter (in dB/m) values were obtained by FibroScan (Echosens, Paris, France).

Follow-up and diagnostic criteria for HCC

The end point of this study was the first occurrence of HCC or death among the enrolled subjects by the end of April 2020, and all patients received nurse counseling, clinical visit and laboratory assessment (biochemistry, blood routine test, bio-markers for HCC, *etc.*) at baseline and every 3-6 mo. According to the diagnostic criteria for HCC of EASL guidelines [4], patients were screened for HCC by ultrasound (US, Philips, No. IU22, 22100 Bothell Everett Highway Bothell, WA, United States) or AFP every 3-6 mo. When HCC was suspected, further examination such as enhanced computed tomography (CT) (Philips Row 64, Haifa, Israel), Gd-EOB-DTPA enhanced magnetic resonance imaging (MRI) (Siemens Skyra3.0T, Germany), hepatic digital subtraction angiography (Artis Zee 3, Fochheim County, Bavaria, Germany), or pathological examination were taken. The duration of follow-up was calculated from reaching SVR after DAA treatment to the diagnosis of HCC, death, or the end of follow-up (Figure 2).

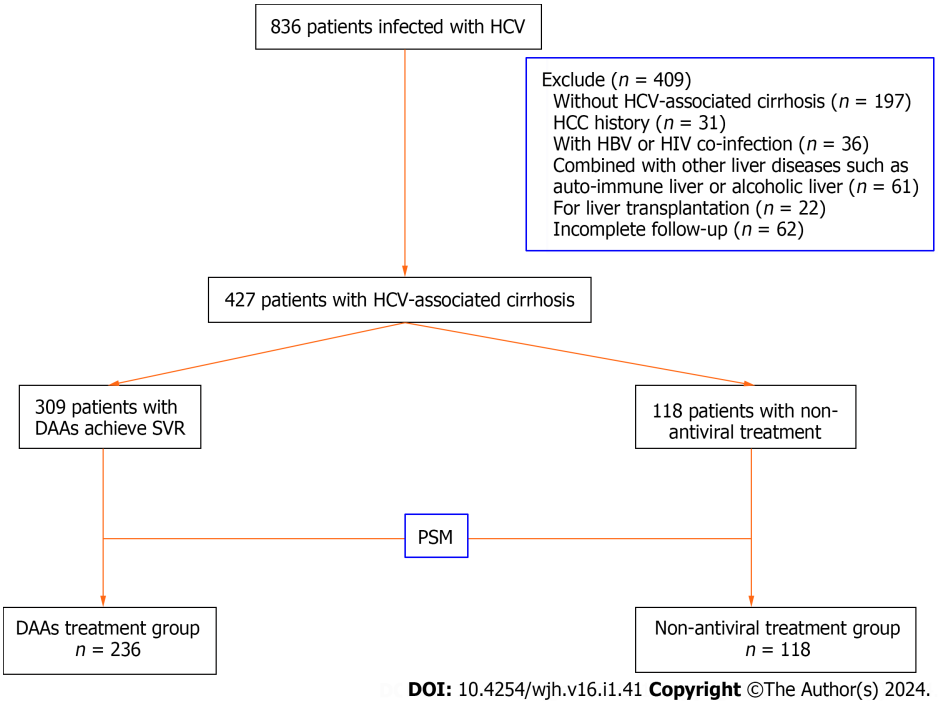

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Figure 1 Flow chart of patients included in this study. HCV: Hepatitis C virus; HCC: hepatocellular carcinoma; DAA: Direct-Acting Antiviral Agents; SVR: sustained virologic response; PSM: propensity score matching.

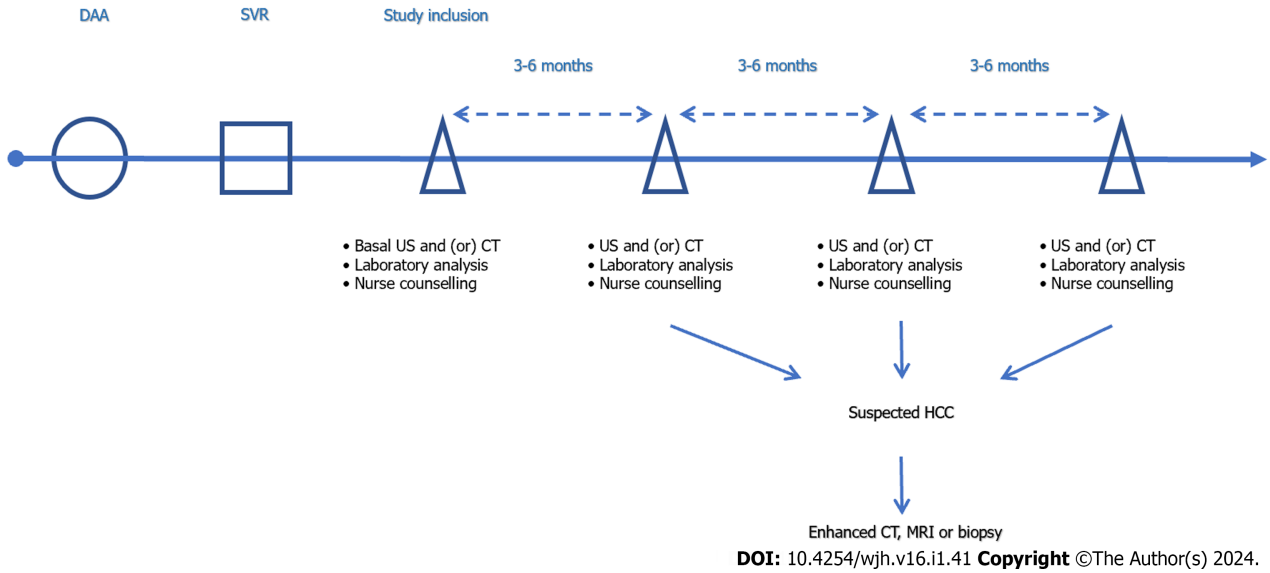

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Figure 2 Study time points and follow-up of this study. DAA: Direct-Acting Antiviral Agents; SVR: sustained virological response; US: Ultrasound; CT: Computed tomography; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging.

Definitions of SVR and nonspecific liver nodules

SVR definition: according to EASL guidelines[18], SVR is defined as 12 wk after the end of treatment (SVR 12), and HCV RNA is not detected in serum or plasma, evaluated by highly sensitive molecular methods, additionally, the detection limit is 15 IU/mL.

Nonspecific liver nodules were defined[19] as ≤ 10 mm or nodules > 10 mm but in which HCC diagnosis was ruled out before starting DAA by contrast enhanced US (CEUS), CT, or MRI.

Statistical analysis

Data conforming to normal distribution were represented by (mean ± SD), and HCV RNA was calculated by denary logarithm. Independent sample *t* test was used to analyze and compare the two groups. The skewness distribution of measurement data was represented by *M* (P25, P75). The comparison between the two groups was analyzed and compared by Mann-Whitney *U* test, and the paired samples were analyzed and compared by Wilcoxon signed rank test.

The statistical data were expressed as percentages, and analyzed by Chi-square test or Fisher's exact probability method. Kaplan-Meier curve and log-rank test were used to compare the difference in the cumulative incidence of HCC between the two groups. All clinical data were included in binary logistic regression analysis for univariate and multivariate analysis to evaluate the influencing factors of HCC occurrence and obtain a regression equation. Cox proportional risk regression was used to re-evaluate the risk factors for HCC before and after DAA treatment, and the independent predictors of HCC were obtained by incorporating the indicators with statistical differences in univariate analysis into multivariate analysis. Meanwhile, risk ratio and 95% confidence interval (CI) were calculated. $P < 0.05$ was considered to indicate that all analyses were statistically significant. Incidences, expressed as 100 patient-years (100PY), relative risks (RR) and their 95%CI were estimated utilizing Poisson regression models, using as offset the logarithm of radiological follow-up.

Statistical analyses were performed using SPSS version 26 (SPSS, Inc., Chicago, IL, United States), GraphPad Prism Version 9.0H (GRAPH PAD Software, Inc., La Jolla, CA, United States) and R Version 3.1.2 (R Core Development Team, 2010).

RESULTS

Baseline characteristics of patients

A total of 427 patients were included in this study from January 2014 to April 2020 (409 patients were not included according to the exclusion criteria) and completed follow-up. Among them, 309 patients were treated with DAAs and 118 patients were not treated with antiviral therapy. Considering the influence of gender and age on the results of this study, we used the propensity score matching (R Version 3.1.2) to adjust for age and sex, and divided patients into DAAs treatment group ($n = 236$) and non-antiviral treatment group ($n = 118$) (Figure 1). The baseline characteristics of the two groups of patients were introduced in Table 1.

Efficiency of DAAs treatment on HCV-associated cirrhosis

After DAAs treatment, all patients in the DAAs treatment group achieved SVR (patients who did not achieve SVR were not included in this study). After achieving SVR, the LSM value (20.55 ± 16.95 kPa) was significantly lower than baseline (26.15 ± 16.90 kPa) ($t = 3.499$, $P = 0.001$), and the values of ALT, AST, γ-GT, ALP and TP were significantly decreased compared with those at baseline ($P < 0.05$), and WBC and PLT were significantly increased ($P < 0.05$), as shown in Table 2.

Comparison of HCC incidence between DAAs treatment group and non-antiviral treatment group

During the follow-up, 27 cases of HCC occurred in the DAAs treatment group (236 cases), while 11 cases of HCC occurred in the non-antiviral treatment group (118 cases), and there was no significant difference in the total incidence of HCC between the two groups ($\chi^2 = 0.369$, $P = 0.544$). In the DAAs treatment group, HCC incidence was 4.68/100PY (95%CI, 3.09-6.81), while it was 3.00/100PY (95%CI, 1.50-5.37) in the non-antiviral treatment group. Indeed, its RR was 1.82 (95%CI, 0.93-3.53, $P > 0.05$). The duration of follow-up in the DAAs treatment group was 1-84 mo (29.33 ± 16.20), the median follow-up time was 27 months and the time of HCC occurrence in the DAAs treatment group was 5-66 mo. The cumulative incidence of HCC at 12, 24, 36 and 48 mo was 3.39%, 6.36%, 8.47% and 10.17%, respectively. The duration of follow-up in the non-antiviral treatment group was 1-84 mo (37.25 ± 15.94), the median follow-up time was 41 months ($t = -4.359$, $P = 0.000$) and the time of HCC occurrence in the DAAs treatment group was 26-48 mo. The incidence of HCC at 12, 24 mo, 36 mo and 48 mo was 0%, 0%, 3.39% and 9.32%, respectively. There was significant difference in the incidence of HCC at 12, 24, and 36 mo between the two groups ($P = 0.048$, $P = 0.003$, and $P = 0.025$), while there was no significant difference in the cumulative incidence of HCC at 48 mo between the two groups ($P = 0.388$) (Figure 3).

Log-rank test was used to compare and analyze the cumulative incidence of HCC between the two groups (log-rank test, $P = 0.107$). Kaplan-Meier curve was used to show the cumulative incidence of HCC between the two groups after adjusting for age and gender factors in Figure 4.

Risk factors for HCC occurrence in patients with HCV-associated cirrhosis

Table 3 shows the risk factors associated with the development of HCC in all patients ($n = 426$). Univariate analysis identified DAAs treatment, their age, cirrhosis (compensate/decompensate), nonspecific liver nodules, Child-Pugh (A/B/C), FIB-4 index, ALB, TBIL, PT, PLT and LSM as factors significantly associated with HCC. According to the multivariate analysis, age [hazard ratio (HR) = 1.089; 95%CI, 1.033-1.147; $P = 0.002$] and LSM (HR = 1.043; 95%CI, 1.022-1.065; $P = 0.000$) were independent factors significantly associated with HCC. The optimal cut-off value of age was 0.249, and the value was 58 years. And for LSM, the optimal cut-off value was 0.466, and the value was 27.85 kPa.

DISCUSSION

A model-based study conducted in 2015 found that about 9.8 million people in China are chronic HCV infection, ranking first in the world[20]. HCV infection is one of the major risk factors for HCC occurrence, and data analysis in 2018 showed that 21% of new HCC cases and deaths were attributed to HCV infection[21]. The relative risk of HCV-infected patients developing HCC is 15-20 times larger than that of uninfected persons[22,23]. The incidence of HCV-associated HCC is mostly based on cirrhosis, and the annual incidence of HCC in non-sclerotic patients (pre-sclerotic) is only 0.68%

Table 1 Baseline characteristics of the direct-acting antiviral agents treatment group (*n* = 236) and the non-antiviral treatment group (*n* = 118), *n* (%)

Variables	DAA treatment (*n* = 236)	Non-antiviral (*n* = 118)	*t*/*Z*/χ^2	*P* value
Ages (yr)	55.01 ± 9.52	53.69 ± 10.07	1.208	0.228
Male	120 (50.85)	68 (57.63)	1.452	0.228
Cirrhosis (compensate)	187 (79.24)	90 (76.27)	0.407	0.524
Nonspecific liver nodules	191 (80.25)	100/18 (15.13)	0.782	0.377
Child-Pugh (A/B/C)	177/51/8	83/30/5	0.887	0.642
Genotype (1a/1b/2a/3a/3b/6a)	1/156/48/11/11/9	0/73/21/7/7/10	4.663	0.458
lg (HCV RNA) IU/mL	6 (5.6)	5 (4.6)	-2.191	0.028
Along with the disease				
Diabetes	51 (21.61)	30 (25.42)	0.648	0.421
Fatty liver	52 (22.03)	13 (11.02)	6.370	0.012
Hypertension	50 (21.19)	23 (19.49)	0.138	0.710
FIB-4	5.27 (3.51, 8.16)	4.94 (2.30, 6.38)	-0.032	0.975
CEA (ng/mL)	3.27 (2.22, 4.86)	2.76 (1.45, 4.70)	-1.015	0.310
AFP (ng/mL)	9,28 (5.15, 19.60)	9.58 (4.27, 17.89)	-2.919	0.004
PIVKA-II (mAU/mL)	24 (18, 32)	24 (21, 35.5)	-1.190	0.234
PLT (10^9/L)	108.02 ± 61.66	110.66 ± 65.68	-0.342	0.733
CAP (dB/m)	232.76 ± 45.35	241.15 ± 53.59	-0.986	0.325
LSM (kPa)	26.15 ± 16.90	29.50 ± 16.61	-1.269	0.206

DAAs: Direct-acting antiviral agents; HCV RNA was calculated by denary logarithm expressed as lg (HCV RNA); FIB-4: The Fibrosis-4 index; ALBI: The albumin-Bilirubin score; CEA: Carcinoembryonic antigen; AFP: Alpha fetoprotein; PIVKA-II: Vitamin K Absence or Antagonist-II; PLT: Platelet; CAP: Controlled attenuation parameter; LSM: Liver stiffness measurement; PSM: Propensity score matching.

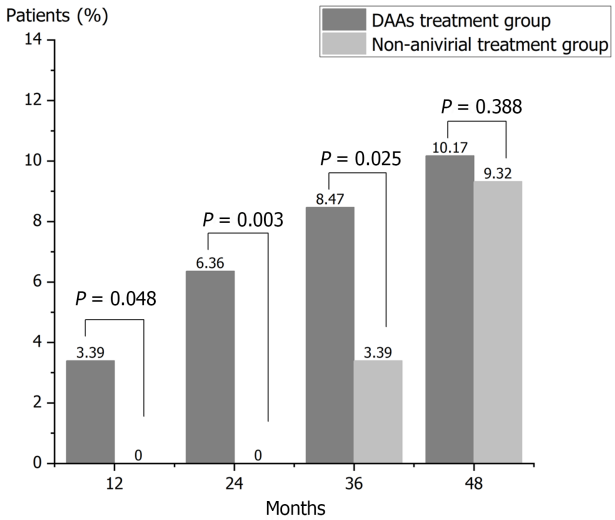

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Figure 3 The incidence of hepatocellular carcinoma at 12 mo, 24 mo, 36 mo, and 48 mo between the direct-acting antiviral agents treatment group and the non-antiviral treatment group. DAA: Direct-acting antiviral agents.

Table 2 Changes of laboratory parameters before and after sustained virologic response in the direct-acting antiviral agents treatment group (*n* = 236)

Variables	Before DAA	After DAA	$t/Z/\chi^2$	*P* value
ALT (U/L)	52 (34, 84.25)	20.5 (16, 32)	12.962	0
AST (U/L)	59.5 (40, 83.5)	26 (20.2, 39)	14.351	0
γ-GT (U/L)	58 (34.75, 108.5)	34 (23, 54)	8.691	0
ALP (U/L)	86 (65.75, 113.25)	83.9 (64, 115)	2.236	0.025
TP (g/L)	71.93 ± 8.23	73.62 ± 7.88	-2.267	0.024
ALB (g/L)	38.78 ± 6.22	42.93 ± 7.08	-6.755	0
TBIL (μmol/L)	18.6 (14.5, 27.5)	23.7 (13.3, 41)	-1.452	0.146
BUN (mmol/L)	4.98 ± 2.24	5.83 ± 3.70	-2.969	0.003
GLU (mmol/L)	6.34 ± 1.98	6.93 ± 2.44	-2.717	0.007
PT (s)	14.41 ± 2.16	14.30 ± 6.35	0.16	0.873
INR	1.27 ± 1.22	1.21 ± 0.56	0.444	0.557
WBC (10^9/L)	4.39 ± 1.69	4.93 ± 2.13	-3.066	0.002
RBC (10^{12}/L)	4.14 ± 0.75	4.27 ± 0.79	-1.803	0.072
HGB (g/L)	130.33 ± 23.05	131.91 ± 25.07	-0.706	0.481
PLT (10^9/L)	108.20 ± 61.66	123.45 ± 65.98	-2.57	0.01
CEA (ng/mL)	3.3 (2.22, 4.89)	3.34 (2.3, 5.27)	-0.111	0.911
AFP (ng/mL)	9.38 (5.31, 19.67)	5.81 (3.6, 9.02)	9.683	0
PIVKA-II (mAU/mL)	24 (18,32)	28 (20,41)	-1.958	0.05
CAP (dB/m)	232.76 ± 45.35	239.56 ± 45.35	-2.062	0.04
LSM (kPa)	26.15 ± 16.90	20.55 ± 16.95	3.499	0.001

SVR: Sustained virologic response; DAA: Direct-acting antiviral agents; HCV RNA was calculated by denary logarithm expressed as lg (HCV RNA); CEA: Carcinoembryonic antigen; AFP: Alpha fetoprotein; PIVKA-II: Vitamin K Absence or Antagonist-II; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: γ-alanine transferase; ALP: Alkaline phosphatase; TP: Total protein; ALB: Albumin; TBIL: Total bilirubin; BUN: Renal function; GLU: Blood glucose; PT: Prothrombin time; INR: International standardized ratio of prothrombin time; WBC: White blood cell; RBC: Red blood cell; HGB: Hemoglobin; PLT: Platelet; CAP: Controlled attenuation parameter; LSM: Liver stiffness measurement.

[24], while the annual average incidence of HCC in patients with HCV-associated cirrhosis is 1%-4%, and even 7% in the Asia-Pacific region[25]. With the introduction of various DAAs, their superior antiviral efficacy, low adverse reactions, and the increasing availability of drugs driven by our medical insurance policy, the HCV antiviral treatment strategy has changed completely, and IFN is no longer the first-line treatment for HCV infection[16]. However, HCV clearance does not mean a decrease in HCV-associated HCC. The current literature mainly studied the influence of DAAs on HCC occurrence in patients with hepatitis C. In this study, we focused on patients with HCV-associated cirrhosis because they are in higher risk of progression to HCC than chronic hepatitis C.

In this study, liver function indicators and LSM became significantly better after DAA treatment in the DAAs treatment group ($P < 0.05$), indicating that DAAs can effectively improve liver function and alleviate liver fibrosis in patients with HCV-associated cirrhosis. Deterding *et al*[26], in a single-center study, showed that interferon-free DAAs treatment effectively improved liver function in patients with HCV-associated cirrhosis, and Quaranta *et al*[27] observed improvement in liver function after HCV eradication in most patients with cirrhosis. Gentile *et al*[28] and Flisiak *et al*[29] confirmed this trend by observing similar changes in their study. Chan *et al*[30] enrolled a total of 70 CHC patients treated with DAAs, and results showed that 34 patients (48.6%) were worthy of significant improvement in LSM value at the end of treatment (relative to the baseline LSM value improvement > 30%). In another study, Curry *et al*[31] also found that at least 85% of patients with liver cirrhosis had a 40% reduction in LSM after HCV elimination by DAAs treatment, although 10% of patients still had an increase. Hence, DAAs treatment should be used as early as possible in CHC patients. In general, the elimination of HCV by DAAs improved the degree of liver fibrosis in most patients, and even reversed cirrhosis in a few patients[32]. In a 5-years follow-up study of CHC patients, Flisiak *et al*[33] found that DAAs treatment could alleviate liver inflammation and fibrosis after HCV eradication, and suggested that the improvement in LSM might be related to the reduction of liver inflammation, which is consistent with our results. It is well known that hypersplenism as a complication happens in patients with cirrhosis, especially in decompensated cirrhosis, then the three systems of blood cells will decrease, and PLT and WBC are laboratory parameter closely related to the development of hypers-

Table 3 Factors of hepatocellular carcinoma occurrence in all patients with hepatitis C virus associated cirrhosis (*n* = 427)

Variables	Univariate			Multivariate		
	HR	95%CI	*P* value	HR	95%CI	*P* value
DAA treated	0.478	0.245-0.933	0.03			
Ages (yr)	1.052	1.019-1.087	0.002	1.089	1.033-1.147	0.002
Gender (M/F)	1.297	0.750-2.242	0.352			
Cirrhosis (compensate/de)	2.312	1.271-4.207	0.006			
nonspecific nodules (Y/N)	3.112	1.734-5.586	0			
Child-Pugh (A/B/C)	2.184	1.416-3.367	0			
Fatty liver	0.686	0.271-1.738	0.427			
FIB-4	1.063	1.026-1.101	0.001			
AFP (ng/mL)	0.994	0.981-1.008	0.419			
AST (U/L)	1.005	0.999-1.010	0.126			
ALB (g/L)	0.922	0.885-0.961	0			
TIBL (μmol/L)	1.015	1.007-1.022	0			
PT	1.23	1.083-1.398	0.001			
PLT	0.992	0.986-0.997	0.005			
LSM	1.038	1.018-1.058	0	1.043	1.022-1.065	0

HCC: Hepatocellular carcinoma; HR: Hazard ratio; DAA: Direct-acting antiviral agents; FIB-4: The Fibrosis-4 index; ALBI: The albumin-Bilirubin score; AFP: Alpha fetoprotein; AST: Aspartate aminotransferase; ALB: Albumin; TBIL: Total bilirubin; EOT: End of treatment; PLT: Platelet; PT: Prothrombin time; LSM: Liver stiffness measurement.

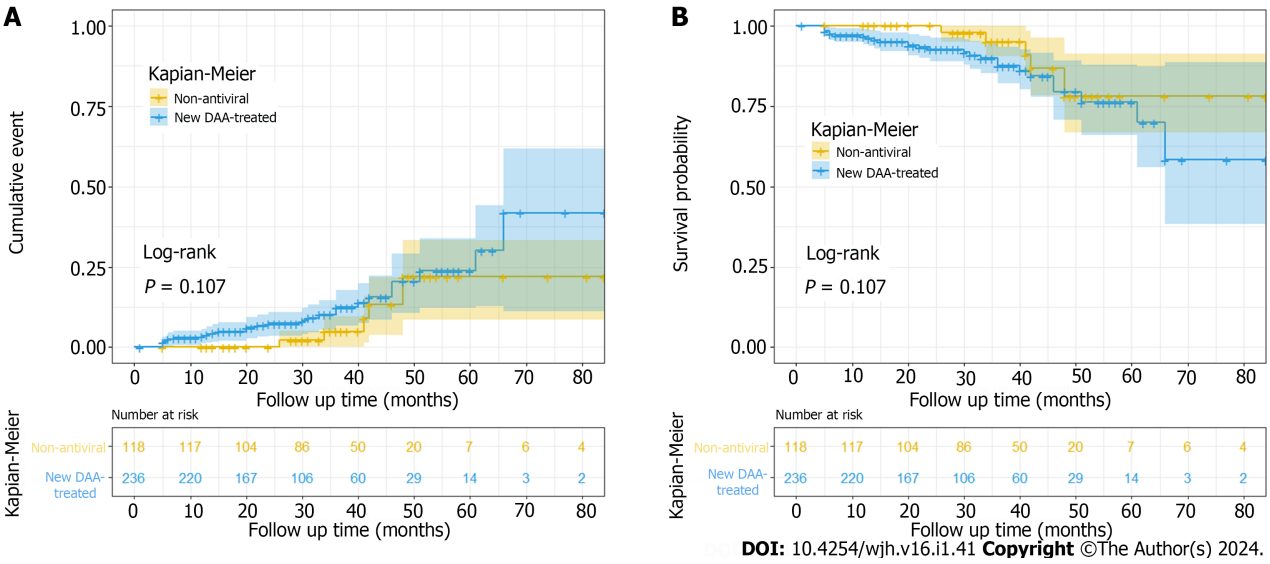

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Figure 4 Cumulative incidence and Survival probability of hepatocellular carcinoma between the direct-acting antiviral agents treatment group and the non-antiviral treatment group. A: Cumulative incidence of hepatocellular carcinoma (HCC) between direct-acting antiviral agents (DAAs) treatment group and non-antivial treatment group; B: Survival probability of HCC between new DAAs treatment group and non-antivial treatment group.

plenism[34]. This study implyed that HCV eradication could alleviate hypersplenism.

The effect of interferon-free DAAs treatment on the occurrence of HCC is controversial, and some recent studies have explored the topic[10,12-15,19,35]. A study[36] from Egypt showed that the incidence of HCC was significantly lower in patients with HCV-associated advanced fibrosis and cirrhosis treated with DAAs than in a historical cohort of untreated patients. A long-term follow-up study from Poland[33] showed that DAAs treatment reduced the risk of HCC, whereas a Spanish study[37] included data from approximately 4000 DAA-treated patients and reported an annual HCC incidence of 0.93% within 18 months of initiation of DAAs treatment. They found that the incidence of HCC in patients with

cirrhosis was higher regardless of their response to DAAs. A cohort of studies[38] from France revealed that the apparent increase in HCC incidence observed in patients with cirrhosis treated with DAAs compared with patients who achieved SVR following an IFN therapy could be explained by patient characteristics (age, diabetes, reduced liver function) and lower screening intensity. The results of this study indicating that DAAs treatment seems unable to reduce the risk of HCC in patients with HCV-associated cirrhosis. Furthermore, we calculated the HCC incidence of DAAs treatment group and non-antiviral treatment group at 12 mo, 24 mo and 36 mo. During this period, the incidence of HCC in the DAAs treatment group was higher than that in the non-antiviral treatment group ($P < 0.05$), which indicated that DAAs treatment may lead to an increased risk of short-term HCC occurrence, and this was consistent with the results of Mettke *et al*[35]. The reason might be that DAA treatment weakens the body's ability to immune surveillance and control tumors. Due to the existence of an effective immune system, the tiny primary tumor has been under the strong surveillance of the immune system. However, the rapid eradication of HCV may lead to the sudden weakening or withdrawal of immune surveillance, which is conducive to the proliferation and growth of isolated tumor cells. Serti *et al*[39] have also reported that DAA therapy can affect the composition of the innate immune system. Furthermore, Faillaci *et al*[40] showed that DAAs-mediated increase of vascular endothelial growth factor favors HCC recurrence/occurrence in susceptible patients, i.e. those with more severe fibrosis and splanchnic collateralization, who already have abnormal activation in liver tissues of neo-angiogenetic pathways, as shown by increased Angiopoietin-2. However, there was no significant difference in HCC incidence between the two groups at 48 months, which may be due to the fact that DAAs treatment can reduce the long-term incidence of HCC, but it is also likely to be limited by the follow-up period of this study. Therefore, a large sample with long-term follow-up should attract the attention of researchers.

In this study, we found old age and high LSM value were factors of HCC in all patients with HCV-associated cirrhosis, and the results shew that patients with HCV-associated cirrhosis had a higher risk for HCC with age ≥ 58 years and baseline LSM ≥ 27.85 kPa. Research by Asahina *et al*[41] also showed that elderly people have a higher risk of HCC. The reason may be that the older the patient is, the worse the physical function is, the more the relative underlying diseases are, and of course, the incidence of HCC is also higher. The best cut-off value of age obtained in this study is 58 years old which we should pay more attention to screen HCC and carry out some drug interventions to minimize the occurrence of clinical HCC when older than that. Hepatic decompensation, liver failure and HCC are more likely to occur in patients with HCV-associated cirrhosis with high LSM. Morisco *et al*[42] also concluded that baseline LSM ≥ 20 kPa identifies HCV cirrhotic subjects at higher risk of liver-related events after SVR. In clinical practice, we usually perform liver function and ultrasound examinations on patients, which suggests that we also should pay more attention to LSM. It is worth mentioning that DAAs treatment showed a statistical difference in univariate analysis, while there was no statistical difference in multivariate analysis. In this study, after adjusting for age and sex, Kaplan-Meier curve and Cox analysis showed no statistical difference in cumulative HCC incidence between DAAs treatment group and non-antiviral treatment group (P =0.107). This result proved that DAAs therapy didn't reduce the occurrence of HCC in patient with HCV-associated cirrhosis in a median 4 years. So, combining immunopotentiator agents or optimizing better DAAs might be considered.

Despite the important findings of this study, there are also limitations: First, this study was a single-center study. Second, the results of this study only reflect the events during the follow-up period of 1-84 mo, extending the follow-up time may have different results. Third, the non-antiviral treatment group is higher than the average follow-up time DAA treatment group, which may affect the results. Last, the DAA treatment regimen in this study is not uniform, so the influence of DAAs factors on the results cannot be excluded.

CONCLUSION

Our study shows that DAAs improved liver function, alleviated hepatic fibrosis and hypersplenism in patients with HCV-associated cirrhosis. This study found that DAAs did not reduce the incidence of HCC in HCV-associated cirrhosis compared without antiviral therapy, suggesting that the priority of DAAs for HCV patients in the clinic is reasonable. However, we should explore solutions to optimize DAAs treatment to reduce the occurrence of HCC in HCV-associated cirrhosis patients, and continued careful follow-up is necessary.

ARTICLE HIGHLIGHTS

Research background

Direct-acting antivirals (DAAs) revolutionized the treatment of chronic hepatitis C virus (HCV)-associated disease achieving high rates of sustained virological response (SVR). However, whether DAAs can reduce the occurrence of hepatocellular carcinoma (HCC) in patients with HCV-associated cirrhosis who are at high risk have not been concluded.

Research motivation

The key to the retrospective cohort study is to explore DAA treatment in HCV-associated cirrhosis patients with HCC. Solutions to optimize DAAs treatment are explored to reduce the occurrence of HCC in patients with HCV-associated cirrhosis, and careful follow-up is needed.

Research objectives

To investigate the effect of DAAs on the occurrence of HCC in patients with HCV-associated cirrhosis after achieving SVR.

Research methods

427 inpatients with HCV-associated cirrhosis were enrolled in Tianjin Second People's Hospital from January 2014 to April 2020. 118 patients weren't received antiviral treatment with any reasons named non-antiviral treatment group, and 236 patients obtained from the 309 DAAs treatment patients according to the propensity score matching named DAAs treatment group. Demographic information and laboratory data were collected from baseline and the following up. Kaplan-Meier curve and Log-Rank test were used to compare the incidence and cumulative incidence of HCC between the two groups. Cox proportional risk regression was used to re-evaluate the risk factors for HCC.

Research results

The DAA treatment group was followed up for 1-84 mo, with a median follow-up of 28 mo, while the non-antiviral treatment group was followed up for 5-84 mo, with a median follow-up of 37 mo. Age > 58 [hazard ratio (HR) = 1.089; 95% confidence interval (CI), 1.033-1.147; P = 0.002] and liver stiffness measurement > 27.85 kPa (HR = 1.043; 95%CI, 1.022-1.065; P = 0.000) were risk factors for HCC in all patients (n = 427), and DAA treatment didn't show protective efficacy. After adjusting for confounding factors (age and sex), 27 cases of HCC occurred in the new DAA treatment group (236 cases), and there was no significant difference in the total incidence of HCC between the two groups (χ^2 = 0.369, P = 0.544). In the new DAA treatment group, HCC incidence was 4.68/100PY (95%CI, 3.09-6.81), while it was 3.00/100PY (95%CI, 1.50-5.37) in the non-antiviral treatment group. The follow-up time of the new DAA treatment group was 1-84 mo (29.33 ± 16.20), the median follow-up time was 27 mo and the time of HCC occurrence in the new DAA treatment group was 5-66 mo. The incidence of HCC at 12, 24, 36 and 48 mo was 3.39%, 6.36%, 8.47% and 10.17% in the new DAA treatment group, and it was 0%, 0%, 3.39% and 9.32% in the non-antiviral treatment group, respectively.

Research conclusions

This is a novel assessment that provides theoretical insight into the impact of achieving SVR after DAA on HCC development in patients with HCV-associated cirrhosis. This study found that DAAs did not reduce the incidence of HCC in HCV-associated cirrhosis compared with no antiviral therapy, suggesting that the clinical priority of DAAs for patients with HCV is justified. We should also explore solutions to optimize DAAs therapy to reduce the occurrence of HCC in patients with HCV-associated cirrhosis.

Research perspectives

In future study, we should be focused on the research of the multicenter, large data, in order to more accurately assess DAAs influence on HCV-associated liver diseases in patients with HCC, thereby reducing the occurrence of HCC, and it can from common biochemical indicator, liquid biopsy, multiple sets of multi-angle discussion such as HCV-associated liver disease risk factors in patients with HCC.

FOOTNOTES

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Author contributions: Xu L, Mi YQ and Tao XM designed the study; Tao XM, Zeng MH and Zhao YF and Han JX performed the research; Tao XM carried out statistical analysis; Tao XM wrote the manuscript; Xu L and Mi YQ critical revised of the manuscript. All the authors have read and approved the final revision to be published. Xu L and Mi YQ, as co-corresponding authors, played an important and indispensable role in the experimental design, data interpretation, and manuscript writing. Xu L and Mi YQ applied for and received funding for this research project. Mi YQ was responsible for the conceptualization, design and supervision of the overall project and the revision of the article. Xu L was responsible for the design of the experiment, revision of the article, preparation and submission of the current version of the manuscript.

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Metabolic puzzle: Exploring liver fibrosis differences in Asian metabolic-associated fatty liver disease subtypes

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Abstract

BACKGROUND

Metabolic-associated fatty liver disease (MAFLD) is a liver condition marked by excessive fat buildup in the absence of heavy alcohol use. It is primarily linked with metabolic issues like insulin resistance, obesity, and abnormal lipid levels, and is often observed with other conditions such as type 2 diabetes and cardiovascular disease. However, whether the subtypes of MAFLD based on the metabolic disorder differentially impact liver fibrosis is not well explicated, especially in the Asian population.

AIM

To compare the severity of liver fibrosis among different MAFLD subtypes.

METHODS

A total of 322 adult patients of either gender with fatty liver on ultrasound were enrolled between January to December 2021. MAFLD was defined as per the Asian Pacific Association for the Study of the Liver guidelines. Fibrosis-4 index (Fib-4) and nonalcoholic fatty liver disease fibrosis score (NFS) were employed to evaluate liver fibrosis.

RESULTS

The mean age was 44.84 ± 11 years. Seventy-two percent of the patients were female. Two hundred and seventy-three patients were classified as having MAFLD, of which 110 (40.3%) carried a single, 129 (47.3%) had two, and 34 (12.5%) had all three metabolic conditions. The cumulative number of metabolic conditions was related to elevated body mass index, triglyceride (TG) levels, and

glycated hemoglobin, lower high-density lipoprotein (HDL) levels, higher liver inflammation (by aspartate aminotransferase and γ-glutamyl transferase), and higher likelihood of fibrosis (by NFS and Fib-4 scores) ($P < 0.05$ for all). The proportion of advanced fibrosis also increased with an increase in the number of metabolic conditions (4.1%, 25.5%, 35.6%, and 44.1% by NFS and 6.1%, 10.9%, 17%, and 26.5% by Fib-4 for no MAFLD and MAFLD with 1, 2, and 3 conditions, respectively). Among MAFLD patients, those with diabetes alone were the eldest and had the highest mean value of NFS score and Fib-4 score ($P < 0.05$), while MAFLD patients diagnosed with lean metabolic dysfunction exhibited the highest levels of TG and alanine aminotransferase but the lowest HDL levels ($P < 0.05$).

CONCLUSION

The study suggests that the severity of liver fibrosis in MAFLD patients is influenced by the number and type of metabolic conditions present. Early identification and management of MAFLD, particularly in patients with multiple metabolic conditions, are crucial to prevent liver-related complications.

Key Words: Metabolic syndrome; Diabetes; Fatty liver disease; Dyslipidemia; Obesity

Core Tip: This is the first study on the South-Asian population on assessment of fibrosis among metabolic-associated fatty liver disease (MAFLD) patients. The study highlights that as the number of risk factors increases in a patient with MAFLD, it is more likely to have progression of liver fibrosis.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of diseases ranging from benign accumulation of excessive fat in the liver (steatosis) to the inflammation of liver cells [nonalcoholic steatohepatitis (NASH)]. It can lead to advanced fibrosis, cirrhosis, and subsequent hepatocellular carcinoma (HCC). NAFLD is now one of the common indications for liver transplantation from Western data. It is primarily a diagnosis of exclusion that needs to exclude other causes of liver fat accumulation, for instance, alcohol intake above a certain quantity, medications, viral hepatitis, and autoimmune liver disease[1]. The disease progression from benign fatty liver to inflammation and, ultimately, liver fibrosis is linked with the co-existence of diabetes mellitus (DM), obesity, and metabolic syndrome (MS)[2]. This has resulted in the proposal of this terminology change from NAFLD to metabolic-associated fatty liver disease (MAFLD) (metabolic malfunction associated fatty liver disease)[3]. The Asian Pacific Association for the Study of the Liver (APASL) also endorsed this amendment in nomenclature and the development of "diagnostic criteria" for MAFLD, unlike NAFLD, a diagnosis of exclusion[4].

When evaluating fatty liver and fibrosis, liver biopsy remains the gold standard. Due to its invasive nature, various noninvasive diagnostic tools (based on imaging or biomarkers) are now being used. Among them are the NAFLD fibrosis score (NFS) and fibrosis-4 index (Fib-4), endorsed by various guidelines as preference screening panels for predicting advanced fibrosis[4,5]. A strong body of evidence suggests that MAFLD is more effective than NAFLD in identifying significant liver fibrosis[6,7]. However, whether the subtypes of MAFLD differentially influence liver fibrosis is not very well understood, especially in the Asian population. Therefore, given the recent notion of MAFLD, our objective was to compare the severity of liver fibrosis among different MAFLD subtypes.

MATERIALS AND METHODS

This cross-sectional study was conducted at the National Institute of Liver and GI Diseases, located at Dow University Hospital in Karachi, Pakistan. Patients (ranging in age between 18 and 65 years, including both males and females) diagnosed with fatty liver disease between January and December 2021 were included. Those patients with decompensated liver disease, HCC, acute hepatitis, acute-on-chronic liver disease, and other concomitant liver disease (chronic active viral, alcohol, autoimmune, or metabolic liver diseases) were excluded from this study. Pregnant or lactating female patients and patients with concomitant systemic diseases such as tuberculosis, autoimmune disorders, and extrahepatic malignancies were also excluded.

The demographic, clinical, and laboratory data of the patients were collected and analyzed. The main indications to perform an ultrasound examination were symptoms of dyspepsia and right upper quadrant abdominal pain and an evaluation showing deranged liver function tests. The fatty liver finding was confirmed on ultrasound examination based on the diffuse increased hepatic parenchymal echogenicity or "bright texture of liver parenchyma"[8]. According to the APASL guidelines, MAFLD was defined as the presence of fatty liver in conjunction with at least one of the following three conditions: Overweight/obesity, type 2 DM, or evidence of metabolic dysfunction (MD) such as increased waist circumference or an abnormal lipid or glycemic profile[4]. Fib-4 and NFS were noninvasive tools used to assess liver fibrosis in this population with fatty liver disease. Asian cutoffs for body mass index (BMI) were used to classify the subjects as overweight/obese *vs* lean/normal weight among different MAFLD groups. Figure 1 describes the study flow chart.

The study was approved by the Institutional Review Board of Dow University of Health Sciences (IRB-1842). Informed consent was obtained from all eligible participants. The methods employed in this study were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000.

The statistical analyses were executed using SPSS software version 26.0. Quantitative variables are expressed as the mean ± SD, while categorical variables are represented as frequencies and percentages. The chi-square test was used to assess categorical variables. The Mann-Whitney *U*-test was applied to compare the difference between two groups, while the Kruskal-Wallis test was performed to evaluate the difference among three groups. A P value of 0.05 or less was considered significant.

RESULTS

A total of 322 patients with fatty liver were included, with a mean age of 44.84 ± 11 years. The majority were female (72%). The mean BMI was 29.83 ± 5.53 kg/m^2, 29.8% had DM, and 9.6% had hypertension.

Out of 322 patients with fatty liver, 273 were classified as having MAFLD. The MAFLD patients were further classified into three categories corresponding to their components of metabolic conditions (*i.e.*, one, two, and three). Out of 273 participants with MAFLD, 110 (40.3%) had a single metabolic condition, 129 (47.3%) had two metabolic conditions, and 34 (12.5%) had all three metabolic conditions (Figure 1).

With the increasing number of metabolic conditions, more patients were diabetic and obese, with the worsening of liver enzymes and lipid profile, as well as increasing hepatic fibrosis scores. With an increase in the cumulative number of metabolic conditions, the patients exhibited a significant elevation in their metabolic parameters such as BMI (28.99 ± 5.19 *vs* 31.63 ± 5.19 *vs* 33.59 ± 4.75; $P < 0.001$) and glycated hemoglobin (Hb1Ac) (5.97 ± 1.13 *vs* 6.82 ± 1.86 *vs* 8.22 ± 1.58, $P < 0.001$). Significant worsening of lipid profile was also noted with the increasing number of metabolic conditions as triglyceride (TG) levels rose (182.45 ± 109.5 *vs* 198.13 ± 98.8 *vs* 221.85 ± 102.38, $P = 0.002$), while high-density lipoprotein (HDL) levels showed a negative trend among MAFLD patients (41.65 ± 15.08 *vs* 36.05 ± 8.93 *vs* 32.38 ± 6.62, $P < 0.001$).

As a consequence of these findings, increasing liver inflammation (as reflected by aspartate aminotransferase (AST) 28.62 ± 20.74 *vs* 32.29 ± 23.36 *vs* 40.06 ± 26.74, $P = 0.021$ and γ -glutamyl transferase 34.93 ± 21.08 *vs* 51.50 ± 36.44 *vs* 65.41 ± 38.02, $P < 0.001$) and liver fibrosis (reflected by the NFS score -2.59 ± 1.59 *vs* -2.00 ± 1.69 *vs* -1.39 ± 1.60, $P = 0.002$ and Fib-4 score 0.79 ± 0.45 *vs* 0.94 ± 0.86 *vs* 1.11 ± 0.66, $P = 0.041$) were seen as the trends of different metabolic categories (Table 1). The proportion of significant fibrosis was also established with the collective number of metabolic conditions. For the NFS score, advanced fibrosis was present in 4.1% of subjects with no fulfilled criteria for MAFLD and in 25.5%, 35.6%, and 44.1% with 1, 2, and 3 MAFLD conditions, respectively, while for the Fib-4 score, advanced fibrosis was present in 6.1% of subjects without MAFLD, and in 10.9%, 17%, and 26.5% with 1, 2, and 3 MAFLD conditions, respectively (Figure 2).

The age of the patients increases somewhat as the number of metabolic diseases increases, with more men afflicted, but these results were not statistically significant across the categories. There was also no significant difference in ALT, platelets, total cholesterol, low-density lipoprotein (LDL) cholesterol, total bilirubin, or alkaline phosphatase levels.

Furthermore, MAFLD patients with a single metabolic condition ($n = 110$, 40.3%) were sub-classified into three categories: Obesity alone ($n = 61$, 55.5%), lean MD ($n = 34$, 30.9%), and DM alone ($n = 15$, 13.6%). Among MAFLD patients with a single metabolic condition, those established with DM alone were the oldest and those with obesity alone were the youngest (mean age 50.73 ± 9.04 for DM *vs* 45.53 ± 10.60 for lean MD and 41.72 ± 10.03 for obesity alone, $P = 0.005$). Similarly significant differences were noted in platelet count, which was within the normal range but the lowest in the DM group (245.40 ± 50.70 *vs* 275.44 ± 81.92 in lean MD *vs* 314.85 ± 97.95 in obesity alone, $P = 0.004$), TG levels, which were the highest in the lean MD group (269.02 ± 120.03 *vs* 176.13 ± 132.33 in DM *vs* 135.75 ± 57.72 in obesity, $P < 0.001$), HDL levels, which were the lowest in the lean MD group (39.96 ± 21.71 *vs* 42.30 ± 8.73 in obesity *vs* 42.50 ± 11.14 in DM, $P = 0.026$), and ALT levels, which were the highest in lean MD (43.94 ± 28.41 *vs* 34.13 ± 19.04 in DM *vs* 33.89 ± 30.47 in obesity, $P = 0.043$). Similarly, diabetic MAFLD had the highest Hb1Ac levels (8.03 ± 1.71 *vs* 5.77 ± 0.48 *vs* 5.56 ± 0.46, $P < 0.001$) than others (Table 2).

When compared among the three subtypes of MAFLD, the proportion of advanced liver fibrosis was significantly higher among diabetic MAFLD patients according to the NFS score (46.6% *vs* 26.5% for MD alone and 19.7% for obesity alone), whereas patients with lean MD had the highest proportion of advanced fibrosis according to the Fib-4 score (14.7% *vs* 9.8% for obesity alone *vs* 6.7% for DM alone) (Figure 3).

No significant differences were observed in gender distribution, education awareness, history of hypertension, blood pressure, and blood levels of cholesterol, LDL, bilirubin, albumin, AST, and alkaline phosphate between these respective

Table 1 Demographic, clinical, and laboratory characteristics of metabolic-associated fatty liver disease patients with different metabolic conditions

Characteristic	Total (*n* = 322)	No MAFLD (*n* = 49)	MAFLD (*n* = 273)			*P* value			
			Single condition (*n* = 110; 40.3%)	Two conditions (*n* = 129; 47.3%)	Three conditions (*n* = 34; 12.5%)	Overall	Single *vs* two	Single *vs* three	Two *vs* three
Age (yr)	44.84 ± 11	42.69 ± 12	44.13 ± 10.47	45.53 ± 10.80	47.65 ± 10.32	0.332	0.474	0.138	0.321
BMI (kg/m^2)	29.83 ± 5.53	34.38 ± 2.20	28.99 ± 5.19	31.63 ± 5.19	33.59 ± 4.75	< 0.001	< 0.001	< 0.001	0.064
Female gender (%)	232 (72)	32 (65.3)	85 (77.3)	94 (72.9)	21 (61.8)	0.201	0.434	0.073	0.206
Hypertension (%)	31 (9.6)	5 (10.2)	14 (12.7)	12 (9.3)	0 (0.0)	0.086	0.397	0.029	0.065
Diabetes (%)	96 (29.8)	0 (0)	15 (13.6)	47 (36.4)	34 (100)	< 0.001	< 0.001	< 0.001	< 0.001
NFS score	-2.41 ± 1.71	-3.76 ± 1.19	-2.59 ± 1.59	-2.00 ± 1.69	-1.39 ± 1.60	0.002	0.033	< 0.001	0.043
Fib-4 score	0.88 ± 0.67	0.75 ± 0.47	0.79 ± 0.45	0.94 ± 0.86	1.11 ± 0.66	0.041	0.771	0.009	0.031
DBP	85.22 ± 12.07	82.26 ± 11	85.32 ± 13.22	86.34 ± 11.59	84.91 ± 11.80	0.631	0.333	0.643	0.958
SBP	133.86 ± 19.18	129.34 ± 16	133.00 ± 17.73	134.83 ± 20.8	139.56 ± 20.55	0.133	0.148	0.071	0.376
Platelet count	295.77 ± 90.85	305.91 ± 73	293.20 ± 91.20	294.06 ± 92.0	295.97 ± 110.13	0.888	0.644	0.856	0.811
Total cholesterol	183.29 ± 45.18	184.3 ± 47.11	181.03 ± 42.35	182.94 ± 48.3	190.41 ± 39.58	0.375	0.705	0.266	0.161
LDL	124.81 ± 39.85	120.58 ± 38.16	123.85 ± 39.87	126.52 ± 41.3	127.44 ± 37.20	0.859	0.949	0.648	0.573
HDL	38.55 ± 12.08	43.01 ± 11.93	41.65 ± 15.08	36.05 ± 8.93	32.38 ± 6.62	< 0.001	< 0.001	< 0.001	0.038
TG	184.79 ± 101.2	125.57 ± 51.8	182.45 ± 109.5	198.13 ± 98.8	221.85 ± 102.38	0.002	0.022	0.001	0.050
Total bilirubin	0.54 ± 0.37	0.55 ± 0.26	0.53 ± 0.35	0.57 ± 0.43	0.49 ± 0.31	0.284	0.358	0.287	0.157
Direct bilirubin	0.21 ± 0.24	0.21 ± 0.23	0.19 ± 0.09	0.24 ± 0.35	0.18 ± 0.08	0.601	0.417	0.732	0.417
Serum albumin	4.41 ± 0.38	4.51 ± 0.34	4.41 ± 0.37	4.37 ± 0.38	4.40 ± 0.43	0.552	0.305	0.481	0.906
ALT	40.67 ± 31.69	42.2 ± 29	37.03 ± 28.70	42.30 ± 36.49	44.09 ± 24.15	0.058	0.229	0.022	0.097
AST	31.84 ± 22.6	32.22 ± 20	28.62 ± 20.74	32.29 ± 23.36	40.06 ± 26.74	0.021	0.361	0.004	0.040
GGT	46.02 ± 34.77	42.45 ± 43.65	34.93 ± 21.08	51.50 ± 36.44	65.41 ± 38.02	< 0.001	< 0.001	< 0.001	0.016
ALP	110.82 ± 51.76	105.3 ± 40	111.90 ± 59.42	108.22 ± 46.1	125.24 ± 58.81	0.169	0.802	0.089	0.070
HbA1c	6.44 ± 1.65	5.32 ± 0.4	5.97 ± 1.13	6.82 ± 1.86	8.22 ± 1.58	< 0.001	< 0.001	< 0.001	< 0.001

MAFLD: Metabolic associated fatty liver disease; BMI: Body mass index; NFS: Nonalcoholic fatty liver disease fibrosis score; Fib-4: Fibrosis-4 index; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; HbA1c: Glycated hemoglobin.

Table 2 Comparison of demographic, clinical, and laboratory characteristics among metabolic associated fatty liver disease with a single metabolic condition alone

Characteristic	Obesity alone (*n* = 61)	Lean MD (*n* = 34)	DM alone (*n* = 15)	*P* value			
				Overall	Obesity *vs* lean MD	Obesity *vs* DM	Lean MD *vs* DM
Age (yr)	41.72 ± 10.03	45.53 ± 10.60	50.73 ± 9.04	0.005	0.082	0.002	0.116
BMI (kg/m^2)	32.33 ± 4.66	24.86 ± 1.63	24.81 ± 1.58	< 0.001	< 0.001	< 0.001	0.965
Female Gender (%)	51 (83.6)	23 (67.6)	11 (73.3)	0.191	0.072	0.358	0.691
Hypertension (%)	8 (13.1)	6 (17.6)	0 (0.0)	0.230	0.551	0.138	0.082
NFS score	-2.86 ± 1.74	-2.50 ± 1.40	-1.61 ± 0.81	0.017	0.309	0.005	0.054
Fib-4 score	0.68 ± 0.35	0.92 ± 0.56	0.95 ± 0.48	0.027	0.050	0.017	0.761
DBP	84.26 ± 15.41	87.03 ± 10.43	85.73 ± 8.61	0.499	0.262	0.548	0.728
SBP	131.03 ± 17.46	135.82 ± 19.94	134.60 ± 12.89	0.306	0.255	0.181	0.828
Platelet count	314.85 ± 97.95	275.44 ± 81.92	245.40 ± 50.70	0.004	0.032	0.002	0.298
Total cholesterol	176.91 ± 37.71	190.97 ± 49.33	175.00 ± 41.93	0.336	0.160	0.879	0.313
LDL	120.53 ± 35.87	126.76 ± 44.83	130.73 ± 44.80	0.626	0.473	0.433	0.688
HDL	42.50 ± 11.14	39.96 ± 21.71	42.30 ± 8.73	0.026	0.010	0.678	0.079
TG	135.75 ± 57.72	269.02 ± 120.03	176.13 ± 132.33	< 0.001	< 0.001	0.213	0.001
Total bilirubin	0.52 ± 0.43	0.55 ± 0.24	0.50 ± 0.22	0.171	0.055	0.698	0.467
Direct bilirubin	0.19 ± 0.10	0.18 ± 0.08	0.20 ± 0.09	0.613	0.480	0.549	0.424
Serum albumin	4.41 ± 0.34	4.40 ± 0.33	4.44 ± 0.59	0.731	0.779	0.526	0.428
ALT	33.89 ± 30.47	43.94 ± 28.41	34.13 ± 19.04	0.043	0.016	0.264	0.288
AST	25.75 ± 15.38	34.76 ± 29.26	26.33 ± 13.60	0.086	0.025	0.724	0.302
GGT	32.32 ± 21.22	37.85 ± 20.37	39.15 ± 22.28	0.139	0.083	0.169	0.849
ALP	113.61 ± 68.86	107.03 ± 39.59	116.00 ± 58.23	0.948	0.880	0.734	0.888
HbA1c	5.56 ± 0.46	5.77 ± 0.48	8.03 ± 1.71	< 0.001	0.006	< 0.001	< 0.001

MD: Metabolic dysfunction; DM: Diabetes mellitus; BMI: Body mass index; NFS: Nonalcoholic fatty liver disease fibrosis score; Fib-4: Fibrosis-4 index; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; HbA1c: Glycated hemoglobin.

three groups.

DISCUSSION

The present study provides valuable insights into the progression of MAFLD and its subtypes in the Pakistani population. Our findings demonstrated that as the cumulative number of metabolic conditions increased, there was a corresponding escalation in the NFS and Fib-4 scores. This trend aligns with the work of Yamamura *et al*[6], which also reported that patients with multiple metabolic conditions exhibited a higher risk of advanced fibrosis.

In our study, around 60% of patients had more than one metabolic condition, which is comparable to a recent study of the NHANES III database in which more than 70% of all patients with MAFLD had more than one metabolic condition. Additionally, having more than one metabolic condition was associated with abnormal liver function tests and kidney diseases[9]. The same study found that there were an increasing number of metabolic conditions in the higher age group. In our study, there was only a non-significant association between older age and comorbidities. This may be attributed to the different demographic spectra in our population (North American *vs* Southeast Asian). Recent meta-analytical evidence lends further credence, delineating the clinical characteristics of NAFLD in Asian populations. It demonstrates that the pooled mean age of NAFLD patients was 52.07 years (95%CI: 51.28-52.85), which contrasts with a notably younger mean age of 42.66 years (95%CI: 32.23-53.11) observed in patients from Southeast Asia, indicating regional age-related disparities among NAFLD patients[10].

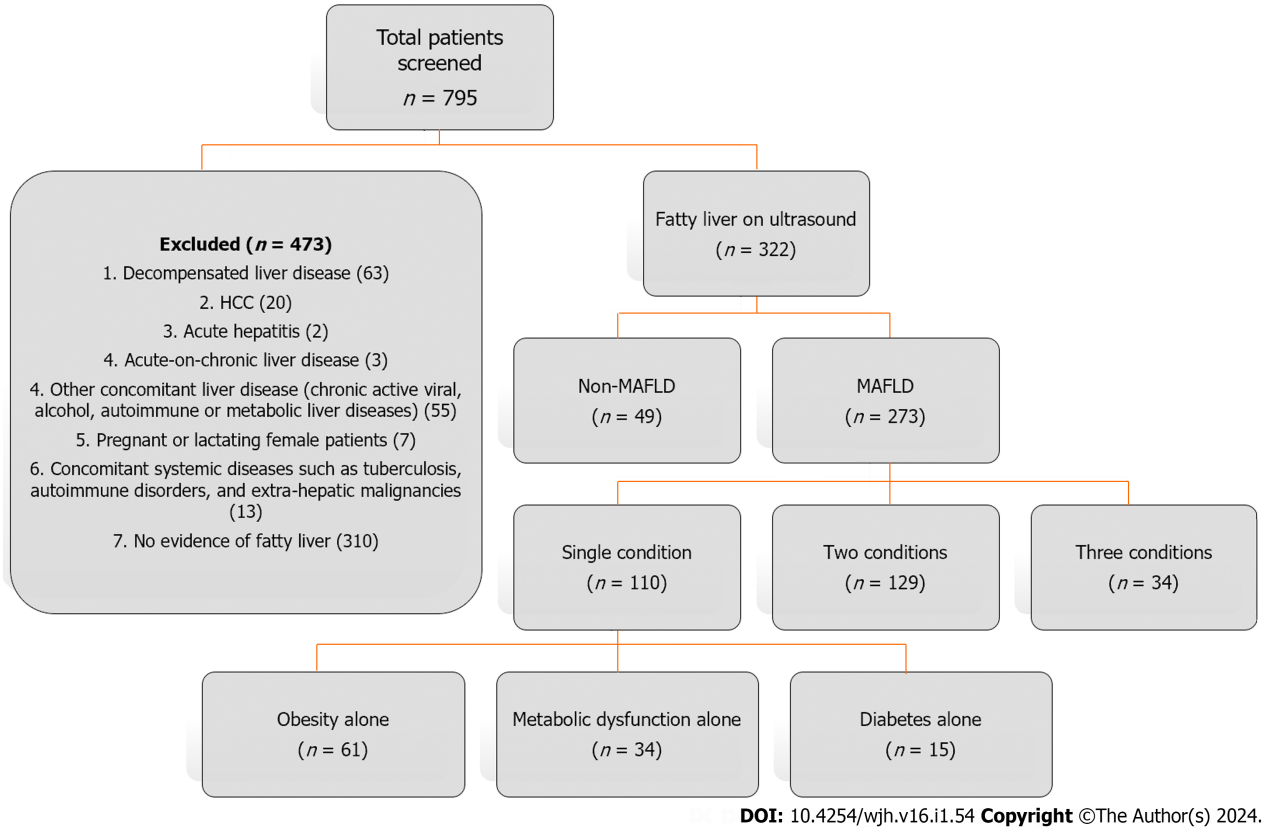

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Figure 1 Flow chart of sample selection. HCC: Hepatocellular carcinoma; MAFLD: Metabolic-associated fatty liver disease.

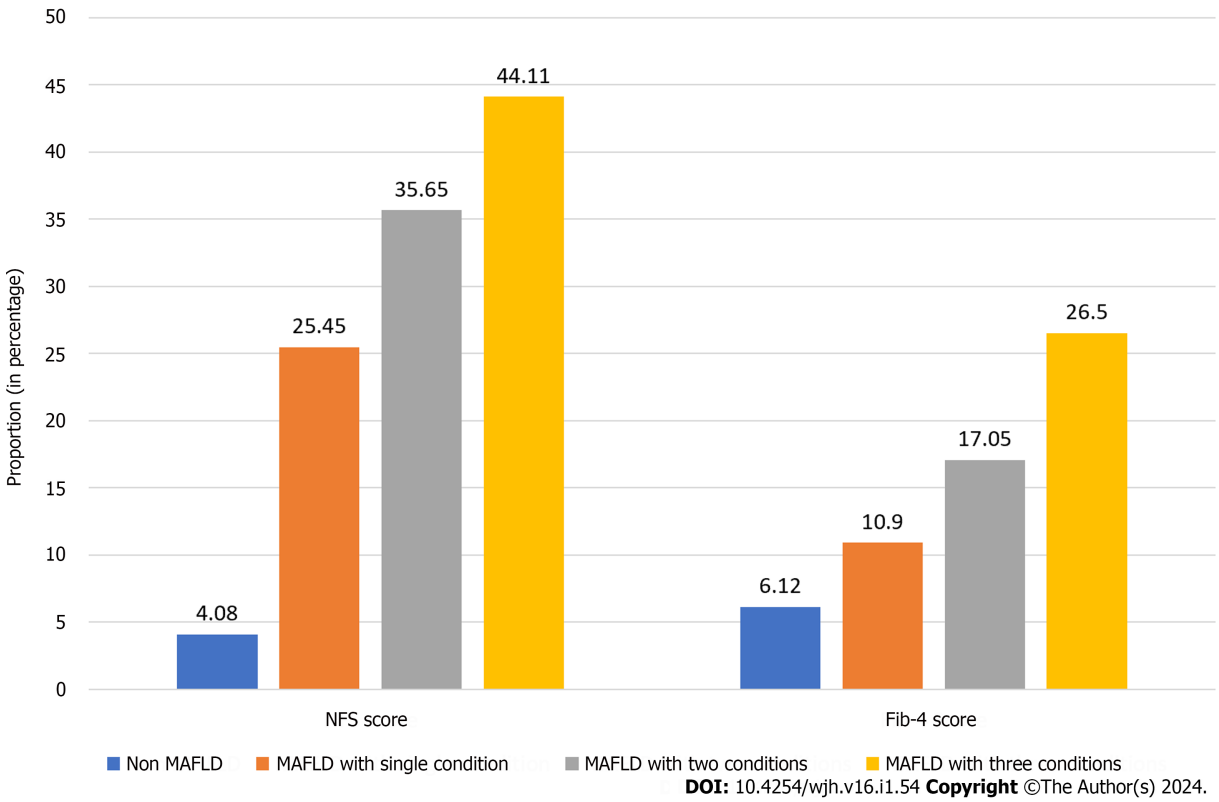

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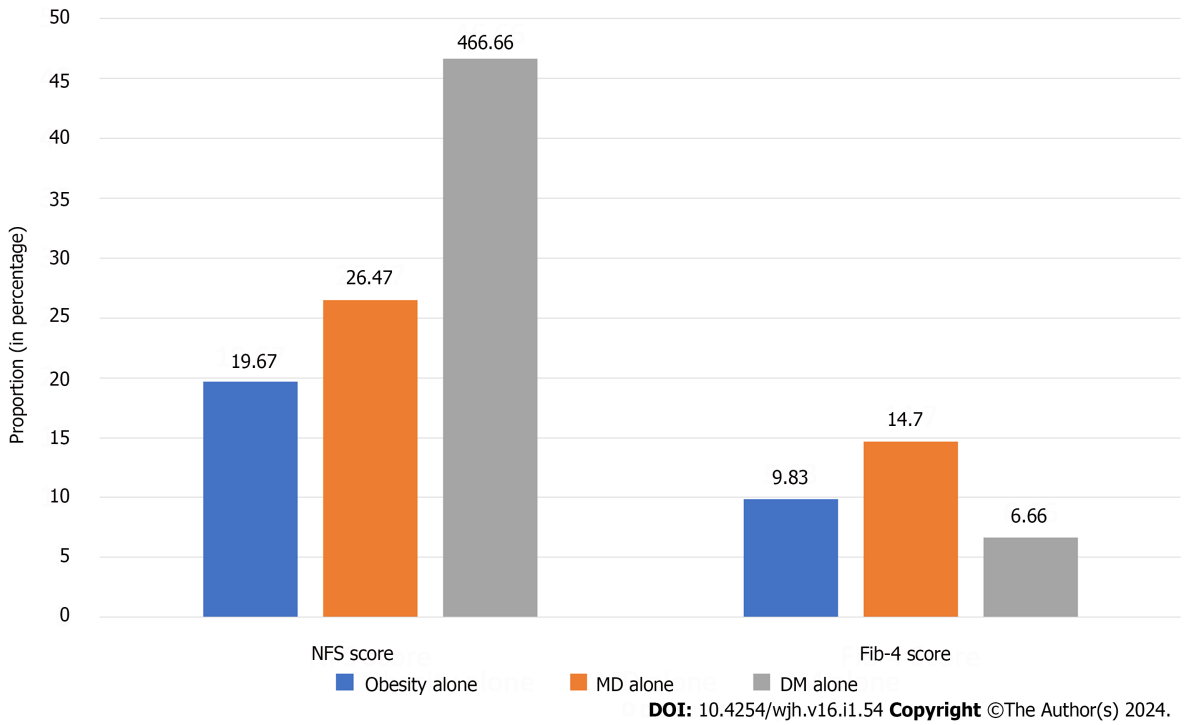
Figure 2 Proportion of advanced liver fibrosis in relation to the cumulative number of metabolic conditions. Fib-4: Fibrosis-4 index; NFS: Nonalcoholic fatty liver disease fibrosis score; MAFLD: Metabolic-associated fatty liver disease.



DOI: 10.4254/wjh.v16.i1.54

Figure 3 Proportion of advanced liver fibrosis in patients with single metabolic conditions. Fib-4: Fibrosis-4 index; NFS: Nonalcoholic fatty liver disease fibrosis score; MD: Metabolic dysfunction; DM: Diabetes mellitus.

The degree of liver fibrosis varies across MAFLD subtypes[11], with an increased risk of liver-related death as fibrosis progresses[12]. Hence, we further classified the MAFLD into subtypes according to the type of metabolic conditions. Interestingly, the sub-classification of MAFLD based on individual metabolic conditions revealed distinct profiles. Diabetic MAFLD patients tended to be older, have higher TG levels, and exhibit more pronounced fibrosis compared to those with other MAFLD subtypes, echoing the finding of Chhabra *et al*[13] that diabetes is a strong predictor of advanced fibrosis in MAFLD. Studies have consistently shown a higher proportion of advanced liver fibrosis in diabetic MAFLD patients compared to other MAFLD subtypes[9,14]. The relationship between DM, MAFLD, and advanced fibrosis is likely a multifactorial chronic process, with insulin resistance and older age playing significant roles[15,16]. This relationship is reflected in the Fib-4 and NFS scores, which incorporate age as a variable, leading to higher scores in older individuals. These findings underscore the importance of considering diabetes as a risk factor for advanced fibrosis in MAFLD patients, particularly among older individuals. Early identification and management of diabetes and MAFLD are crucial to prevent liver-related complications and improve patient outcomes.

Elevated TG, ALT, and AST levels in lean MAFLD indicate that lean MAFLD has clinical implications and is associated with liver inflammation or injury. Lean MAFLD patients have a more detrimental metabolic profile compared to lean non-MAFLD patients[17]. Lean MAFLD is independently associated with an increased risk of overall mortality [hazard ratio (HR): 1.296; 95%CI: 1.064-1.578][18], as well as liver-specific mortality (HR: 2.84; 95%CI: 2.72-2.97) as compared to other MAFLD subtypes[19]. Furthermore, this impact was also observed in post-liver transplant, as lean NASH patients have worse post-liver transplant overall survival compared to non-lean NASH (HR: 0.17; 95%CI: 0.03-0.86, $P = 0.0142$) [20]. These findings highlight the importance of recognizing lean MAFLD as a distinct clinical entity with significant adverse health outcomes. Early identification and management of lean MAFLD are crucial to prevent liver-related complications and improve patient outcomes.

On the other hand, individuals with obesity as the sole metabolic condition presented with a younger age and less severe fibrosis, suggesting a potential protective effect of youth or a longer disease trajectory before significant fibrosis develops, which has been suggested by Yang *et al*[21].

Our study did not find statistically significant differences in ALT, total cholesterol, LDL, bilirubin, or alkaline phosphatase levels across the MAFLD subtypes, which diverges from the findings of Wong *et al*[22], who reported dyslipidemia and elevated liver enzymes as common features in MAFLD patients. This discrepancy could be attributed to the genetic or dietary factors unique to our study population, underlining the complexity of MAFLD phenotypes as noted by Eslam *et al*[23].

The diagnostic performance of Fib-4 and NFS for advanced fibrosis can be influenced by various factors, including age, DM, and BMI. In particular, the inclusion of overweight or obesity as a criterion for MAFLD has impacted the BMI component in NFS, leading to differences in the sensitivity and specificity of the two scores in identifying advanced fibrosis. A recent study found that although the overall performance of Fib-4 and NFS in diagnosing liver fibrosis was similar between lean and non-lean individuals, the sensitivity and specificity of NFS varied according to BMI quartile ranges. Specifically, NFS was found to be less sensitive in lean individuals compared to Fib-4[24]. Another study found that the diagnostic ability of NFS was lower among individuals with diabetes compared to Fib-4 [area under the receiver

operating characteristic curve (AUROC) 0.717 *vs* 0.809; P = 0.002]. This suggests that NFS may not be as effective in identifying advanced fibrosis in patients with diabetes[25]. A recent study also found that Fib-4 was superior to NFS in accurately classifying non-obese NAFLD patients with F2–4 fibrosis (AUROC 81.5% *vs* 73.7%, P < 0.001). This suggests that Fib-4 may be a better choice for diagnosing advanced fibrosis in this patient population[26]. Overall, the evidence suggests that Fib-4 may be a more reliable tool for diagnosing advanced fibrosis than NFS, particularly in lean individuals and patients with diabetes. Further research is needed to confirm these findings and to determine the optimal use of both scores in clinical practice.

The strengths of this study are multifaceted, encompassing stringent participant selection, methodological robustness, ethical integrity, and analytical rigor. First, the study employed rigorous inclusion and exclusion criteria, ensuring a well-defined study population that accurately represented the target demographic for MAFLD. This strategic participant selection minimized confounding variables, thereby enhancing the validity of the findings. Second, the adoption of a cross-sectional study design facilitated the examination of the prevalence and association patterns of liver fibrosis with metabolic conditions at a specific point in time. Moreover, this is the first study on the South Asian population to highlight the importance of subtyping MAFLD, validating previous reports from the Western world that the severity of liver fibrosis varies across the MAFLD subtypes and is linked with mortality in fatty liver disease[27,28]. The ample sample size of 322 patients provided sufficient statistical power to the findings. The subclassification of MAFLD patients based on the presence of metabolic conditions permitted a nuanced analysis of the data. Lastly, the real-world clinical setting at Dow University Hospital ensured that the research findings were applicable and relevant to clinical practice, enhancing the external validity and generalizability of the study.

The present study, while contributing valuable insights into the long-term implications of MAFLD subtypes on hepatic fibrosis, is not without its limitations that merit acknowledgment. First, the study's design was observational, precluding any assertions of causality between MAFLD subtypes and the progression of hepatic fibrosis. Second, the reliance on existing clinical datasets limits the scope to fully capture the nuances of patients' longitudinal metabolic changes and their direct impact on liver pathology. Another constraint is the study's dependence on non-invasive markers of hepatic fibrosis, which, while clinically relevant, cannot substitute for the histopathological assessment through liver biopsy or transient elastography, the gold standard for fibrosis evaluation. The use of surrogate endpoints, therefore, necessitates cautious interpretation of the findings. However, Fib-4 and NFS are widely used and endorsed by various guidelines for screening MAFLD patients for advanced fibrosis, and they are superior to other scores like aspartate aminotransferase to platelet ratio index (APRI) and BMI, AST/ALT ratio, and diabetes mellitus (BARD)[29,30]. Even though, they may not be as effective due to limitations by risk factors like age and BMI scores[1]. Lastly, the study's geographic and demographic concentration may restrict the generalizability of the findings across different populations and ethnicities.

These limitations highlight areas for future research, emphasizing the need for prospective studies, using a longitudinal study design, larger sample size with a more diverse demographic distribution, integration of transient elastography with existing non-invasive markers like NFS and Fib-4, and comparative analyses juxtaposing patients with MAFLD against control groups without MD, to delineate the specific contributory pathways leading to fibrosis within the context of MS.

CONCLUSION

This research has rigorously demonstrated that the severity of liver fibrosis in MAFLD patients is influenced by the number and type of metabolic conditions present. Early identification and management of MAFLD, particularly in patients with multiple metabolic conditions, are crucial to prevent liver-related complications.

ARTICLE HIGHLIGHTS

Research background

Metabolic-associated fatty liver disease (MAFLD) is a medical condition characterized by the presence of fatty liver along with overweight/obesity and/or diabetes and/or metabolic dysfunction. However, whether the subtypes of MAFLD based on the metabolic disorder differentially impact on liver fibrosis is not well explicated, especially in the Asian population.

Research motivation

Different subgroups of MAFLD present distinct clinical spectra and risks of advanced liver fibrosis, which can influence their treatment strategies. Metabolic syndrome is related to higher deaths in nonalcoholic fatty liver disease (NAFLD) patients. Moreover, the high fibrotic burden in fatty liver disease is associated with a higher risk of development of hepatocellular carcinoma, liver-related mortality, and cardiovascular disease. Hence, it is worth classifying the MAFLD patients depending on the number of metabolic conditions at the beginning. This helps to stratify patients with MAFLD according to the long-term risk of significant liver fibrosis.

Research objectives

To compare the severity of liver fibrosis among different MAFLD subtypes.

Research methods

This was a cross-sectional investigation carried out at the National Institute of Liver and GI Diseases, located at Dow University Hospital in Karachi, Pakistan. All patients aged between 18 and 65 years, irrespective of gender, who were diagnosed with fatty liver between January and December 2021 were included. Patients with decompensated liver disease, hepatocellular carcinoma, acute hepatitis, acute-on-chronic liver disease, and other concomitant liver disease (chronic active viral, alcohol, autoimmune, or metabolic liver diseases) were excluded. Pregnant or lactating female patients and patients with concomitant systemic diseases such as tuberculosis, autoimmune disorders, and extra-hepatic malignancies were also excluded from the study. MAFLD was defined according to the Asia Pacific Association for the Study of the Liver guidelines, and fibrosis-4 index (Fib-4) and NAFLD fibrosis score (NFS) were used to assess liver fibrosis. Asian cutoffs were used for body mass index to classify the subjects into overweight/obese *vs* lean/normal weight MAFLD groups.

Research results

Out of 322 patients with fatty liver, 273 were classified as having MAFLD. The MAFLD patients were segregated into three categories according to their number of metabolic conditions (*i.e.*, one, two, and three). Out of 273 participants with MAFLD, 110 (40.3%) carried a single metabolic condition, 129 (47.3%) had two metabolic conditions, and 34 (12.5%) had all the three metabolic conditions. The proportion of significant fibrosis increased with the cumulative number of metabolic conditions. For the NFS score, advanced fibrosis was 4.1%, 25.5%, 35.6%, and 44.1% for no MAFLD and MAFLD with 1, 2, and 3 conditions, respectively, while for Fib-4 score, the proportion of advanced fibrosis was 6.1%, 10.9%, 17%, and 26.5% for no MAFLD and MAFLD with 1, 2, and 3 conditions, respectively. Furthermore, MAFLD patients with a single metabolic condition (n = 110, 40.3%) were sub-classified into three categories: Obesity alone (n = 61, 55.5%), lean metabolic dysfunction (MD) (n = 34, 30.9%), and diabetes mellitus (DM) alone (n = 15, 13.6%). When compared among the three subtypes of MAFLD, the proportion of advanced liver fibrosis was significantly higher among diabetic MAFLD patients according to the NFS score (46.6% *vs* 26.5% for MD alone and 19.7% for obesity alone), whereas patients with lean MD had the highest proportion of advanced fibrosis according to the Fib-4 score (14.7% *vs* 9.8% for obesity alone *vs* 6.7% for DM alone).

Research conclusions

The increased number of metabolic conditions increases the likelihood of fibrosis in patients with MAFLD. The severity of liver fibrosis varies among different subtypes of MAFLD. Patients with diabetes and MAFLD have the highest risk of developing fibrosis.

Research perspectives

The direction of future research in this area involves several key questions that need to be addressed. Investigating the specific diagnostic markers for different subgroups within MAFLD, such as those with obesity, lean individuals, and those with type 2 diabetes. Further exploration is needed regarding the pathogenesis of MAFLD/metabolic dysfunction-associated steatohepatitis (MASH). By conducting thorough investigations into these areas, researchers can gain a better understanding of the complexities surrounding non-alcoholic fatty liver disease and its associated MD. Future research should focus on identifying effective pharmacotherapeutic interventions for MAFLD/MASH, as there is currently no approved treatment for this condition.

FOOTNOTES

Author contributions: Shaikh SS designed the research study; Shaikh SS and Nafay S participated in the data acquisition; Shaikh SS and Zaheer S participated in the analysis and interpretation of the data; Shaikh SS and Qazi-Arisar FA drafted the initial manuscript; Qazi-Arisar FA, Shaikh H, and Azam Z revised the article critically for important intellectual content.

Institutional review board statement: The study was reviewed and approved by the Dow University of Health Sciences Institutional Review Board (IRB-1842).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: There are no conflicts of interest to report.

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Subcellular distribution of prohibitin 1 in rat liver during liver regeneration and its cellular implication

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Abstract

BACKGROUND

The function of prohibitin 1 (Phb1) during liver regeneration (LR) remains relatively unexplored. Our previous research identified downregulation of Phb1 in rat liver mitochondria 24 h after 70% partial hepatectomy (PHx), as determined by subcellular proteomic analysis.

AIM

To investigate the potential role of Phb1 during LR.

METHODS

We examined changes in Phb1 mRNA and protein levels, subcellular distribution, and abundance in rat liver during LR following 70% PHx. We also evaluated mitochondrial changes and apoptosis using electron microscopy and flow cytometry. RNA-interference-mediated knockdown of Phb1 (PHBi) was performed in BRL-3A cells.

RESULTS

Compared with sham-operation control groups, Phb1 mRNA and protein levels in 70% PHx test groups were downregulated at 24 h, then upregulated at 72 and 168 h. Phb1 was mainly located in mitochondria, showed a reduced abundance at 24 h, significantly increased at 72 h, and almost recovered to normal at 168 h. Phb1 was also present in nuclei, with continuous increase in abundance observed 72 and 168 h after 70% PHx. The altered ultrastructure and reduced mass of mitochondria during LR had almost completely recovered to normal at 168 h. PHBi in BRL-3A cells resulted in increased S-phase entry, a higher number of apoptotic cells, and disruption of mitochondrial membrane potential.

CONCLUSION

Phb1 may contribute to maintaining mitochondrial stability and could play a role in regulating cell proliferation and apoptosis of rat liver cells during LR.

Key Words: Prohibitin 1; Liver regeneration; Subcellular proteomic analysis; Mitochondrial stability; Cell proliferation

Core tip: Using subcellular proteomic analysis, we previously found that prohibitin 1 (Phb1) was downregulated in rat liver mitochondria at 24 h after 70% partial hepatectomy (PHx). Phb1 has various functions, but little is known about its role during liver regeneration (LR). To explore the function of Phb1 in mitochondria during LR, we investigated the changes of Phb1 expression, the alterations of mitochondrial mass and ultrastructure, and the subcellular distribution of Phb1 at 24, 72 and 168 h in rat liver after 70% PHx. Using RNA-interference-mediated knockdown of Phb1, the potential functions of Phb1 were analyzed. Phb1 was differentially expressed during LR.

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INTRODUCTION

It is well known that the liver has the capacity to regenerate and restore its original size and function after 70% partial hepatectomy (PHx), or injury[1,2]. It would be important clinically to develop therapeutic strategies to enhance liver regeneration (LR) or support the liver in its attempt to restore its functional integrity under pathophysiological circumstances[3,4]. However, the complexity of the regulatory mechanisms of LR, together with our limited understanding of the functional priorities of the hepatocytes have rendered difficult the identification of targets for therapeutic interventions.

As the hub of energy metabolism, mitochondria have been investigated due to their direct involvement in the process of LR[5]. In an attempt to identify mitochondrial proteins that are correlated with the early phase of LR, using subcellular proteomic analysis in our recent study, our recent study revealed that Prohibitin 1 (Phb1), a potential tumor suppressor protein, was downregulated in rat liver mitochondria at 24 h after 70% PHx[6].

Phb1 is a ubiquitously expressed highly conserved protein among eukaryotes. Previous research has proposed that Phb1 is involved in many cellular processes, such as cell cycle regulation, senescence, transcription regulation, tumor suppression and apoptosis[7-11]. Phb1 is reported to mainly localize in mitochondria, with its expression upregulated by mitochondrial stress and downregulated during cellular senescence[12]. Therefore, Phb1 is thought to have a crucial role in mitochondria function. One study identified a novel function of Phb1 in the maintenance of mitochondrial DNA (mtDNA). In Phb1-knockdown cells, the status of mtDNA is altered in several ways[13]. Despite such information, our understanding of the overall functions of Phb1 in mitochondria remains incomplete and its potential role during LR is largely unexplored. LR is a complicated biological procedure involving various signal transduction pathways and molecular events[14,15]. Thus, we hypothesized that Phb1 could play a crucial role during LR. This study aimed to investigate the function of Phb1 in mitochondria during changes in Phb1 expression, mitochondrial mass and ultrastructure, and the subcellular distribution of Phb1 at 24, 72 and 168 h post 70% PHx in rat liver. Using RNA-interference-mediated knockdown of Phb1 (PHBi), we also analyzed the potential functions of Phb1. Our results revealed differential expression of Phb1 during LR, with its primary localization in mitochondria, where its altered expression may be associated with the recovery of mitochondrial mass and ultrastructure. Phb1 was also present in the nuclei, with increased abundance during LR. PHBi in BRL-3A cells, a widely used cell line in liver research, led to increased S-phase entry and apoptotic cell count. We also observed disruption of mitochondrial membrane potential following Phb1 knockdown in BRL-3A cells, mirroring our previous findings. Collectively, these results suggest that Phb1 may contribute to maintaining mitochondrial stability and regulating the cell cycle and apoptosis during LR.

MATERIALS AND METHODS

Animals and surgery

Adult male Sprague–Dawley rats (220–250 g) were obtained from the Experimental Animal House at Second Military Medical University (Shanghai, China). The rats were randomly divided into two groups: Five served as the sham-operation control group and the other five comprised the 70% PHx test group. PHx (~70%) was performed according to the method of Higgins *et al*[16]. The experimental rats were anesthetized by intraperitoneal injection of 2% pentobarbital (40 mg/kg). In the test group, the median and left lateral lobes were removed without injuring the remaining liver tissue.

The control group underwent a sham operation identical to the test group procedure, but without liver removal. After surgery, the rats were kept on a standard diet until they were killed by cervical dislocation under anesthesia.

Electron microscopy

Liver specimens were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 h at 4C. After fixation, they were washed overnight in sodium cacodylate buffer at 4C. The specimens were then postfixed with 1% osmium tetroxide in sodium cacodylate buffer for 1 h at 4C, dehydrated in alcohol, embedded in araldite resin, and semithin sections were removed for optical microscopy. Ultrathin sections were mounted on copper mesh grids and stained with uranyl acetate and lead citrate as described[17] before examination with a Hitachi H-800 electron microscope.

Separation of rat liver subcellular fractions and protein preparation

Rat livers were removed and treated as previously reported[18] for isolation of nuclei, cytosol and mitochondria. Livers were collected and homogenized. Subsequent centrifugation at increasingly higher speeds at 4C yielded the following fractions: Nuclear fraction at 1000 *g* for 10 min; mitochondrial fraction at 15 000 *g* for 15 min; and microsomes at 144 000 *g* for 90 min. The final supernatant was the cytosolic fraction. Purification of mitochondria was performed by Nycodenz density gradient purification[19]. The mitochondrial pellets obtained from differential centrifugation were suspended in 12 mL 25% Nycodenz and placed onto a discontinuous Nycodenz gradient consisting of 5 mL 34% Nycodenz and 8 mL 30% Nycodenz, followed by 8 mL 23% Nycodenz, and finally, 3 mL 20% Nycodenz. The sealed tubes were centrifuged for 90 min at 52 000 *g* at 4C. The mitochondria were in the band at the 25%/30% interface which was collected and diluted with the same volume of homogenization buffer and then centrifuged twice at 15 000 *g* for 20 min. The preparation of each subcellular fraction protein of rat livers was performed as previously described[19]. Protein concentration of each fraction was determined with a Quick Start Bradford Assay Kit (Bio-Rad).

Western blotting

Protein extracts of each sample were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The blots were probed by anti-Phb1 antibody (Neomarker) and proteins were normalized with anti--actin antibody (Neomarker) or anti-COX IV antibody (Cell Signaling) or anti-histone H3 antibody (Cell Signaling) and were visualized by Amersham ECL system. The digital image was obtained by scanning the membrane, and then subjected to gray value analysis. For a better understanding of western blotting results and derived ratio changes, a detailed methodology introduction can be found in the subsequent figures and legends.

Cell culture

The normal rat liver cell line BRL-3A was obtained from the Shanghai Institute of Biochemistry and Cell Biology. The BRL-3A cells were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 mg /mL streptomycin. The cells were maintained at 37C in an atmosphere with 5% CO_2.

RNA interference

Duplex siRNA was obtained from GeneChem (Shanghai, China). The siRNA sequence targeting rat Phb1 was 5′-GCCAGAUUUGUGGUGGAAAtt-3′ (sense) and 5′-UUUCCACCACAAAUCUGGCtt-3′ (antisense). A nonsense duplex was used as the control (mock). BRL-3A cells were plated on six-well plates with antibiotic-free DMEM overnight and transfected with siRNA by Lipofectamine2000 (Invitrogen). The final concentration of siRNA duplex was 100 nM. Six hours after transfection, the medium was switched to DMEM supplemented with antibiotics.

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR

Total RNA of each sample was isolated by TRIzol reagent (Invitrogen). After treatment with DNase I, each RNA sample was reverse-transcribed with random primers (dN6) (MBI Fermantas, Vilnius, Lithuania). The single-stranded cDNA was used in quantitative real-time PCR to evaluate the relative expression levels of Phb1 (5′-GCGGTGGAAGCCAAACAG-3′ and 5′-TTCTTCTGCTGCTCAGCCTTT-3′), compared to -actin (5′-ATGGTGGGTATGGGTCAGAAG-3′ and 5′-TGGCTGGGGTGTTGAAGGTC-3′) used as an internal control for determining cell number and metabolic status. Quantitative real-time PCR (ABI7300, Applied Biosystems) was done with the SYBR Green I reagents (TOYOBO) and the primers were designed according to the ABI manufacturer's protocol. Forty cycles of PCR were performed with cycling conditions of 15 s at 95C and 60 s at 60C. The real-time PCR signals were analyzed with LightCycler 3.5 software (Roche Diagnostics).

Flow cytometry

Cells were stained with propidium iodide (PI; BD Clontech) as previously described[20]. A suspension of 10^4 cells was analyzed for each DNA histogram, and from the analysis of DNA histograms, the percentages of cells in different phases of cell cycle were evaluated. Flow cytometry was performed on a FACSCalibur and analyzed using CellQuest software (BD Bioscience). The Annexin V/PI method was used to quantify numbers of apoptotic cells. Cells were washed twice with phosphate-buffered saline and stained with Annexin V and PI for 20 min at room temperature. The level of apoptosis was determined by measuring the fluorescence of the cells by flow cytometry analysis.

Statistical analysis

The data presented are the means ± SD of three independent experiments. Statistical significance was estimated with Student's *t*-test for unpaired observations. $P < 0.05$ was considered significant.

RESULTS

Altered expression of Phb1 mRNA and protein abundance during LR

Phb1 mRNA expression was examined by real-time PCR. Compared with the control group, expression of Phb1 mRNA in the 70% PHx test group was 0.46-fold decreased at 24 h, and 1.54-fold and 1.89-fold increased at 72 and 168 h, respectively (Figure 1A). Western blotting showed that Phb1 protein expression during LR was 0.54-fold decreased at 24 h, and 1.48-fold and 1.73-fold increased at 72 and 168 h, respectively, after 70% PHx (Figure 1B), which was consistent with the expression of Phb1 mRNA.

Subcellular distribution and protein abundance alteration in each subcellular fraction of Phb1 during LR

Previous observations suggest that examining the subcellular distribution of Phb1 might yield important information about physiological or pathological processes that are taking place in cells. To verify the cellular distribution pattern of Phb1 during LR, we fractionated cytosolic, mitochondrial and nuclear fractions of rat liver cells and performed western blotting analysis. The purity of subcellular fractionation was controlled by several marker proteins (Figure 2A). Phb1 was mainly located in mitochondria and its abundance was reduced 0.47-fold at 24 h, and induced 1.47-fold at 72 h and almost recovered to normal at 168 h after 70% PHx (Figure 2B). Phb1 was also located in nuclei and its abundance was increased during LR after 70% PHx (Figure 2C). No Phb1 was found in the cytosol.

Alterations of mitochondria during LR

Mitochondrial mass and ultrastructural alterations during LR were observed to determine whether Phb1 changes were associated with mitochondrial stabilization or biogenesis. The mitochondrial mass was quantified by examining the protein contents of mitochondrial fractions[21] extracted from equivalent weights (1 g) of liver tissues from each experimental group. The results indicated an increase in mitochondrial protein contents during LR with 5.37 ± 0.08, 6.38 ± 0.10 and 7.16 ± 0.16 mg at 24 , 72 and 168 h respectively, after 70% PHx. The mitochondrial protein contents at 168 h in the 70% PHx test group closely mirrored that of the control group (Figure 3A).

Mitochondrial ultrastructural alterations during LR were observed by electron microscopy. The mitochondrial morphologies of control livers (Figure 3B upper panel) (SH 24 h, SH 72 h, SH 168 h), were characterized by a consistent basic architecture featuring a folded internal membrane and a dense matrix. The alterations in mitochondrial ultrastructure following 70% PHx are showed in Figure 3B bottom panel. At 24 h after 70% PHx (PH 24 h), the mitochondria displayed significant swelling, reduction in the number of cristae, dilated and pale matrix, absence of dense granules, and clear matrix compartment vacuolization. At 72 h after 70% PHx (PH 72 h), only slight changes with moderate distension were seen in mitochondrial morphology. By 168 h after 70% PHx (PH 168 h), the mitochondria had mostly returned to their normal morphology, and were rich in cristae, with an electron-dense matrix. These alterations in mitochondrial ultrastructure have been associated with changed in mitochondrial function during LR[17].

PHBi leads to an increase in the number of apoptotic cells

To downregulate Phb1 cellular expression, PHBi was performed in BRL-3A cells. PHBi resulted in a dramatic reduction in both Phb1 mRNA and protein level compared with that of the control group (mock). Detailed results are available in our previous publication[4].

Previous reports have suggested that Phb1 could serve an antiapoptotic role in undifferentiated granulosa cells[22]. In this study, to evaluate whether Phb1 was involved in modulating apoptosis in rat liver cells, flow cytometry was used to evaluate percentage of apoptotic cells by Annexin V/PI staining. Phb1 knockdown cells displayed a 1.56-fold increase in the percentage of apoptotic cells compared with controls (Figure 4A).

PHBi leads to increased S-phase entry

We investigated whether the decrease of Phb1 by PHBi had any effect on cell growth and proliferation. The cell cycle distribution in Phb1 knockdown cells showed a 1.26-fold increase in the S-phase compared to control cells (Figure 4B). Although the increase in the S-phase was not dramatic, the difference was significant. Nuell *et al*[23] also previously reported a cell cycle modulatory role of Phb1, indicating that Phb1 could function as a negative cell cycle regulator.

DISCUSSION

Phb1, a potential tumor suppressor protein, was initially cloned due to its ability to induce G1/S phase arrest. Phb1 is proposed to be involved in numerous cellular processes. However, most studies to date have focused on the role of Phb1 in various types of tumors, with its role during LR remaining largely unexplored. In recent years, some studies have explored the role of Phb1 in liver injury and liver cancer[10,24-27]. However, the role of Phb1 in LR remained unstudied.

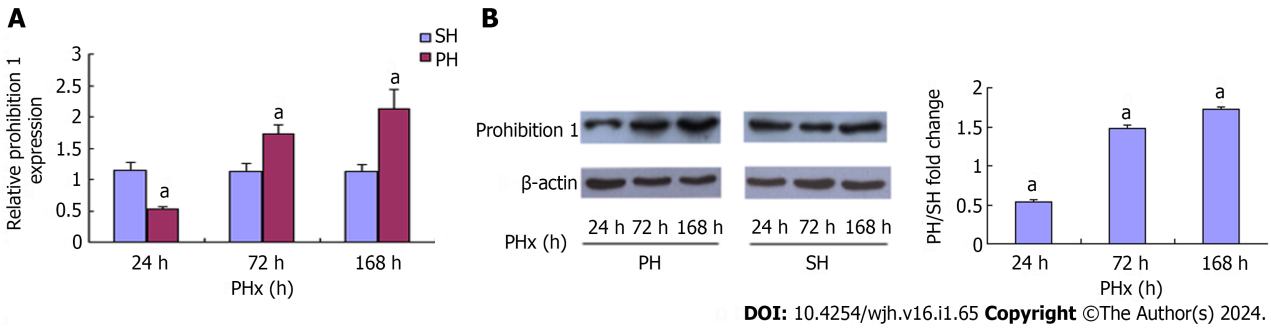

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Figure 1 Altered expression of prohibitin 1 mRNA and protein during liver regeneration. A: Altered expression of prohibitin 1 (Phb1) mRNA during liver regeneration (LR). Rat livers were homogenized and harvested with TRIzol reagent. Real-time polymerase chain reaction (PCR) amplifications were performed, standardized by the amounts of β-actin. The real-time PCR signals were analyzed with LightCycler 3.5 software; B: Altered expression of Phb1 protein during LR. Equal amounts of protein from each sample were separated on 12% SDS-PAGE and immunoblotted with antibodies against Phb1 and control protein, β-actin. On the left are the western blotting results, and on the right is a gray analysis of the results showing the fold changes of Phb1 expression in PH groups in comparison with SH groups, standardized by the amounts of β-actin. All grouping of gels/blots were cropped from different parts of the same gel. PH: 70% PHx test groups; PHx: Partial hepatectomy; SH: Sham-operation control groups;. Results are from three independent experiments and data are represented as means ± SD. $^{a}P < 0.05$, *versus* control group, significant difference.

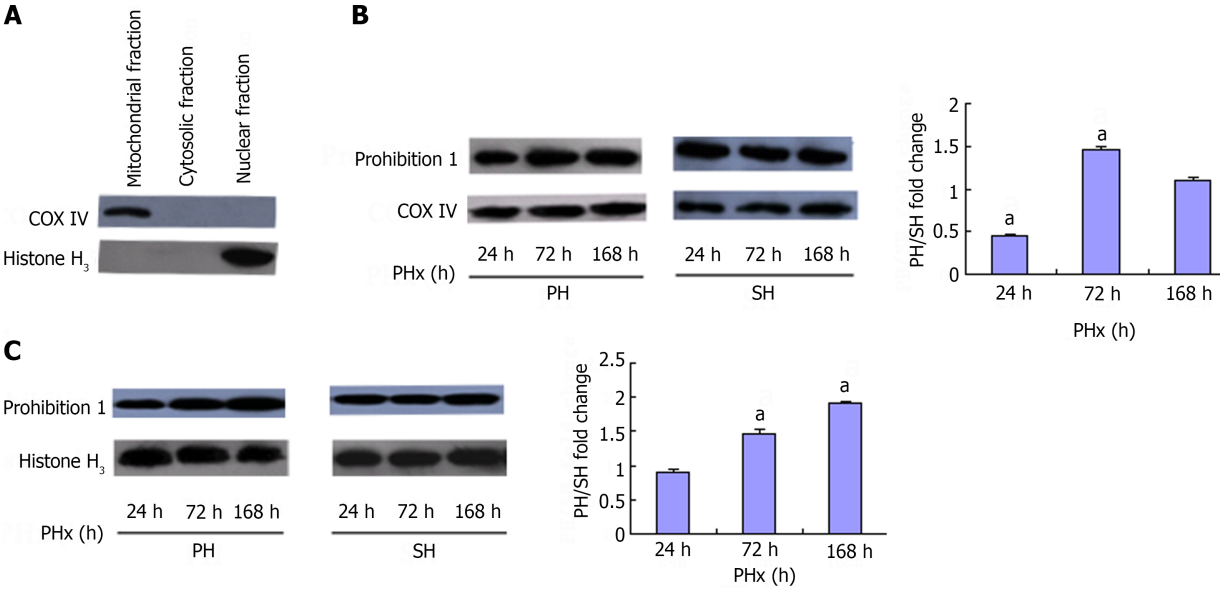

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Figure 2 Subcellular distribution and protein abundance alterations of prohibitin 1 during liver regeneration. Equal amounts of protein extracts obtained from each subcellular fraction were separated on 12% SDS-PAGE and immunoblotted with antibodies against prohibitin 1 (Phb1), cyclooxygenase IV (COX IV) and histone H3. A: Control western blotting for subcellular fractionation (COX IV for mitochondria and Histone H3 for nuclei). The grouping of gels/blots were cropped from different parts of different gels; B: Changes in Phb1 protein abundance in mitochondria. On the left are the western blotting results, and on the right is a gray analysis of the results showing the fold changes of Phb1 expression in mitochondria in PH groups in comparison with SH groups, standardized by the amounts of COX IV. The grouping of gels/blots in PH groups were cropped from different parts of the same gel. The grouping of gels/blots in SH groups were cropped from different parts of the same gel; C: Changes of Phb1 protein abundance in nucleus. On the left are the Western blotting results, and on the right is a gray analysis of the results showing the fold changes of Phb1 expression in nucleus in PH groups in comparison with SH groups, standardized by the amounts of Histone H3. The grouping of gels/blots for Phb1 were cropped from different parts of the same gel. The grouping of gels/blots for histone H3 were cropped from different parts of the same gel. PH: 70% PHx test groups; PHx: Partial hepatectomy; SH: Sham-operation control groups. Results are from three independent experiments and data are represented as means ± SD. $^{a}P < 0.05$, *versus* control group, significant difference.

In this study, Phb1 mRNA and protein expression underwent concordant changes during LR after 70% PHx. Compared to sham-operation control groups, 70% PHx test groups showed downregulation of Phb1 mRNA and protein expression at 24 h, and upregulation at 72 and 168 h (Figure 1). A previous study found that the gene encoding Phb1 might have additional antiproliferative effects that do not require translation[11]. Manjeshwar *et al*[28] reported that the 3′ untranslated region of the *Phb1* gene encoded a functional RNA that arrested cell-cycle proliferation between the G1 and S phases. In light of previous reports, we propose that Phb1 might regulate cell proliferation during LR in a complex manner, potentially involving mechanisms mediated by both Phb1 mRNA and protein.

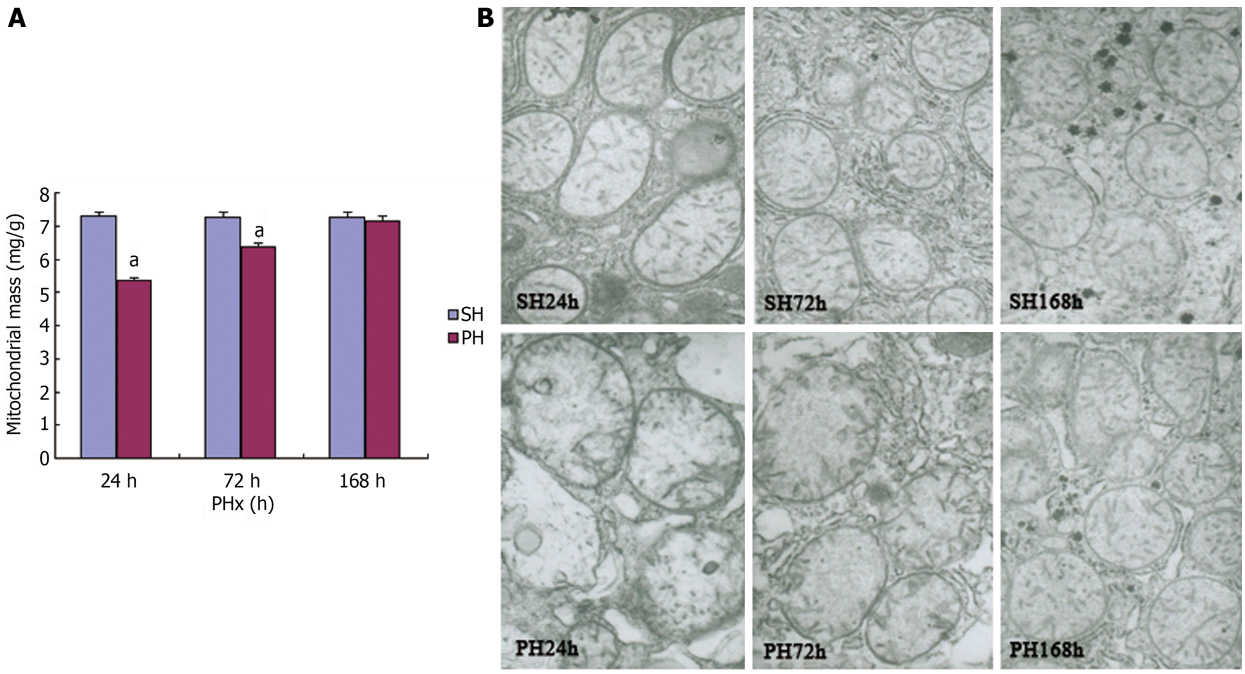

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Figure 3 Mitochondrial mass and ultrastructural alterations during liver regeneration. A: Changes of mitochondrial mass during liver regeneration (LR). Mitochondrial mass was measured by examining the protein contents of mitochondrial fractions extracted from the same weight (1 g) liver tissues of each experimental group; B: Electron micrographs of mitochondria in liver tissues during LR. Three maps in the upper panel are detail of hepatocytes in control groups, showing normal mitochondria. The other three maps in the bottom panel are detail of hepatocytes after 70% PHx, showing altered mitochondria during LR. PH: 70% PHx test groups; PHx: Partial hepatectomy; SH: Sham-operation control groups; PH: 70% PHx test groups. Ten randomly selected electron micrographs of the same magnification (15 000×) were examined from one hepatic lobule of five rats for each experimental group.

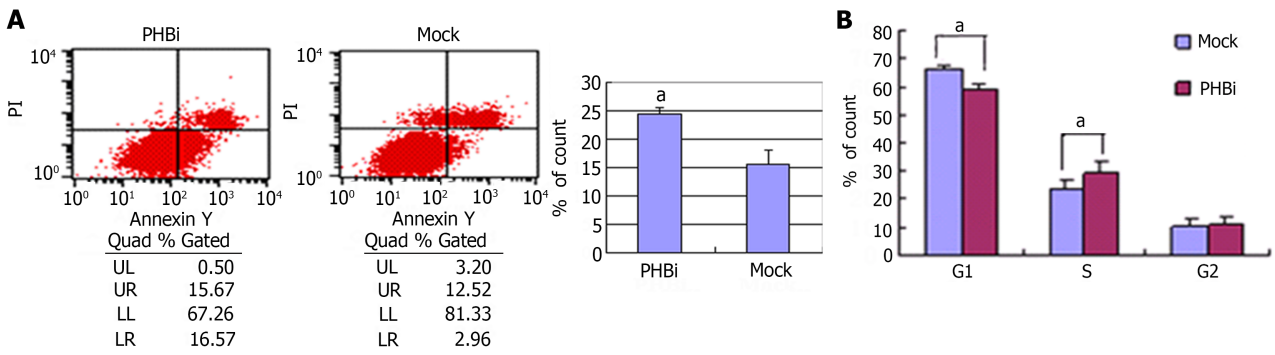

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Figure 4 RNA-interference-mediated knockdown of Phb1 induced an increase in the number of apoptotic cells and increased S-phase entry. A: An increase in the number of apoptotic cells in RNA-interference-mediated knockdown of Phb1 (PHBi) cells. At 72 h post-transfection, stained with Annexin V and propidium iodide (PI), PHBi cells showed a significant increase in the percentage of Annexin V/PI positive cells compared with controls (cells transfected with nonsensing duplex); B: Increased S-phase entry in PHBi cells. At 48 h post-transfection, FACS analysis was performed with PHBi and mock (cells transfected with nonsensing duplex, used as control). Results are from three independent experiments and data are represented as means ± SD. $^{a}P < 0.05$, *veruss* mock, significant difference. UL: Upper left; UR: Upper right; LL: Lower left; LR: Lower right; G1: G1 phase; S: S phase; G2: G2 phase.

The well-characterized function of Phb1 is as a chaperone involved in the stabilization of mitochondrial proteins. Mitochondrial-localized Phb1 is confirmed as a high-molecular-weight hetero-complex (ring-shaped structure) by single-particle structures[7]. The interaction of no assembled respiratory chain subunits with the Phb1 complex has led to the proposal of a chaperone activity of Phb1 during the biogenesis of the respiratory chain[29]. Recently, Phb1 was reported to be essential for normal mitochondrial development, and Phb1 deficiency was showed to be associated with deficient mitochondrial biogenesis[30]. PHBi showed enhanced sensitivity to anthralin-induced cell death due to enhanced loss of mitochondrial membrane potential in psoriatic lesions[31]. Mitochondria are the center of energy metabolism and play a crucial role in regulating cell life. Various stimuli can induce dysfunction and structural injury in mitochondria, which triggers a series of cellular events ultimately leading to apoptosis or necrosis. We found that Phb1 was mainly located in the mitochondria in rat liver, and its abundance underwent a 0.47-fold reduction at 24 h, a 1.47-fold induction at 72 h, and nearly recovered to normal level at 168 h after 70% PHx (Figure 2B). Mitochondria showed significant changes in the

ultrastructure at 24 h, and nearly recovered to normal at 168 h after 70% PHx (Figure 3B). The reduced mitochondrial mass also nearly recovered to normal at 168 h after 70% PHx. Mitochondrial membrane potential is an important parameter of mitochondrial function. In our previous study, we found that knockdown of Phb1 in BRL-3A cells resulted in disruption of mitochondrial membrane potential, implying a potential role of Phb1 in maintaining mitochondrial integrity[6]. Ross *et al*[32] also reported that siRNA-mediated knockdown of Phb1 in Kit225 cells resulted in disruption of mitochondrial membrane potential and Phb1 proteins were novel phosphoproteins upregulated during T-cell activation that function to maintain mitochondrial integrity. In this study, using PHBi, we also observed that Phb1 knockdown cells exhibited a 1.56-fold increase in the number of apoptotic cells (Figure 4A). Although these results provide evidence for a functional role of Phb1 in suppressing apoptosis in rat liver cells, the involved molecular mechanisms remain unknown. It is likely that the mechanism by which knockdown of Phb1 results in apoptosis targets the mitochondria in agreement with previous findings[22]. All these results suggest that Phb1 has a role in regulating stabilization of mitochondria during LR, which might affect mitochondrial function.

Although it has been reported that Phb1 is primarily located in mitochondria[12,30,33,34], other studies have reported that Phb1 is also located in the nuclei[35,36]. We found that Phb1 was located in nuclei as well as mitochondria in rat liver and its abundance increased during LR (Figure 2B). Previous studies reported that Phb1 was present in the nuclei and interacted with transcription factors important in cell-cycle progression[35,36]. In this study, using PHBi, we observed that Phb1 knockdown cells showed an increase in S-phase entry (Figure 4B). The involvement of Phb1 in the cell cycle was also observed in a prostate cancer cell line, in which downregulation of Phb1 led to an increase in cell-cycle entry from G1 to S phase[30]. Although most data suggest that Phb1 has an antiproliferative effect by interacting with the p53 and pRb pathways in the nuclei[9,37], it appears that Phb1 can also have antiapoptotic effects. In osteosarcoma cells, *Phb1* was identified as a gene with downregulated expression in response to cytotoxic drugs, and the transient overexpression of the Phb1 coding sequence significantly reduced cytotoxic drug-induced apoptosis in these cells[38]. In this study, we also observed that Phb1 knockdown cells showed an increase in the number of apoptotic cells (Figure 4A). It has been reported that the subcellular localization of Phb1 may depend on the cell type examined and its physiological status, and Phb1 might have distinct but overlapping functions in each of these cellular compartments[39]. Although there is controversy concerning the function of nuclear-localized Phb1, in combination with previous reports, we suggest that the upregulated Phb1 in the nuclei in rat liver cells might have a function, at least in part, in regulating cell-cycle progression of rat liver cells. It might regulate the balance between proliferation and apoptosis during LR after 70% PHx, but this needs further investigation.

CONCLUSION

In summary, our results demonstrate that Phb1 plays two roles in the LR process: one is to regulate cell cycle and apoptosis, and the other is to regulate and maintain mitochondrial stability. Whether the two effects are directly linked or show two different effects remains unclear. Further in-depth studies will aid in us better understanding the complexities and roles of Phb1 in the LR process.

ARTICLE HIGHLIGHTS

Research background

It is clinically important to develop therapeutic strategies to enhance liver regeneration (LR) or support the liver in its attempt to restore its functional integrity under pathophysiological circumstances. However, the complexity of the regulatory mechanisms of LR, together with our limited understanding of the functional priorities of the hepatocytes have rendered difficult the identification of targets for therapeutic interventions.

Research motivation

Prohibitin 1 (Phb1) is a ubiquitously expressed highly conserved protein among eukaryotes. Previous research has proposed that Phb1 was involved in many cellular processes. Phb1 was reported to mainly localize in mitochondria, with its expression upregulated by mitochondrial stress and downregulated during cellular senescence. Therefore, Phb1 is thought to have a crucial role in mitochondrial function. One study identified a novel function of Phb1 in the maintenance of mitochondrial DNA (mtDNA). In Phb1-knockdown cells, the status of mtDNA is altered in several ways. Despite such information, our understanding of the overall functions of Phb1 in mitochondria remains incomplete and its potential role during LR is largely unexplored. LR is a very complicated biological procedure involving various signal transduction pathways and molecular events. Thus, we hypothesized that Phb1 could play a crucial role during LR.

Research objectives

This study aimed to further investigate the function of Phb1 in mitochondria during changes in Phb1 expression, mitochondrial mass and ultrastructure, and the subcellular distribution of Phb1 at 24, 72 and 168 h post 70% partial hepatectomy (PHx) in rat liver. Using RNA-interference-mediated knockdown of Phb1 (PHBi), we also analyzed the potential functions of Phb1.

Research methods
We examined changes in Phb1 mRNA and protein levels, subcellular distribution, and abundance in rat liver during LR following 70% PHx. We also evaluated mitochondrial changes and apoptosis levels using electron microscopy and flow cytometry. PHBi was performed in BRL-3A cells.

Research results
Compared with sham-operation control groups, Phb1 mRNA and protein levels in 70% PHx test groups were downregulated at 24 h, then upregulated at 72 and 168 h. Phb1 was mainly located in mitochondria, showed a reduced abundance at 24 h, significantly increased at 72 h, and almost recovered to normal at 168 h. Phb1 was also present in nuclei, with continuous increase in abundance observed at 72 and 168 h after 70% PHx. The altered ultrastructure and reduced mass of mitochondria during LR had almost completely recovered to normal at 168 h. PHBi in BRL-3A cells resulted in increased S-phase entry, a higher number of apoptotic cells, and disruption of mitochondrial membrane potential.

Research conclusions
In summary, our results demonstrate that Phb1 plays two roles in the LR process: one is to regulate cell cycle and apoptosis, and the other is to regulate and maintain mitochondrial stability.

Research perspectives
Whether the two effects are directly linked or show two different effects remains unclear. Further in-depth studies will aid in us better understanding the complexities and roles of Phb1 in the LR process.

FOOTNOTES

Author contributions: Sun QJ designed the research study; Sun QJ and Liu T performed the research; Sun QJ and Liu T analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Rifaximin on epigenetics and autophagy in animal model of hepatocellular carcinoma secondary to metabolic-dysfunction associated steatotic liver disease

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Abstract

BACKGROUND

Prevalence of hepatocellular carcinoma (HCC) is increasing, especially in patients with metabolic dysfunction-associated steatotic liver disease (MASLD).

AIM

To investigate rifaximin (RIF) effects on epigenetic/autophagy markers in animals.

METHODS

Adult Sprague-Dawley rats were randomly assigned (n = 8, each) and treated from 5-16 wk: Control [standard diet, water plus gavage with vehicle (Veh)], HCC [high-fat choline deficient diet (HFCD), diethylnitrosamine (DEN) in drinking water and Veh gavage], and RIF [HFCD, DEN and RIF (50 mg/kg/d) gavage]. Gene expression of epigenetic/autophagy markers and circulating miRNAs were obtained.

RESULTS

All HCC and RIF animals developed metabolic-dysfunction associated steatohepatitis fibrosis, and cirrhosis, but three RIF-group did not develop HCC. Comparing animals who developed HCC with those who did not, miR-122, miR-34a, tubulin alpha-1c *(Tuba-1c)*, metalloproteinases-2 *(Mmp2)*, and metalloproteinases-9 *(Mmp9)* were significantly higher in the HCC-group. The opposite occurred with *Becn1*, coactivator associated arginine methyl-transferase-1 (*Carm1*), enhancer of zeste homolog-2 (*Ezh2*), autophagy-related factor LC3A/B *(Map1 Lc3b)*, and *p62/* sequestosome-1 (*p62*/SQSTM1)-protein. Comparing with controls, *Map1 Lc3b*, *Becn1* and *Ezh2* were lower in HCC and RIF-groups (P < 0.05). *Carm1* was lower in HCC compared to RIF (P < 0.05). Hepatic expression of *Mmp9* was higher in HCC in relation to the control; the opposite was observed for *p62/Sqstm1* (P < 0.05). Expression of p62/SQSTM1 protein was lower in the RIF-group compared to the control (P = 0.024). There was no difference among groups for *Tuba-1c*, Aldolase-B, alpha-fetoprotein, and *Mmp2* (P > 0.05). miR-122 was higher in HCC, and miR-34a in RIF compared to controls (P < 0.05). miR-26b was lower in HCC compared to RIF, and the inverse was observed for miR-224 (P < 0.05). There was no difference among groups regarding miR-33a, miR-143, miR-155, miR-375 and miR-21 (P > 0.05).

CONCLUSION

RIF might have a possible beneficial effect on preventing/delaying liver carcinogenesis through epigenetic modulation in a rat model of MASLD-HCC.

Key Words: Animal model; Autophagy; Epigenetic; Hepatocellular carcinoma; Metabolic dysfunction-associated steatotic liver disease; Rifaximin

Core Tip: Managing metabolic dysfunction-associated steatotic liver disease (MASLD)-hepatocellular carcinoma (HCC) is a clinical challenge, with many unanswered questions, as autophagy and epigenetics appear to contribute to drug resistance. Additionally, the broad-spectrum oral antibiotic Rifaximin influences inflammation, energy metabolism, and fat storage. Utilizing animal models for MASLD-HCC is crucial in understanding pathophysiological mechanisms and potential therapeutic targets. Furthermore, Rifaximin may have a beneficial effect in rats by possibly preventing or delaying hepatic carcinogenesis through epigenetic modulation.

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INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) comprises a spectrum of histological abnormalities, ranging from isolated steatosis to steatohepatitis, characterized by inflammation, necrosis, and hepatocellular ballooning, and progression to fibrosis, cirrhosis, liver failure and/or hepatocellular carcinoma (HCC)[1,2]. Along with diet and sedentary lifestyle, many other factors often determine the progression of MASLD and the development of MASLD-associated carcinogenesis[3]. Although only 2.4% to 12.4% of cirrhotic MASLD patients develop HCC, it is expected that it will become the leading HCC cause by 2030[4,5].

Several MASLD-associated oncogenesis mechanisms, such as structural genomic defects, epigenetic alterations and autophagy promote significant changes in regulatory and signaling pathways, creating a favorable hepatic microenvironment for lesion progression and HCC development, which can occur with or without cirrhosis[6-8]. Among the epigenetic mechanisms, microRNAS act on the expression or suppression of genes responsible for the worsening of liver damage, that is, they have the potential to be used as new biomarkers for early diagnosis and/or therapeutic targets to HCC[8-10]. Autophagy is recognized for playing a beneficial role in the initial liver injury by contributing to the removal of protein aggregates, damaged organelles, and lipid droplets, preventing the formation of pre-tumor cells[6,7]. However, during tumor promotion, autophagy predominantly acts on the adaptive mechanism, contributing to tumor maintenance and growth through the supply of energy substrates and metabolic adaptation, which improves their survival ability in hypoxic and low-nutrient environments, promoting tumor progression[6,11]. Autophagy tends to play a complicit role in HCC treatment resistance, the interface between these two processes being multifactorial and therefore crosstalk can occur in different target proteins. In this sense, therapies for the epigenetic control of autophagy are promising targets for treatment of liver injury[6,11].

Currently, there is no approved pharmacological therapy for steatohepatitis, and several studies are being conducted with different targets. Dietary management, such as flavonoids, has shown beneficial effects on the epigenetic mechanisms of MASLD-HCC[12]. On the other hand, although microbiota plays an important role in MASLD pathogenesis, there is no definite evidence of a positive effect of its modulation in human steatohepatitis[13,14]. Rifaximin (RIF) is a minimally absorbed, broad-spectrum oral antibiotic that have a positive modulation on components of gut microbiota, improving endotoxemia, exerting an influence on inflammation, energy metabolism and fat storage[14-16]. Therefore, this study was designed to assess the effect of RIF treatment on the underlying pathophysiological mechanisms associated with epigenetic changes and autophagy in MASLD associated hepatocarcinogenesis.

MATERIALS AND METHODS

Animals and ethical procedures

Twenty-four adult (60-d-old) male Sprague Dawley rats weighing 250–400 g was included for this study. The animals were group-housed in polypropylene cages with sawdust-covered floors. Rats were maintained on a standard 12-h light/dark cycle, in a temperature-controlled environment (22 ± 2 °C). The Institutional Ethics Committee approved all experiments and procedures for the Use of Animals, No. 2021-0105. The procedures for scientific animal's use were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed, 2011) and law No. 11.794 (Brazil, 2008).

Study design

After acclimatization to the environment, the animals were randomized by weight into a control group (n = 8) that received a standard diet, water free of diethylnitrosamine (DEN, catalog number N0756, Sigma-Aldrich, United States) and gavage with vehicle (Veh) solution from the 5th week of the experiment until the 16th week; HCC-group (n = 8) that received a high-fat and choline-deficient diet (HFCD, catalog number RH19576, Rhoster, Brazil), 135 mg/L DEN in drinking water and gavage with Veh solution, during the same period previously described; and the RIF-group (n = 8) that received HFCD diet plus DEN and RIF (catalog number R9904, Sigma-Aldrich, United States) by gavage from the 5th week of the experiment until the 16th week of the experiment. The DEN dose and the experimental period for the development of this protocol were based on a previous study, which combine a dietary model of fatty liver based on high trans-fat with exposure to a known hepatic carcinogen as a means of provoking and accelerating more severe injury. The model replicated many features of MASLD including steatohepatitis with ballooning, fibrosis, cirrhosis, and HCC[17]. The experimental study design is presented in Figure 1. The animals were weighed twice a week during the experimental period. After 16 wk of the experiment, all the animals were euthanized by cardiac exsanguination. Serum samples and liver fragments were collected aseptically, frozen in liquid nitrogen, and stored in an ultra-freezer at -80 °C until the experimental procedures were carried out. A portion of each liver sample was fixed in 10% formalin for histological analysis.

Experimental diets

Animals in the control group received a standard rodent diet (Nuvilab CR-1; Quimtia SA, Brazil) with an energy value of 2.93 kcal/g. The diet consisted of 55.0% carbohydrates, 22.0% protein, 4.5% fat, and 18.5% of other nutrients such as fibers and vitamins. Animals in the intervention groups received an HFCD diet with an energy value of 4.3 kcal/g. This product consisted of 54.5% carbohydrates, 14.0% protein, and 31.5% fat (enriched with 54.0% trans fatty acids). The diet of the intervention group was chosen as such because it mirrors many of the phenotypes observed in humans with MASLD, as previously demonstrated by our research group[18]. The diet offered to the animals in the control and intervention

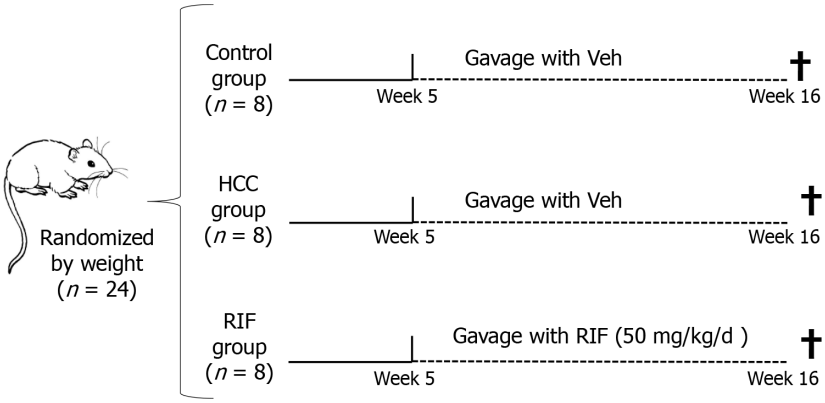

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Figure 1 Experimental design. The control group (*n* = 8) received a standard diet, water without diethylnitrosamine (DEN) and gavage with vehicle (Veh) solution; hepatocellular carcinoma-group (*n* = 7) received a high-fat and choline-deficient (HFCD) diet, water with DEN and gavage with Veh solution; rifaximin (RIF)-group (*n* = 7) received a HFCD diet, water with DEN and gavage with RIF. After all the animals were euthanized. DEN: Diethylnitrosamine; HCC: Hepatocellular carcinoma; HFCD: High-fat and choline-deficient; RIF: Rifaximin; Veh: Vehicle.

groups was replaced every two days. Both the groups received water and food *ad libitum* during the experimental period.

Rifaximin treatment

The therapeutic intervention through the administration of daily gavage with RIF or the offer of Veh solution, also by gavage, in the respective experimental groups occurred daily from the 5th week of the experiment until the date of euthanasia. The animals in the control group and HCC-group received daily gavage with a Veh solution (0.5 mL/kg distilled water) in order to undergo the same stress conditions as those in the RIF-group. The RIF-group received a daily dose of 50 mg/kg/d of RIF by daily gavage from the 5th week of the experiment until the 16th week of the experiment. The dose of RIF used was in accordance with a previous study published in the literature[19].

Assessment gene expression of hepatocarcinogenesis and autophagy markers

The total RNA was extracted from fragments of the liver tissue using TRIzol (catalog number 15596026, Invitrogen, United States). The cDNA conversion, from 2 µg of RNA, was performed using the High-capacity cDNA Reverse Transcription kit (catalog number 4368814, Applied Biosystems, United States). Quantitative (q) real-time polymerase chain reaction (RT-PCR) with TaqMan assay (Applied Biosystems, United States) was used to assess the gene expression of aldolase-B (*Aldob*), tubulin alpha-1c *(Tuba1c)*, alpha-fetoprotein (*Afp)*, metalloproteinases-2 *(Mmp2), Mmp9*, autophagy-related factor LC3A/B *(Map1 Lc3b), p62*/sequestosome-1 (*p62/Sqstm1*), beclin-1 (*Becn1*), enhancer of zeste homolog-2 (*Ezh2*) and coactivator associated arginine methyltransferase-1 (*Carm1*) in liver tissue. The *Actb* gene was used as a normalizer in both tissues. The probes used are described in Supplementary Table 1. Values were calculated with the formula $2^{-\Delta\Delta Ct}$.

Analysis of the circulating microRNAs

To analyze the circulating microRNAs from serum, total RNA was extracted using the miRNeasy serum/plasma kit (catalog number 217184, Qiagen, United States). Then, cel-miR-39 (1.6 × 10^8 copies) spike in control (catalog number 21961, Qiagen, United States) was added to provide an internal reference. cDNA conversion was performed from 10 ng of total RNA using the TaqMan microRNA Reverse Transcription kit (catalog number 4366597, Applied Biosystems, United States). Analysis of the gene expression of miR-122, miR-34a, miR-26b, miR-224, miR-33a, miR-143, miR-155, miR-375 and miR-21, together with the cell-miR-39 normalizer, was performed by RT-qPCR using TaqMan assay (Applied Biosystems, United States). The sequences and codes of the assessed microRNAs are described in Supplementary Table 1. Values were calculated by formula $2^{-\Delta\Delta Ct}$.

Analysis of protein expression of hepatic autophagy markers

For western blotting analysis, protein extraction was performed on samples of liver tissue from rats. The samples were homogenized in a solution containing Triton X-100, β-mercaptoethanol, tris-buffered saline (TTBS), ethylenediaminetetraacetic acid and proteases inhibitor cocktail (catalog number 29131, Santa Cruz Biotechnology, United States). The samples were then normalized to 40 µg of protein. Subsequently, proteins were separated by electrophoresis using a 12% w/v polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was washed with TTBS, and then incubated for one hour in a blocking solution containing 3% bovine serum albumin in TTBS. Following the blocking step, the membrane was washed three times with TTBS and incubated overnight at 4 °C in a blocking solution containing the following primary antibodies: Anti-actin (catalog number A5060, Sigma-Aldrich, United States), anti-LC3B (catalog number ab128025, Abcam, United Kingdom), and anti-SQSTM1 (catalog number ab56416, Abcam, United Kingdom). The primary antibodies were used at a dilution of 1:1000. After the overnight incubation, the membrane was washed three times with TTBS and then incubated for two hours in a solution containing a horseradish peroxidase-conjugated anti-IgG

secondary antibody in TBBS, at a concentration of 1:2000. For band detection, Clarity Western ECL Substrate (catalog number 1705062, BioRad, United States) was used, and the resulting image was captured using an ImageQuant LAS 500 (GE Healthcare Lifesciences) imaging system. Band intensities were quantified using the ImageJ software, and actin was used as a constitutive protein reference.

Correlations between the analyzed markers

For this analysis, in relation to markers related to hepatocarcinogenesis we selected *Mmp2 Mmp9, Afp, Tuba1c* and *Aldob* and associated with the autophagy and epigenetic process we selected *Becn1, p62/Sqstm1, Map1 Lc3b, Ezh2*, *Carm1*, p62 (protein ratio), and LC3B (protein ratio). Regarding microRNAs, we selected miR-122, miR-26b, miR-224 and miR-34a, which are markers related to liver disease and which showed a significant difference between the experimental groups.

Histopathological analysis

Formalin-fixed liver tissue samples were embedded in paraffin and stained with hematoxylin and eosin (H&E) and picrosirius red. Histopathological lesions of the different evolutionary stages of nonalcoholic fatty liver disease were assessed according to the score by Liang *et al*[20], which is a highly reproducible scoring system applicable to experimental rodent models[20]. This scoring system was developed through the assessment of various experimental models, aiming to establish generic criteria for analysis. To validate the proposed scoring, biological material from rodents was evaluated by blinded pathologists in two separate assessments, with an interval of over 3 mo between them. In this validation, observers estimated the percentage of macrovesicular steatosis, microvesicular steatosis, hypertrophy, and the number of inflammatory foci per field[20]. The degree of fibrosis was evaluated using the slides stained with picrosirius red, and cancerous lesions were graded according to the Edmondson & Steiner classification[21]. The analysis was performed by an experienced pathologist, who was blinded to the experimental groups.

Sample size calculation and statistical analysis

The sample size estimation was performed using the WINPEPI 11.20 software (Brixton Health, Israel), based on a previously published study by the research group demonstrating HCC development in an experimental model[17]. Considering a power of 80% and a significance level of 5%, it was determined that 8 animals per experimental group would be required for conducting this study. The outcome used for the calculation was the prevention of HCC.

Normality was verified for all variables using Shapiro-Wilk test and histograms. Nonparametric data were analyzed using the Kruskal-Wallis followed by Dunn test. Quantitative variables were expressed as median and interquartile ranges (25^{th}–75^{th}) and percentage. Spearman's correlation coefficient was performed, with a moderate ($0.3 < r < 0.6$), strong ($0.6 < r < 0.9$) or very strong ($0.9 < r < 1.0$) correlation were adopted. Statistical significance was set at $P < 0.05$. Data were analyzed using the Statistical Package for Social Sciences (version 28.0; SPSS Inc., United States).

RESULTS

During the 16 wk of the experiment, one animal from the HCC-group and another from the RIF-group died, totaling 7 animals per experimental group. There were no deaths in the control group ($n = 8$) during the study period.

Expression of genes involved in hepatic disease pathogenesis

The data of gene expression of hepatocarcinogenesis markers are shown in Table 1. There was no difference between the experimental groups for the gene expression of *Tuba1c* ($P = 0.839$), *Aldob* ($P = 0.595$), *Afp* ($P = 0.837$) *and Mmp2* ($P = 0.101$). There was a significant difference between the experimental groups in the gene expression of *Mmp9* ($P = 0.035$). For this marker, there was a significant increase in its expression in the HCC-group compared to the control group ($P = 0.013$). However, there was no significant difference in the expression of *Mmp9* between the HCC-group and RIF-group ($P = 0.071$).

Data obtained for the gene expression of markers of autophagy are shown in Figure 2. There was a significant decrease in *Map1 Lc3b* gene expression in the HCC and RIF-groups compared to the control group ($P = 0.002$ and $P = 0.001$, respectively). There was no significant difference in *p62/Sqstm1* expression in the RIF-group compared to the control ($P = 0.078$) and HCC-group ($P = 0.890$); however, the HCC-group showed a significant decrease in its expression compared to the control ($P = 0.010$). *Becn1* and *Ezh2* showed a significant decrease in their expression in the HCC-group ($P = 0.001$ and $P < 0.001$, respectively) and RIF-group ($P = 0.009$ and $P = 0.010$, respectively) compared to the control. There was a significant reduction in *Carm1* expression in the HCC-group compared to the RIF-group ($P = 0.004$).

Gene expression of the circulating microRNAs

The results obtained from the gene expression of the circulating microRNAs related to liver damage are demonstrated in Table 1. There was a significant increase in miR-122 gene expression in the HCC-group compared to the control group ($P < 0.001$), however the RIF-group did not differ significantly from the control ($P = 0.118$). There was a significant difference between the experimental groups in the gene expression of miR-34a ($P = 0.007$). The expression of miR-34a was significantly lower in the RIF-group compared to the control ($P = 0.005$). There was a significant difference between the experimental groups in the gene expression of miR-26b ($P = 0.026$). The HCC-group showed a significant decrease in miR-26b expression compared to the RIF-group ($P = 0.008$), which was like the control ($P = 0.469$). The gene expression of miR-224 was significantly higher in the HCC-group compared to the RIF-group ($P < 0.001$), which is like the control

Table 1 Gene expression of markers related to hepatocarcinogenesis

Variables[1]	Control (*n* = 8)	HCC (*n* = 7)	RIF (*n* = 7)	*P* value
Tuba1c	1.28 (0.18-2.76)	4.27 (0.00-9.69)	1.38 (0.29-19.8)	0.839
Aldob	2.46 (0.01-5.78)	4.24 (0.00-9.92)	0.12 (0.00-2.69)	0.595
Afp	1.28 (0.18-2.76)	2.33 (0.00-9.69)	1.38 (0.29-19.8)	0.837
Mmp2	1.81 (0.03-5.15)	31.4 (0.25-62.2)	0.92 (0.16-18.4)	0.101
Mmp9	2.36 (0.02-4.25)[a]	23.6 (2.69-76.8)[b]	14.6 (1.32-28.7)[a,b]	0.035
miR-122	1.14 (0.34-2.34)[a]	7.07 (1.41-11.8)[b]	2.62 (1.20-6.00)[a,b]	0.005
miR-34a	0.94 (0.63-1.70)[b]	0.39 (0.33-0.44)[a]	0.33 (0.13-0.89)[a]	0.007
miR-26b	1.04 (0.61-1.57)[a,b]	0.36 (0.21-1.85)[a]	2.22 (1.46-2.96)[b]	0.026
miR-224	1.05 (0.41-2.45)[a,b]	2.97 (1.96-5.94)[b]	0.58 (0.13-0.85)[a]	0.005
miR-33a	0.98 (0.53 – 2.16)	0.60 (0.54-1.17)	1.4 (0.70-1.60)	0.445
miR-143	1.18 (0.25-3.67)	1.40 (0.54-5.16)	0.62 (0.17-2.54)	0.471
miR-155	1.46 (0.32-1.96)	0.63 (0.01-1.18)	0.34 (0.04-1.46)	0.074
miR-375	0.79 (0.61-2.42)	0.85 (0.17-1.09)	0.38 (0.14-1.76)	0.216
miR-21	1.12 (0.34-2.13)	1.49 (0.91-6.08)	1.69 (0.84-3.68)	0.188

[1]Variables expressed by median (25^{th}–75^{th} percentiles).
[a]$P < 0.05$. Different letters indicate a significant difference between groups.
[b]$P < 0.05$. Different letters indicate a significant difference between groups.
HCC: Hepatocellular carcinoma; RIF: Rifaximin; *Afp*: Alpha-fetoprotein; *Aldo-B*: Aldolase-B; *Mmp*: Metalloproteinases; *Tuba1c*: Tubulin alpha-1c.

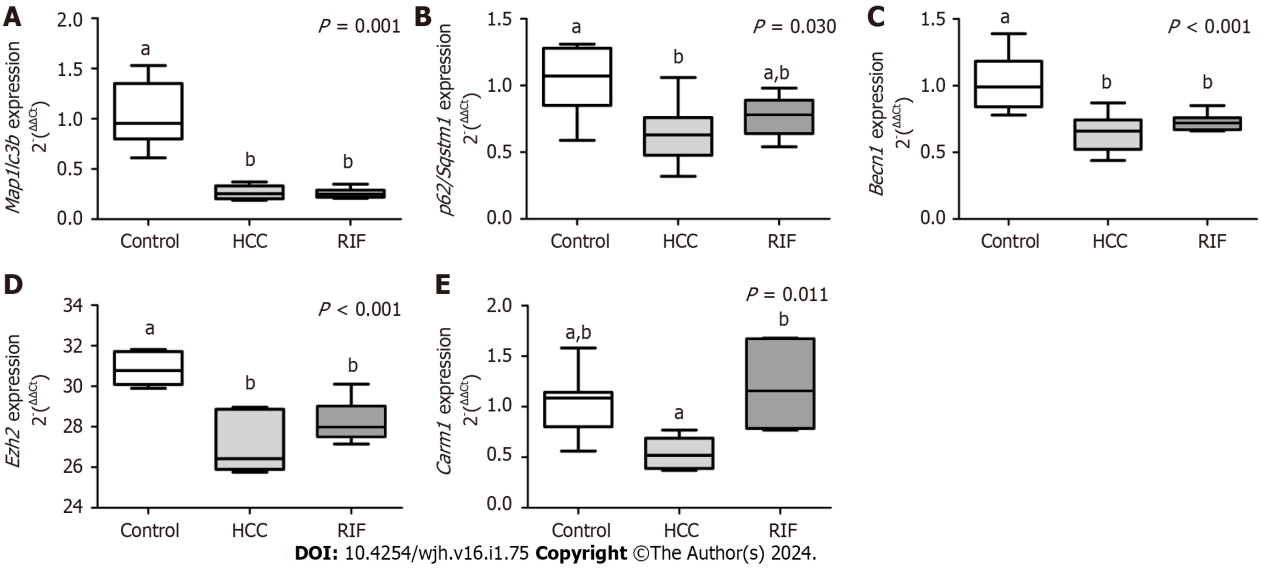

Figure 2 Hepatic gene expression of autophagy markers. A: Autophagy-related factor LC3A/B; B: P62/sequestosome-1; C: Beclin-1; D: Enhancer of zeste homolog-2; E: Coactivator associated arginine methyltransferase-1. Data expressed as median (25^{th}-75^{th} percentile), Kruskal-Wallis test. Different letters indicate a significant difference between groups ($P < 0.05$). [a]$P < 0.05$, [b]$P < 0.05$. Different letters indicate a significant difference between groups. *Becn1*: Beclin-1; *Carm1*: Coactivator associated arginine methyltransferase-1; *Ezh2*: Enhancer of zeste homolog-2; HCC: Hepatocellular carcinoma; *Map1 Lc3b*: Autophagy-related factor LC3A/B; *p62/Sqstm1*: Sequestosome-1; RIF: Rifaximin.

group ($P = 0.099$). There was no significant difference between groups in the gene expression of miR-33a ($P = 0.445$), miR-143 ($P = 0.471$), miR-155 ($P = 0.074$), miR-375 ($P = 0.216$) and miR-21 ($P = 0.188$).

Protein expression of autophagy markers

The data obtained from the protein expression analysis of p62/SQSTM1 and MAP1LC3B, conducted through the Western blot technique, are represented in Figure 3. A difference in the expression of the MAP1LC3B protein between the experimental groups was observed ($P = 0.024$). A significant decrease in its expression was noted in the RIF-group animals

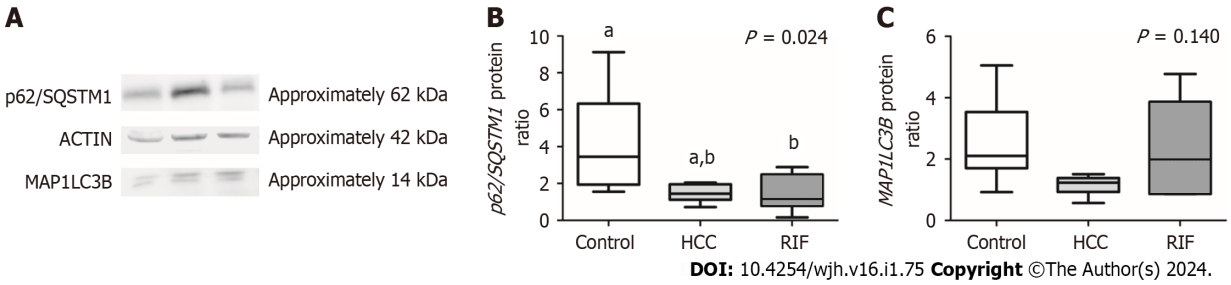

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Figure 3 Protein concentration of autophagy markers. A: Protein expression bands obtained through the Western blot technique; B: p62/SQSTM1 expression (densitometry analysis); C: MAP1LC3B expression (densitometry analysis). Data expressed as median (25th-75th percentile), Kruskal-Wallis test. Different letters indicate a significant difference between groups ($P < 0.05$). $^{a}P < 0.05$, $^{b}P < 0.05$. Different letters indicate a significant difference between groups. HCC: Hepatocellular carcinoma; MAP1LC3B: Autophagy-related factor LC3A/B, p62/SQSTM1: Sequestosome-1; RIF: Rifaximin.

compared to the control group ($P = 0.039$). No significant differences in MAP1LC3B expression were found between the animals receiving RIF treatment and the animals in the HCC-group ($P > 0.05$). Regarding the protein expression of p62/SQSTM1, no significant differences were observed between the experimental groups ($P = 0.140$).

Correlations between hepatocarcinogenesis, autophagy and epigenetic markers

The values obtained from the correlations between markers of hepatocarcinogenesis, autophagy and epigenetic are described in Table 2. There was a negative correlation between the expression of miR-122 and miR-34a, both related to the severity of liver injury compared to the autophagy markers *Map1 Lc3b*, *p62/Sqstm1* and *Becn1* that act in the development of the autophagosome. Additionally, this negative correlation was also demonstrated between these microRNAs and *Ezh2*, an enzyme that modulates the expression of autophagy genes. There was a positive correlation between the three autophagy indicators (*Map1 Lc3b*, *p62/Sqstm1* and *Becn1*), however there was no correlation between *Ezh2* and *Carm1*, demonstrating that these enzymes that perform epigenetic control of autophagy act in different ways. *Ezh2* positively correlated with *Map1 Lc3b*, *p62/Sqstm1* and *Becn1*. While the correlations were strong between the autophagy markers *Map1 Lc3b*, *p62/Sqstm1* and *Ezh2*, it was only moderate with *Becn1*. As *Ezh2* can catalyze the methylation of lysine to histone (H3K27), which can decrease the expression of target genes, an attempt was made to assess whether *Becn1* would be on the target of *Ezh2*-H3K27 axis. The result was described in Supplementary Figure 1. Metalloproteinases were positively correlated with the expression of *Afp* and *Tuba1c*, markers related to hepatocarcinogenesis. *Aldob* expression was positively correlated with *Mmp2, Afp* and *Tuba1c*. The protein expression of p62 correlated positively with the gene expression of the markers *Becn1*, *p62/Sqstm1*, and *Map1 Lc3b*.

Liver histopathological analysis

No hepatic histopathological changes were observed in the liver tissue of the control group (Figure 4A and B). The animals in the HCC-group had predominantly macrovesicular steatosis along with microvesicular steatosis of moderate intensity, with inflammatory activity and mild hypertrophy (Figure 4C and D). The RIF-group presented mild macrovesicular and microvesicular steatosis, no inflammation was observed and in only two animals the presence of hypertrophy was evidenced. In the staging of the histopathological lesion, seven animals in the HCC-group and RIF-group developed steatohepatitis (Table 3). In the evaluation of hepatic fibrosis, through H&E and picrosirius red staining respectively, five animals from the HCC-group and RIF-group developed fibrosis with multiple septa without the presence of cirrhosis and two animals from both experimental groups developed liver cirrhosis (Figure 4E and F). The breakdown by experimental group observed for microvesicular steatosis, macrovesicular steatosis, hypertrophy, inflammation, fibrosis, and HCC as a percentage is shown in Figure 4G.

In the evaluation of the tumor classification by the Edmondson & Steiner score, we reported a lesion grade three and four, corresponding to the presence of poorly differentiated and undifferentiated cancer, respectively, in the HCC-group. This finding was replicated for some animals in the RIF-group; however, three animals did not develop cancer.

Comparison between animals that developed or not hepatocellular carcinoma

Additionally, to better detail the results obtained between the autophagy and epigenetics markers with the hepatic histopathological results, we performed the subdivision of the experimental groups. Initially, we stratified the RIF-group, and performed a comparison analysis between the animals that developed HCC ($n = 4$) and those that did not develop HCC ($n = 3$). No significant differences ($P > 0.05$) were observed in gene expression of microRNAs, markers of hepatocarcinogenesis and autophagy between animals that developed or did not develop HCC in the RIF-group, as detailed in Table 4.

Subsequently, we carried out a new stratification of the experimental groups to compare the results obtained between the animals that developed HCC and those that did not develop HCC. The comparison of results is detailed in Table 5. In total, eleven animals developed HCC and eleven did not develop this clinical condition at the end of the study. Regarding epigenetic markers, we reported a significant increase in the gene expression of miR-122 ($P = 0.029$) and miR-34a ($P = 0.012$) in animals with HCC compared to animals without HCC. There was a significant increase in the gene expression of *Mm*^{2} ($P = 0.017$), *Mm9* ($P = 0.013$) and *Tuba1c* ($P = 0.017$), markers related to hepatic hepatocarcinogenesis, in animals with HCC compared to those that did not develop HCC. Regarding autophagy markers, there was a significant reduction in

Table 2 Correlations between hepatocarcinogenesis, autophagy and epigenetic markers

Variables[1]		microRNAs			Hepatocarcinogenesis					Autophagy and Epigenetic						
		miR-26b	miR-224	miR-34a	*Mmp2*	*Mmp9*	*Afp*	*Tuba1c*	*Aldob*	*Becn1*	*p62/Sqstm1*	*Map1lc3b*	*Ezh2*	*Carm1*	p62 protein	MAP1LC3B protein
microRNAs	miR-122	-0.235	0.075	0.400[a]	0.195	0.422	-0.189	0.085	0.030	-0.502[a]	-0.673[b]	-0.554[a]	-0.760[b]	-0.375	-0.245	0.017
	miR-26b		-0.360	0.538[a]	-0.071	0.203	0.199	-0.090	-0.113	-0.089	0.164	-0.047	-0.005	0.525[a]	-0.124	0.186
	miR-224			-0.195	0.041	-0.241	-0.201	0.025	0.153	-0.298	-0.022	0.032	-0.061	-0.335	-0.007	-0.393
	miR-34a				0.110	0.426	0.123	0.110	-0.127	-0.461[a]	-0.340	-0.612[b]	-0.584[b]	0.181	-0.400	-0.191
Hepatocarcinogenesis	*Mmp2*					0.742[b]	0.704[b]	0.957[b]	0.548[b]	-0.321	-0.133	-0.321	-0.361	-0.286	-0.318	-0.368
	Mmp9						0.521[a]	0.703[b]	0.292	-0.398	-0.276	-0.351	-0.586[b]	-0.278	-0.547[a]	-0.229
	Afp							0.702[b]	0.468[a]	-0.086	0.049	-0.173	0.038	-0.073	-0.328	-0.078
	Tuba1c								0.644[b]	-0.324	-0.058	-0.281	-0.350	-0.282	-0.258	-0.246
	Aldob									-0.137	-0.042	0.186	0.111	-0.314	-0.032	0.174
Autophagy and Epigenetic	*Becn1*										0.541[b]	0.771[b]	0.597[b]	0.433	0.631[b]	0.302
	p62/Sqstm1											0.657[b]	0.609[b]	0.459	0.554[a]	0.265
	Map1lc3b												0.718[b]	0.230	0.512[a]	0.343
	Ezh2													0.288	0.342	0.253
	Carm1														0.185	-0.044
	p62 protein															0.406

[1]Variables were evaluated by Spearman's r correlation coefficient: Moderate (0.3 < r < 0.6), strong (0.6 < r < 0.9) or very strong (0.9 < r < 1.0).

[a]$P < 0.05$, correlation significant at the 0.05 level.

[b]$P < 0.01$, correlation significant at the 0.01 level.

Afp: Alpha-fetoprotein; *Aldo-B*: Aldolase-B; *Becn-1*: Beclin-1; *Carm1*: Coactivator associated arginine methyltransferase-1; *Ezh-2*: Enhancer of zeste homolog-2; *Map1lc3b*: Autophagy-related factor LC3A/B; *Mmp*: Metalloproteinases; *p62/Sqstm1*: P62/sequestosome-1; *Tuba1c*: Tubulin alpha-1c.

the gene expression of *Becn1* ($P = 0.004$), *Map1 Lc3b* ($P = 0.004$), *Ezh2* ($P = 0.010$) and *Carm1* ($P = 0.026$) in animals with HCC compared to animals who did not develop this clinical condition. Additionally, we demonstrated a significant decrease in the protein expression of p62/SQSTM1 ($P = 0.013$) in the animals with HCC compared to the animals without HCC in the RIF group.

Table 3 Distribution of liver histopathological findings according to general non-alcoholic fatty liver disease scoring system for rodent models, *n* (%)

Variable[1]	Control (*n* = 8)	HCC (*n* = 7)	RIF (*n* = 7)
No MASLD	8 (100)	0 (0.0)	0 (0.0)
MASLD	0 (0.0)	0 (0.0)	0 (0.0)
MASH	0 (0.0)	7 (100)	7 (100)

[1]Variables described as frequency (%).
HCC: Hepatocellular carcinoma; MASLD: Metabolic-dysfunction associated steatotic liver disease; MASH: Metabolic-dysfunction associated steatohepatitis; RIF: Rifaximin.

Table 4 Comparison between animals in the rifaximin-group that developed or not hepatocellular carcinoma

Variables[1]	Developed HCC (*n* = 4), median (min–max)	Did not developed HCC (*n* = 3), median (min–max)	*P* value
miR-122	2.27 (1.20–4.57)	2.97 (1.51–5.98)	0.571
miR-375	0.42 (0.14–1.76)	0.33 (0.30–0.67)	1.000
miR-26b	1.97 (1.10–3.38)	2.41 (1.97–3.99)	0.571
miR-224	0.46 (0.13–0.85)	0.58 (0.46–0.82)	0.857
miR-34	11.0 (7.17–18.9)	10.0 (8.64–10.5)	0.786
miR-21	2.36 (0.84–3.68)	1.68 (1.00–1.69)	0.700
miR-143	0.76 (0.17–2.53)	0.48 (0.25–1.96)	1.000
miR-155	0.69 (0.06–1.46)	0.13 (0.03–0.36)	0.250
miR-33a	0.88 (0.52–1.86)	1.55 (1.40–1.58)	0.400
Mm2	7.95 (0.58–18.4)	0.71 (0.16–0.92)	0.229
Mm9	24.0 (1.32–28.7)	8.48 (1.73–14.6)	0.400
Afp	6.99 (1.38–19.8)	0.46 (0.29–0.48)	0.057
Tuba1c	4.63 (0.30–7.12)	0.26 (0.25–0.30)	0.057
Aldob	2.32 (0.11–2.69)	0.07 (0.00–0.12)	0.114
Becn1	0.70 (0.67–0.85)	0.74 (0.66–0.76)	1.000
p62/Sqstm1	0.84 (0.54–0.98)	0.72 (0.64–0.79)	0.629
Map1lc3b	0.24 (0.22–0.35)	0.27 (0.21–0.29)	1.000
Ezh2	29.0 (28.0–30.1)	27.5 (27.2–28.0)	0.114
Carm1	0.79 (0.77–1.67)	1.23 (1.08–1.68)	0.400
p62/SQSTM1 protein	1.07 (0.16–2.89)	1.78 (1.19–2.36)	0.355
MAP1LC3B protein	2.00 (0.87–4.78)	2.20 (0.86–3.57)	0.643

[1]Variables expressed by median (25^{th}–75^{th} percentiles).
$P < 0.05$ is considered significant.
A*ldob*: Aldolase B; *Afp*: Alpha-fetoprotein; *Becn-1*: Beclin-1; *Carm1*: Coactivator associated arginine methyltransferase-1; *Ezh-2*: Enhancer of zeste homolog-2; HCC: Hepatocellular carcinoma; *Map1lc3b*: Autophagy-related factor LC3A/B; *Mmp*: Metalloproteinases; *p62/Sqstm1*: P62/sequestosome-1; RIF: Rifaximin; *Tuba1c*: Tubulin alpha 1c.

DISCUSSION

In this study, carried out in an experimental model of HCC secondary to MASLD, with the objective of evaluating the effect of treatment with RIF in relation to autophagy and epigenetic markers, we demonstrated: (1) A positive correlation between *Map1cl3b, p62/Sqstm1* and *Becn1* in HCC, however, there was no significant difference in the expression of these markers between the HCC-group and RIF-group; (2) a lack of correlation between *Ezh2* and *Carm1*, demonstrating that these enzymes regulate different signaling pathways, although RIF promoted a significant increase in *Carm1* expression;

Table 5 Comparison between the animals that developed and did not develop hepatocellular carcinoma

Variables[1]	HCC (*n* = 11), median (min–max)	No HCC (*n* = 11), median (min–max)	*P* value
miR-122	4.49 (1.20–11.8)	1.41 (0.34–5.98)	0.029[a]
miR-375	0.64 (0.14–1.76)	0.72 (0.30–2.42)	0.512
miR-26b	1.10 (0.46–3.38)	1.23 (0.59–3.99)	0.656
miR-224	1.40 (0.13–5.94)	0.82 (0.41–2.45)	0.545
miR-34a	7.73 (2.29–18.9)	1.79 (0.14–10.5)	0.012[a]
miR-21	1.92 (0.84–6.08)	1.46 (0.34–2.13)	0.109
miR-143	1.08 (0.17–5.16)	1.13 (0.25–3.67)	0.756
miR-155	0.66 (0.01–1.46)	0.60 (0.03–1.95)	0.349
miR-33a	0.66 (0.52–1.86)	1.40 (0.34–2.64)	0.200
Mm2	18.4 (0.25–62.2)	1.17 (0.03–5.15)	0.017[a]
Mm9	23.6 (1.32–76.8)	2.76 (0.02–14.6)	0.013[a]
Afp	3.23 (0.00–19.8)	0.62 (0.18–2.76)	0.190
Tuba1c	7.12 (0.12–27.8)	0.76 (0.12–3.71)	0.017[a]
Aldob	2.32 (0.00–9.92)	1.35 (0.00–5.78)	0.549
Becn1	0.68 (0.44–0.87)	0.88 (0.66–1.39)	0.004[a]
p62/Sqstm1	0.65 (0.32–1.06)	0.92 (0.59–1.31)	0.052
Map1lc3b	0.25 (0.19–0.37)	0.86 (0.21–1.53)	0.004[a]
Ezh2	28.4 (25.8–30.1)	30.2 (27.2–31.8)	0.010[a]
Carm1	0.69 (0.37–1.67)	1.10 (0.56–1.68)	0.026[a]
p62/SQSTM1 protein	1.27 (0.16–2.39)	2.87 (1.19–9.12)	0.013[a]
MAP1LC3B protein	1.29 (0.57–4.78)	2.10 (0.86–5.05)	0.183

[1]Variables expressed by median (25^{th}–75^{th} percentiles).

[a]$P < 0.05$ is considered significant.

Aldob: Aldolase B; *Afp*: Alpha-fetoprotein; *Becn-1*: Beclin-1; *Carm1*: Coactivator associated arginine methyltransferase-1; *Ezh-2*: Enhancer of zeste homolog-2; HCC: Hepatocellular carcinoma; *Map1lc3b*: Autophagy-related factor LC3A/B; *Mmp*: Metalloproteinases; *p62/Sqstm1*: P62/sequestosome-1; RIF: Rifaximin; *Tuba1c*: Tubulin alpha 1c.

(3) a significant increase in miR-26b expression in the RIF-group compared to the HCC-group; the inverse was observed for miR-224; (4) a negative correlation between the expression of miR-122 and miR-34a related to liver injury with *Map1 Lc3b, p62/Sqstm1* and *Becn1*; and (5) finally, steatohepatitis developed in all animals of the HCC-group and RIF-group, but three animals treated with RIF did not develop HCC.

RIF, a semisynthetic derivative of rifamycin, has an intestinal absorption of approximately 0.007%, which prevents it from exerting systemic or adverse effects[13,16]. It has broad-spectrum *in vitro* activity against aerobic and anaerobic enteric bacteria and has been used in the treatment of hepatic encephalopathy, traveler's diarrhea, and irritable bowel syndrome[13,22]. Recent studies report that RIF inhibited hepatic fibrosis in rodent models of alcoholic liver injury and steatohepatitis[23,24]. In clinical practice, its use seems to be effective and safe in MASLD, promoting the reduction of serum endotoxemia, improvement of insulin resistance, inflammatory process, and histopathological score[13,16]. To our knowledge, no studies evaluate the effect of RIF on the progression from MASLD to HCC in relation to autophagy and epigenetic markers. We emphasize that the treatment with RIF in the animals started from the fifth week of the experiment when the animals probably already had steatohepatitis, as demonstrated in previous studies[17]. In the histopathological evaluation, all animals in the HCC and RIF groups developed steatohepatitis; however, three animals treated with RIF did not develop HCC. This interesting finding continues to be investigated by our research group and we seek to understand the role of RIF in this process. A multicenter, double-blind, randomized, placebo-controlled study in patients with biopsy-proven steatohepatitis investigated the effects of daily administration of RIF for six months[13]. The results obtained demonstrated that patients treated with RIF showed a significant decline in cytokeratin-18 Levels, a biomarker capable of predicting the histopathological manifestations of steatohepatitis[13]. However, Cobbold *et al*[14] reported that RIF therapy in patients with steatohepatitis for six weeks caused no changes in liver triglyceride content, insulin sensitivity, or systemic inflammation[14]. Due to the lack of studies evaluating the effect of RIF on HCC secondary to steatohepatitis, further studies are needed in the search for new therapies for the prevention of disease progression and future clinical application.

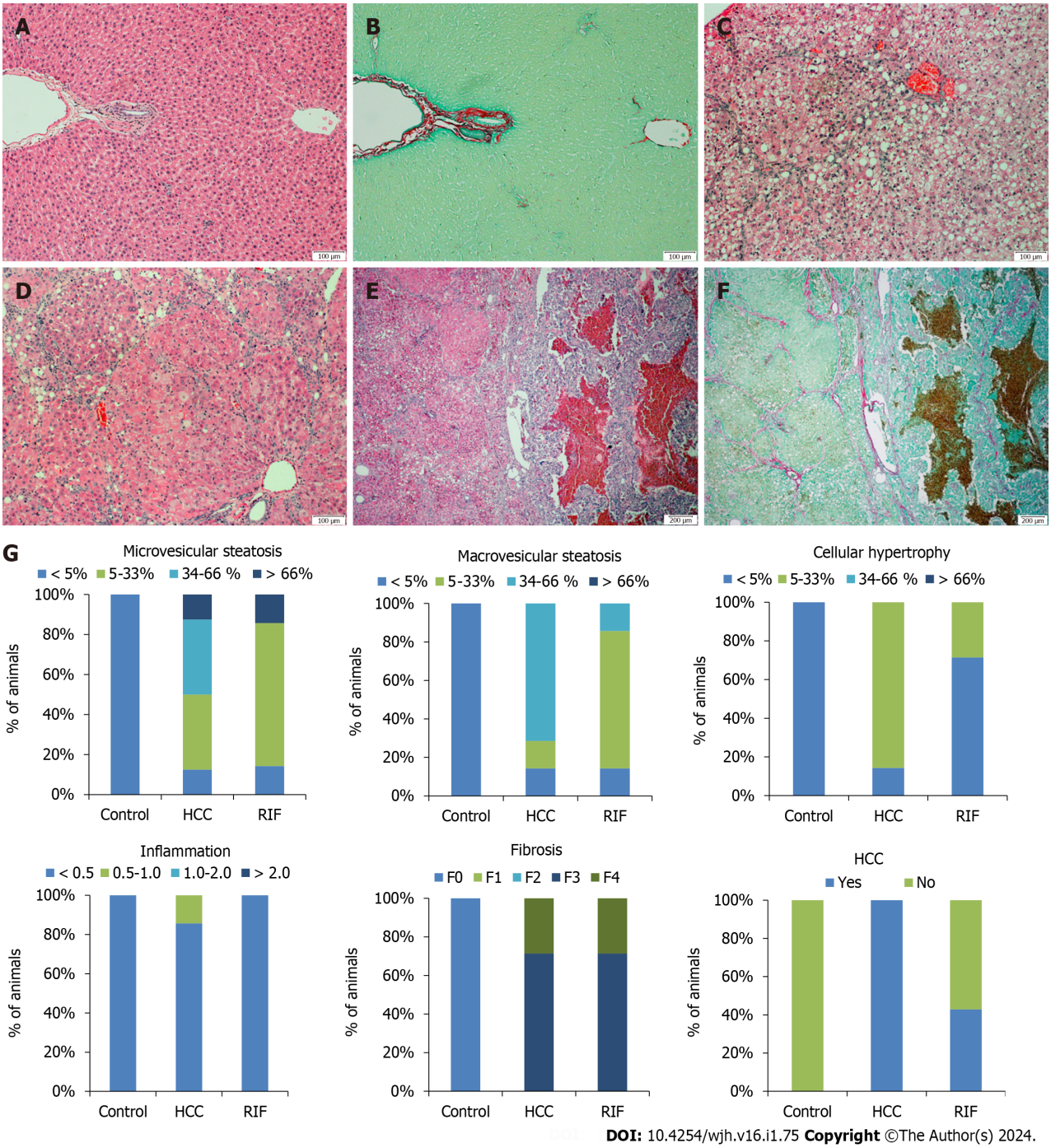

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Figure 4 Histological evaluation of hepatic tissue in different experimental groups and percentage description for the presence of microvesicular steatosis, macrovesicular steatosis, hypertrophy, inflammation, fibrosis and hepatocellular carcinoma. A and B: The images correspond to hematoxylin & eosin (H&E) and picrosirius red staining's in the control group, showing a normal hepatic parenchyma with no significant changes; C and D: In the hepatocellular carcinoma (HCC)-group, animals exhibited macrovesicular steatosis and hypertrophy; E and F: Tumoral lesions with multiple fibrosis septa were observed in the HCC-group and rifaximin-group, as evidenced by H&E and picrosirius stained slides, respectively; G: Percentage description for the presence of microvesicular steatosis, macrovesicular steatosis, hypertrophy, inflammation, fibrosis, and HCC. Images A-D are magnified at 100 ×, and images E-F are magnified at 40 ×. HCC: Hepatocellular carcinoma; H&E: Hematoxylin & eosin; RIF: Rifaximin.

It is known that MASLD-HCC is a tumor of specific molecular characteristics compared to other etiologies of HCC. In the tumor microenvironment, the adaptive response caused by cellular hypoxia promotes the activation of pathways that intensify the process of angiogenesis, inflammation, fibrogenesis, and autophagy[9,25]. This response is dependent on hypoxia-inducible transcriptional factors, such as *Aldob*, *Tuba1c*, and metalloproteinases, among other markers. In this study animals of the HCC-group showed a significant increase in the expression of *Mmp9* in relation to the control group, and the treatment with RIF caused a reduction of this expression, obtaining values like the control. Additionally, we reported an increase in the gene expression of *Mm*^{2}, *Mm9* and *Tuba1c* in animals that developed HCC compared to animals that did not develop this clinical condition. The increased expression of these markers is linked to the process of hepatic hepatocarcinogenesis, as it is known that metalloproteinases are master regulators in the process of cell prolif-

eration and migration, and play a role in cell apoptosis, tissue regeneration, and immune response[26,27]. It is known that inflammation drives the progression of MASLD; however, the mechanisms associated with this process are not fully elucidated. In clinical practice, assessing the dietary inflammatory index and the systemic immunological inflammation index are tools that can be used to predict and evaluate the prognosis of liver disease, considering the associated genetic, environmental, and dietary factors[28,29].

Autophagy contributes with cellular homeostasis, thus avoiding cell transformation and tumor initiation. However, in the advanced stages of the tumor, autophagy acts mainly as a suppressor of cell death, allowing the adaptation of cancer cells to stressful conditions[30,31]. The autophagy process is regulated by different markers, including *Map1 Lc3b*, *p62/Sqstm1*, and *Becn1*[30]. In this study, we demonstrated a significant decrease in the expression of these genes in the HCC-group and RIF-group compared to healthy animals. It is known that there is no pattern in the expression of these markers in different cell types and tissues of origin[10,30]. An example of this process is the expression of *Becn1* which is reduced in glioblastoma, ovarian, lung, and esophageal cancer, but increased in colorectal and gastric cancer cells[32-34]. The results obtained in this study have a similar pattern of autophagy markers in HCC and hepatic fibrosis that was found in previous studies[10,35-38]. *Becn1* acts as an initiator of autophagy and its deregulation increase the susceptibility of cells to transformation, that is, its lower expression is predictive of inferior survival in HCC[10,33,37,38]. *Becn1* deficiency is also associated with increased angiogenesis, which indirectly corroborates the results obtained in this study[39]. Al-Shenawy[10] reports that autophagy and apoptosis in the liver are interrelated processes, in which elevated levels of *Becn1* in patients with chronic hepatitis may limit liver damage and interact with progression to cancer, where *Becn1* Later it is suppressed in aggressive cases of HCC[10]. The absence of microtubule-associated protein-1 Light chain-3 expression, an essential component in the formation of autophagosomes, is predictive of immediate mortality from HCC [35]. Duran *et al*[36] report that *p62/Sqstm1* deficiency increased the activation phenotype of hepatic stellate cells, inhibiting their anti-fibrotic and anti-inflammatory functions[36]. Although we reported a significant decrease in the expression of these autophagy markers in the HCC and RIF groups, we did not observe a significant difference with the treatment of RIF. It is known that the greater the difference in the levels of autophagy between cancer and normal tissue, the worse the prognosis of the lesion, a result observed in this study.

Epigenetics is involved in autophagy "turn on, turn off" along the carcinogenesis, through the activity of metyltransferases such as *Ezh2* and *Carm1*[40-42]. In this study, there was a significant decrease in *Ezh2* expression in the HCC-group and RIF-group compared to the control. Treatment with RIF promoted a significant increase in *Carm1* expression. No significant correlation was observed between the expression of *Ezh2* and *Carm1*, probably because they act in different ways of epigenetic control of autophagy. *Ezh2* represses the expression of genes related to the mammalian target of rapamycin pathway and *Carm1* acts on transcription factors such as p53, and factor nuclear-κB[40,41]. Inhibition of *Ezh2* activity induces autophagy, through the formation of LC3B and consequently the formation of the autophagosome, which corroborates with this study, given the lower expression of *Map1 Lc3b*[40,42]. The function of *Carm1*, like other epigenetic controllers, is dependent on the stage of lesion development, acting as a tumor repressor or promoter[41].

Epigenetic regulation is also carried out by microRNAs, which are short RNA sequences that function as modulators of mRNA expression, by either impairing translation or promoting its degradation[43,44]. Subtle dysregulation of anyone step in microRNAs biogenesis may lead to tumorigenesis. The miR-122 acts in the balance of proliferation and differentiation of hepatocytes, however its physiological role in carcinogenesis is variable and the mechanism by which it contributes to the progression of the lesion is undetermined[43]. In this study, the HCC-group showed a significant increase in miR-122 expression compared to healthy animals, a result that corroborates the literature[43,45]. We emphasize that this increase in gene expression was maintained in animals that developed HCC compared to animals that did not develop this clinical condition, in the stratified analysis of experimental groups. However, contradictory data are also reported, a possible explanation being the heterogeneity of the samples under different environmental conditions [44]. We did not observe a significant difference in the expression of miR-34a between the HCC-group and RIF-group, however there was a significant increase in its expression in the RIF-group compared to healthy animals. This increase in expression may represent a beneficial effect of RIF, as miR-34 inhibits the process of carcinogenesis through the regulation of p53, promoting apoptosis, cell cycle arrest, and senescence[46,47]. This data corroborates the increase in the expression of *Carm1* regulator of autophagy through the expression of p53. Additionally, we reported a significant increase in miR-26b expression in RIF treated animals. We infer that it is a beneficial effect of the treatment since the lower expression of this microRNA is associated with a worse prognosis of HCC[48,49]. The suppression of miR-26 may result in an increase in the expression of *Ezh2*, in opposite to our results. However, we know that the expression of these epigenetic markers is variable according to the tissue and stage of the lesion[50]. Another possible beneficial effect of RIF treatment was the decrease in the expression of miR-224, which acts as an oncomiR in HCC cells, and its upregulation promotes the proliferation and migration of malignant hepatocytes[51,52]. *Carm1* regulates the expression of transcription factors such as p53 and nuclear factor-κB which regulate the expression of miR-224. Possibly this signaling mechanism is activated and RIF acts in its modulation, however further studies are necessary to be carried out for the elucidation of the process[51,52]. Additionally, we compared the results of animals that developed or not HCC, and we found no difference within them in the RIF-group, probably due to the small number of animals. However, analyzing the entire group of animals, there was a difference between miR-122, miR-34a, *Tuba1c*, metalloproteinases and autophagy markers between the groups that developed cancer *vs* those who did not present HCC. These interesting findings also may demonstrate the influence of epigenetic and autophagy markers in the development of HCC in this scenario.

So far, there is no specific medication approved by the Food and Drug Administration in clinical practice for the treatment of MASLD; therefore, counseling focuses on lifestyle to prevent progression to HCC. Biological aging and the progression of liver disease occur through the interference of various factors, including environmental, genetic, and dietary issues[28]. The assessment of these mechanisms can contribute to the search for preventive strategies in the development of MASLD-HCC. In this study, animals were subjected to a HFCD with the aim of developing the liver

condition, allowing the study of the use of RIF as a preventive measure. Since dietary management tools are essential for controlling this clinical condition, one can speculate if including additional dietary intervention in the model, such as high fiber diet or flavonoids, for instance, could alleviate liver damage[12,53,54].

CONCLUSION

In summary, MASLD-HCC management is a clinical challenge, and many questions need to be addressed, including the response to the new immunotherapy agents. The process of autophagy and epigenetics tends to play a complicit role in drug resistance and the interface between the two is multifactorial and crosstalk occurs in different proteins of each process[6,55]. In this study, we observed, mainly in relation to the epigenetic markers evaluated, a possible beneficial effect of the treatment with RIF in rats with MASLD-HCC, suggesting it could be useful to prevent or delay carcinogenesis. On the other hand, the study has some weaknesses to be considered, like the small number of animals and the gene expression markers analysis. Thus, new preclinical studies are needed to evaluate the epigenetic and autophagy mechanisms in MASLD-HCC for a better understanding of the role of RIF, as there are factors that need to be better explored, such as the variability of the course of the disease, the complexity of the autophagy mechanism and the individualized treatment requirements.

ARTICLE HIGHLIGHTS

Research background

Metabolic-dysfunction associated steatotic liver disease (MASLD) incidence is increasing worldwide. Hepatocellular carcinoma (HCC) is a complex and heterogeneous neoplasm, and there's evidence showing MASLD-related HCC has some unique features, including gut microbiota (GM). However, current treatment does not take this heterogeneity into account, dealing with viral and non-viral HCC in the same way. This study is intended to characterize autophagy and epigenetics in experimental MASLD-HCC and its response to rifaximin (RIF), a minimally-absorbed broad-spectrum oral antibiotic, that may interfere in GM-derived inflammation.

Research motivation

Epigenetic changes, autophagy and GM are involved in hepatocarcinogenesis, but there is no definite evidence of a positive effect of its modulation in human steatohepatitis. RIF may influence in these complex mechanisms. Understanding GM influence on epigenetics and autophagy can help not only as a diagnostic tool but also as a target for new therapies.

Research objectives

The main objective was to investigate rifaximin (RIF) effects on epigenetic and autophagy markers in experimental HCC secondary to MASLD. Future research in humans with MASLD can open a new therapeutic pathway to decrease HCC burden in this setting.

Research methods

We conducted an innovative RIF experiment in a MASLD-HCC model with 24 adult Sprague-Dawley rats, randomly assigned in three groups (n = 8, each) and treated from 5-16 wk. We compared the results of control animals to RIF group and MASLD (animals in the last two groups received a high-fat choline deficient diet plus diethylnitrosamine in drinking water. Gene expression of epigenetic and autophagy markers was obtained at the end of experiment.

Research results

All animals in RIF and MASLD groups developed steatohepatitis, fibrosis, and cirrhosis. All MASLD animals also presented HCC, but in RIF group three rats did not develop tumor. Some microRNAs, metalloproteinases and aggressivity markers were higher in rats that developed HCC comparing with those that not developed, and the opposite occurred with the autophagy markers.

Research conclusions

The results suggest that autophagy and epigenetics could exert influence on MASLD-HCC *via* GM interference with RIF and support clinical studies in the area.

Research perspectives

RIF may have effect on autophagy and epigenetic markers as shown in this study. These initial results in animals shall be confirmed in other preclinical and clinical studies before recommending its use in high-risk patients with MASLD cirrhosis.

FOOTNOTES

Co-first authors: Matheus Truccolo Michalczuk and Larisse Longo.

Author contributions: Michalczuk MT and Longo L performed the conceptualization, methodology, formal analysis, investigation, data curation, writing of the original draft, writing-review, and editing; Keingeski MB, Guerreiro GTS, Ferrari JT, Filippi-Chiela E, Uribe-Cruz C and Cerski CTS performed the methodology and formal analysis, writing review and editing; Basso BS performed the methodology; Vargas JE performed the analysis; Oliveira CP performed the methodology and writing review; Álvares-da-Silva MR performed the conceptualization, methodology, formal analysis, investigation, data curation, writing of the original draft, writing-review, editing and research fundraising.

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Institutional animal care and use committee statement: All experimental procedures were approved by the Ethics Committee for the Use of Animals protocol No. 2021-0105, in accordance with international guidelines for animal welfare and measures were taken to minimize animal pain and discomfort.

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META-ANALYSIS

Sorafenib plus transarterial chemoembolization *vs* sorafenib alone for patients with advanced hepatocellular carcinoma: A systematic review and meta-analysis

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Abstract

BACKGROUND

Although the past decade has seen remarkable advances in treatment options for hepatocellular carcinoma (HCC), the dismal overall prognosis still envelops HCC patients. Several comparative trials have been conducted to study whether transarterial chemoembolization (TACE) could improve clinical outcomes in patients receiving sorafenib for advanced HCC; however, the findings have been inconsistent.

AIM

To study the potential synergies and safety of sorafenib plus TACE *vs* sorafenib alone for treating advanced HCC, by performing a systematic review and meta-analysis.

METHODS

This study was conducted following the PRISMA statement. A systematic literature search was conducted using the Cochrane Library, Embase, PubMed, and Web of Science databases. Data included in the present work were collected from patients diagnosed with advanced HCC receiving sorafenib plus TACE or sorafenib alone. Data synthesis and meta-analysis were conducted using Review Manager software.

RESULTS

The present study included 2780 patients from five comparative clinical trials (1

was randomized control trial and 4 were retrospective studies). It was found that patients receiving sorafenib plus TACE had better prognoses in terms of overall survival (OS), with a combined hazard ratio (HR) of 0.65 [95% confidence interval (95%CI): 0.46–0.93, P = 0.02, n = 2780]. Consistently, progression free survival (PFS) and time to progression (TTP) differed significantly between the sorafenib plus TACE arm and sorafenib arm (PFS: HR = 0.62, 95%CI: 0.40–0.96, P = 0.03, n = 443; TTP: HR = 0.73, 95%CI: 0.64-0.83, P < 0.00001, n = 2451). Disease control rate (DCR) was also significantly increased by combination therapy (risk ratio = 1.36, 95%CI: 1.02-1.81, P = 0.04, n = 641). Regarding safety, the incidence of any adverse event (AE) was increased due to the addition of TACE; however, no significant difference was found in grade ≥ 3 AEs.

CONCLUSION

The combination of sorafenib with TACE has superior efficacy to sorafenib monotherapy, as evidenced by prolonged OS, PFS, and TTP, as well as increased DCR. Additional high-quality trials are essential to further validate the clinical benefit of this combination in the treatment of advanced HCC.

Key Words: Hepatocellular carcinoma; Sorafenib; Transarterial chemoembolization; Systematic review; Meta-analysis

Core Tip: No consensus is available in the literature about whether addition of transarterial chemoembolization (TACE) could improve survival in patients receiving sorafenib for advanced hepatocellular carcinoma. This is the first systematic review and meta-analysis comparing sorafenib/TACE combination therapy and sorafenib monotherapy for advanced hepatocellular carcinoma. We investigated these two treatments in terms of overall survival, progression free survival, time to progression, disease control rate, and adverse events.

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INTRODUCTION

As a global health problem, liver cancer, including hepatocellular carcinoma (HCC), represents the sixth most frequent malignancy and the fourth cause of cancer-related death[1]. In particular, the incidence of HCC is rising, with an annual incidence of above 0.6 million patients at present, which is estimated to be > 1 million by 2025 worldwide[2]. There have been remarkable advances in treatment options for HCC, and several treatment options have been adopted as standard of care according to clinical practice guidelines[3-5]. In principle, potentially curative therapies (*i.e.*, surgical resection, local ablation, and liver transplantation) are preferred for early-stage tumours, transarterial chemoembolization (TACE) is recommended for intermediate-stage tumours, and systemic drugs (*i.e.*, sorafenib, and atezolizumab plus bevacizumab) are the mainstay of treatment for advanced tumors. All these therapies have contributed to a progressive improvement in life expectancy of HCC patients[4-7]. However, the dismal overall prognosis still envelops HCC patients primarily because of the late diagnosis and frequent relapse[8].

Because quite many HCCs are diagnosed at an advanced stage, namely, Barcelona Clinic Liver Cancer (BCLC) stage C [9], how to prolong the survival of patients with advanced HCC is more crucial than the treatments for early stage HCC. It has not achieved global consensus on the definition of advanced HCC, which is generally indicated in cases with portal vein infiltration, extrahepatic metastasis, or progression on curative treatments[10]. Sorafenib, an inhibitor of several tyrosine kinases, such as VEGFR-2 (vascular endothelial growth factor receptor-2), PDGFR-β (platelet-derived growth factor receptor-β), and Raf serine/threonine kinases, is the first molecular targeting drug approved for the treatment of advanced HCC, and yet the standard first-line therapy internationally[11,12]. However, the overall survival (OS) outcomes of most patients are still far from satisfactory, and further prolonging survival is challenging.

To augment the clinical benefit of sorafenib, several clinical studies have evaluated the effects of addition of other systemic/locoregional therapies to it[13-15], including TACE[16]. TACE is a vascular interventional surgery which can concentrate chemotherapeutic drugs at tumour site, thus blocking tumour feeding from the primary artery to delay tumor progression. As an effective therapy for unresectable HCC, TACE is recommended by most guidelines for HCC at intermediate stage or multifocal HCC[4,10,12]. Although TACE is preferred for HCC patients at BCLC stage B, in many countries, it is frequently performed across all disease stages as well, including advanced stage[17]. Treatment with TACE leads to VEGF upregulation and thus the increase of tumour angiogenesis, while sorafenib would be expected to strengthen the effectiveness of TACE by suppressing angiogenesis by inhibiting VEGF signaling. Several comparative trials worldwide have been conducted to study whether TACE could provide clinical benefit in patients receiving sorafenib for advanced HCC; however, the findings have been not consistent[18-23]. Hence, the efficacy of the combination therapy of sorafenib plus TACE in patients with advanced disease has not been thoroughly understood. This

systematic review and meta-analysis aimed to assess the potential synergies and safety of sorafenib plus TACE as compared with sorafenib alone in the treatment of advanced HCC.

MATERIALS AND METHODS

Search strategy

This systematic review and meta-analysis was conducted following the PRISMA statement. Four databases (PubMed, the Cochrane Library, EMbase, and Web of Science) were used in systematic search to capture relevant studies from inception to August 18, 2023. Two independent investigators (Yang HJ and Ye B) conducted this search. We used the combinations of the following keywords: Hepatocellular carcinoma/HCC, sorafenib /tyrosine kinase inhibitor/TKI/multikinase inhibitor/MKI, and transarterial chemoembolization/TACE/chemoembolization.

Selection criteria

The criteria for including eligible studies into this meta-analysis were: (1) Study patients were diagnosed with advanced HCC, regardless of the kind of treatment that they have experienced before; (2) at least two intervention arms (TACE plus sorafenib *vs* sorafenib alone) were compared in the study; (3) one of the following outcomes must be included in study: OS, progression free survival (PFS), time to progression (TTP), or disease control rate (DCR). Studies published only as an abstract or those containing unobtainable/unusable data were excluded. Two independent investigators (Liao JX and Lei L) judged the records based on the title/abstract and then full-text. Any disagreement between the two investigators was discussed to reach a consensus.

Data extraction

Two investigators (Yang HJ and Ye B) independently extracted the data of baseline characteristics and outcome measures from eligible studies using a specially-designed standardized extraction form. Study data included first author, year of publication, study design, sample size, age, gender, Eastern Cooperative Oncology Group performance status (ECOG-PS), BCLC stage, Child-Pugh class, alpha-fetoprotein (AFP), portal vein tumor thrombus (PVTT), follow-up, description of interventions, and type of outcome measures. Efficacy outcome measures included OS, PFS, and TTP, described as hazard ratio (HR) with 95% confidence interval (95%CI), and DCR, defined as the percentage of patients whose response was complete response, partial response, or stable disease. Safety outcomes included any adverse event (AE) reported by patients, grade ≥ 3 AEs, and typical AEs. Any controversy between investigators was resolved by discussion.

Quality assessment

The quality of the randomized controlled trials (RCT) was assessed using the Jadad scale, while the retrospective studies were assessed using the Newcastle-Ottawa scale.

Statistical analysis

Meta-analysis was conducted using Review Manager 5.4 (Cochrane Collaboration, Oxford, United Kingdom) using a random-effects model. Pooled continuous data are described as HR while pooled dichotomous data are described as risk ratio (RR), with 95%CI. Heterogeneity was assessed through χ^2 test and I^2 statistic, with values over 60% indicating substantial heterogeneity. Sensitivity analysis was conducted through the leave-one-out approach if needed. Publication bias was not assessed since the number of included studies was too small.

RESULTS

Study selection and characteristics

Overall, a total of 4335 unique studies were captured after removing duplicates, and then nine were retained as potentially eligible trials for full-text assessment. After deleting four ineligible studies (two ongoing trials, one repeated study, and one unobtainable data study), five studies were finally included for meta-analysis[19-23] (Figure 1).

The baseline characteristics of patients from the included studies are summarized in Table 1. The five studies consisted of four retrospective studies[19,21-23] and one RCT[20]. A total of 2780 patients with advanced HCC were included, of which 751 received sorafenib plus TACE and 2029 received sorafenib alone. The participants were at ages of 50 to 70 years mostly, with the majority being male. At baseline, all patients had an ECOG-PS of 0 or 1-2, and most patients had BCLC stage C, and Child-Pugh class A. Four of five trials reported the characteristics of AFP and PVTT in patients[19-22].

The details on intervention characteristics and outcome measures of the included trials are summarized in Table 2. Obvious differences were found in intervention program, namely, the sequence and interval between sorafenib administration and TACE operation in the sorafenib plus TACE arm across studies. Sorafenib treatment was started after TACE operation in two trials[19,22], while sorafenib administration was initiated prior to TACE in another three[20,21,23]. Generally, sorfenib was orally administrated at 400 mg twice daily[20,21]. Among trials reporting the median period of sorafenib administration, it ranged from the minimum 0.1 mo to maximum 48.4 mo across studies. Varied combinations of outcome measures from OS, PFS, TTP, and DCR, were adopted in different trials, with OS adopted in all trials.

Table 1 Baseline characteristics of patients with advanced hepatocellular carcinoma receiving sorafenib with or without add-on transcatheter arterial chemoembolization

Ref.	Study design	Group	Number of cases	Age[1] (yr)	Male	ECOG-PS		BCLC stage			Child-Pugh class		AFP		PVTT
						0	1-2	A	B	C	A	B			
Koch *et al*[19], 2021	Retrospective cohort study	Sorafenib + TACE	54	64 (34-77)	47 (87)	16 (30)	38 (70)	0 (0)	0 (0)	54 (100)	40 (74)	14 (26)	< 400 ng/mL; 36 (66)	≥ 400 ng/mL; 18 (34)	18 (33)
		Sorafenib alone	82	66 (28-85)	72 (88)	37 (45)	45 (55)	0 (0)	0 (0)	82 (100)	61 (74)	21 (26)	52 (64)	30 (36)	27 (33)
Park *et al*[20], 2019	Multi-center RCT phase III	Sorafenib + TACE	170	60 ± 10	136 (80)	136 (80)	34 (20)	3 (2)	39 (23)	128 (75)	148 (87)	22 (13)	< 200 ng/mL; 79 (47)	≥ 200 ng/mL; 91 (54)	68 (40)
		Sorafenib alone	169	61 ± 10	147 (87)	140 (83)	29 (17)	0 (0)	44 (26)	125 (74)	147 (87)	22 (13)	76 (45)	93 (55)	63 (37)
Kok *et al*[23], 2019	Retrospective cohort study	Sorafenib + TACE	426	60 (51-69)	355 (83)	NA		0 (0)	0 (0)	426 (100)	426 (100)	0 (0)	NA		NA
		Sorafenib alone	1686	60 (52-68)	1410 (84)	NA		0 (0)	0 (0)	1686(100)	1686 (100)	0 (0)	NA		NA
Wu *et al*[21], 2017	Retrospective study	Sorafenib + TACE	56	50 ± 12	48 (86)	NA		0 (0)	10 (18)	46 (82)	45 (80)	11 (20)	< 400 ng/mL; 33 (59)	≥ 400 ng/mL; 23 (41)	32 (57)
		Sorafenib alone	48	48 ± 13	46 (96)	NA		0 (0)	16 (33)	32 (67)	46 (96)	2 (4)	23 (49)	24 (51)	24 (50)
Zhang *et al*[22], 2015	Retrospective study	Sorafenib + TACE	45	50 ± 9	43 (96)		45 (100)	NA			34 (76)	11 (24)	< 200 ng/mL; 3 (7)	≥ 200 ng/mL; 42 (93)	45 (100)
		Sorafenib alone	44	54 ± 10	41 (93)		44 (100)	NA			34 (77)	10 (23)	9 (20)	35 (80)	44 (100)

[1]Ages are expressed as the median (range) or mean ± SD.

Data are expressed as *n* (%) for categories. ECOG-PS: Eastern Cooperative Oncology Group performance status; BCLC: Barcelona Clinic Liver Cancer; AFP: Alpha-fetoprotein; PVTT: Portal vein tumor thrombus; TACE: Transcatheter arterial chemoembolization; NA: Not available.

Quality assessment

The quality of the data from four retrospective studies[19,21-23] was evaluated using the Newcastle-Ottawa scale. All retrospective studies received a score of 8, suggesting that the data were of good quality. The quality of the Park *et al*[20]'s study that was a RCT, was evaluated using the Jadad scale. The data were considered of high quality as it received a score of 3. All included studies may have detection bias as the outcome assessors in all trials were not blinded. The details of study quality assessment are summarized in Table 3.

Data synthesis and meta-analysis

OS-primary outcome:OS is objective and clinically relevant, serving as the sole robust endpoint in the management of HCC, and all included trials reported OS as an endpoint. Thus, OS was chosen as the primary outcome in the present study. All five studies[19-23] provided point estimates and 95%CI for HR regarding OS; hence, all were included for the meta-analysis. The results suggested that patients treated with sorafenib plus TACE had better outcomes regarding OS compared to those treated with sorafenib alone: HRs ranged from 0.34 to 1.17, with a combined HR of 0.65 (95%CI:

Table 2 Intervention characteristics and outcome measures of the trials included in this meta-analysis

Ref.	Intervention	Patients	Follow-up (mo)	Sorafenib dose	Sorafenib duration (mo)	OS (mo)		TTP (mo)		PFS (mo)		DCR (%)
						Median	HR (95%CI)	Median	HR (95%CI)	Median	HR (95%CI)	
Koch *et al* [19], 2021	TACE was usually initiated before sorafenib	54	NA	NA	NA	16.5	0.34 (0.23-0.53)	7.0	NA	NA	NA	28/53 (53)
	Sorafenib alone	82	NA		NA	8.4		4.1		NA		17/74 (23)
Park *et al* [20], 2019	Sorafenib initiated within 3 d of randomization, first TACE initiated between 7 and 21 d after randomization	170	14 (4-27)	200-400 mg twice daily, then 400 mg twice daily	5.5 (0.1-41.6)	12.8	0.91 (0.69-1.21)	5.3	0.67 (0.53-0.85)	5.2	0.73 (0.59-0.91)	103/170 (61)
	Sorafenib initiated within 3 d of randomization	169	19 (2-27)		4.3 (0.2-48.4)	10.8		3.5		3.6		80/169 (47)
Kok *et al* [23], 2019	Sorafenib prior to TACE	426	7.4 (4.7-11.5)	NA	4.7 (4.2-5.3)	12.5	0.74 (0.63-0.88)	4.7	0.76 (0.65-0.89)	NA	NA	NA
	Sorafenib alone	1686	4.4 (2.3-8.4)		2.8 (2.6-3.0)	6.7		2.8		NA		
Wu *et al*[21], 2017	Sorafenib prior to TACE	56	NA	400 mg twice daily	NA	22	0.50 (0.28-0.89)	NA	NA	8	0.46 (0.27-0.78)	27/48 (56)[1]
	Sorafenib alone	48	NA		NA	18		NA		6		23/40 (58)
Zhang *et al* [22], 2015	Sorafenib started 1-3 d after TACE	45	7.3 (2-18)	NA	5.6 (1-18)	7	1.17 (0.52-1.81)	3	NA	NA	NA	24/43 (60)
	Sorafenib alone	44			5.4 (1-17)	6		3		NA		18/44 (51)

[1]DCR was measured at the 6th month.

Data are *n* (%) for categories, and median for continuous data. OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; DCR: Disease control rate; TACE: Transarterial chemoembolization; HR: Hazard ratio; 95%CI: 95% confidence interval; NA: Not available.

0.46–0.93, P = 0.02, n = 2780; Figure 2A). Because of high heterogeneity across studies, a sensitivity analysis was conducted. Removal of the study of Koch *et al*[19] caused the heterogeneity to become non-significant, while the results of the pooled OS were almost identical.

PFS-secondary outcome

Only two[20,21] of the five studies reported the point estimate (HR) and 95%CI for PFS. The combined HR showed that the PFS significantly differed between patients treated with sorafenib plus TACE and those treated with sorafenib alone (combined HR = 0.62, 95%CI: 0.40–0.96, P = 0.03, n = 443; Figure 2B).

Table 3 Quality assessment of included trials

Ref.	Newcastle-Ottawa scale				
	Selection	Comparability	Outcome	Score	Quality
Koch *et al*[19], 2021	3	2	3	8	Good
Kok *et al*[23], 2019	3	2	3	8	Good
Wu *et al*[21], 2017	3	2	3	8	Good
Zhang *et al*[22], 2015	3	2	3	8	Good
Park *et al*[20], 2019	Jadad scale				
	Randomization	Double blinding	Withdrawals and dropouts	Score	Quality
	2		1	3	Good

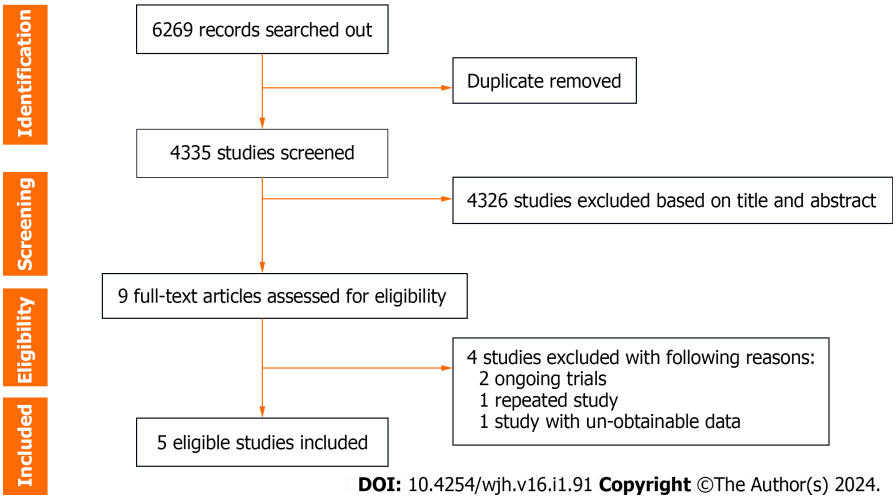

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Figure 1 Flowchart of study selection.

TTP-secondary outcome

Three studies were excluded from the meta-analysis without 95%CI for TTP; hence, only two[20,23] studies were used for the meta-analysis. The pooled result was positive, with an HR of 0.73 (95%CI: 0.64-0.83, $P < 0.00001$, $n = 2451$; Figure 2C), indicating better outcome regarding TTP achieved by sorafenib plus TACE as compared with sorafenib alone.

DCR-secondary outcome

Four[19-22] of five studies were included in meta-analysis for DCR after excluding the study of Kok *et al*[23], which did not provide the relevant data. The meta-analysis yielded positive results for pooled DCR, with a combined RR of 1.36 (95%CI: 1.02-1.81, $P = 0.04$, $n = 641$, Figure 2D), revealing that patients receiving sorafenib plus TACE had better prognoses in terms of DCR, compared to those treated with sorafenib alone. Sensitivity analysis indicated that removal of the study of Koch *et al*[19] eliminated the heterogeneity, while the results of pooled DCR were almost identical.

AE-secondary outcome

The summary of AEs is shown in Table 4. We classified the outcomes of AEs as any AE, grade ≥ 3 AEs, and typical AEs. The meta-analysis for any AE with inclusion of two studies demonstrated that the differences in the incidence of any AE was significant (RR = 1.07, 95%CI: 1.01-1.13, $P = 0.01$, $n = 448$; Figure 3A). Whereas, the incidence of grade ≥ 3 AEs was not statistically significant (RR = 1.09, 95%CI: 0.71-1.67, $P = 0.69$, $n = 321$; Figure 3B), as indicated by meta-analysis including the studies of Koch *et al*[19] and Wu *et al*[21]. The typical AEs across the trials related to sorafenib plus TACE treatment were hand-foot skin reactions (HFSR), diarrhea, hypertension, fatigue, alopecia, abdominal pain, and vomiting. The pooled results of typical AEs are presented in a forest plot in Figure 4. Among these AEs, only abdominal pain showed a significant difference between the sorafenib plus TACE group and sorafenib group (combined RR = 14.95, 95%CI: 1.13-198.39, $P = 0.04$, $n = 641$), while others demonstrated no significant difference (Figure 4).

Table 4 Summary of adverse events occurring in either group of the trials included in this meta-analysis

Ref.	Group	Patients	AE								
			Any	Grade ≥ 3	HFSR	Diarrhoea	Hypertension	Fatigue	Alopecia	Abdominal pain	Vomiting
Koch *et al*[19], 2021	Sorafenib + TACE	50	43 (86)	17 (34)	12 (24)	13 (26)	NA	3 (6)	NA	14 (41)	5 (14)
	Sorafenib alone	78	62 (80)	25 (32)	13 (17)	17 (22)	NA	6 (8)	NA	0 (0)	0 (0)
Park *et al*[20], 2019	Sorafenib + TACE	153	148 (97)	NA	74 (48)	60 (39)	27 (18)	24 (16)	23 (15)	82 (54)	31 (20)
	Sorafenib alone	167	151 (90)	NA	88 (53)	54 (32)	23 (14)	24 (14)	25 (15)	29 (17)	11 (7)
Kok *et al*[23], 2019	Sorafenib + TACE	NA									
	Sorafenib alone	NA									
Wu *et al*[21], 2017	Sorafenib + TACE	56	NA	13 (23)	30 (54)	25 (45)	13 (23)	12 (21)	4 (7)	NA	31 (55)
	Sorafenib alone	48	NA	14 (29)	36 (75)	27 (56)	22 (46)	12 (25)	3 (6)	NA	27 (56)
Zhang *et al* [22], 2015	Sorafenib + TACE	45	NA	12 (27)	29 (64)	20 (44)	1 (2)	11 (24)	25 (56)	26 (58)	21 (47)
	Sorafenib alone	44	NA	6 (14)	26 (59)	19 (43)	2 (5)	12 (27)	22 (50)	0 (0)	0 (0)

AE: Adverse event; HFSR: Hand-foot skin reaction; TACE: Transarterial chemoembolization; NA: Not available.

DISCUSSION

The present work presents the most comprehensive synthesis of data for currently available comparisons of the efficacy and safety of sorafenib plus TACE *vs* sorafenib alone in treating patients with advanced HCC. We identified data for meta-analysis from five studies that enrolled a total of 2780 patients[19-23]. We found that the addition of TACE to sorafenib improved OS, PFS, TTP, and DCR, compared to sorafenib alone. Besides, addition of TACE increased the incidence of any AE but not grade ≥ 3 AEs.

As a multi-kinase inhibitor, sorafenib was speculated to assist TACE in the management of HCC, as it can suppress angiogenesis in tumours by abolishing VEGF upregulation induced by TACE[24]. Therefore, numerous clinical studies have compared the efficacy and safety of sorafenib combined with TACE *vs* TACE alone; however, they yielded inconsistent results. Therefore, the potential synergies remain controversial in treating patients with unresectable HCC. Likewise, since TACE was also suggested as a treatment option for advanced HCC[25,26], investigators worldwide began to study whether TACE could improve the outcomes of patients treated with sorafenib for advanced HCC. Zhang *et al*[22] reported that the addition of TACE to sorafenib did not provide benefit regarding OS and PFS *vs* sorafenib monotherapy (OS: 7.0 mo *vs* 6.0 mo, $P = 5.544$; PFS: 3.0 mo *vs* 3.0 mo, $P = 5.924$). Whereas, the study of Wu *et al*[21] showed that TACE + sorafenib combination yielded better OS (HR = 0.498, 95%CI: 0.278-0.892, $P = 0.019$), based on multivariate Cox regression analysis. The only multi-center phase III trial[20] comprising 339 patients with advanced HCC reported that the addition of TACE to sorafenib did not improve OS (HR = 0.91; 90%CI: 0.69-1.21, $P = 0.290$), but improved PFS and TTP. On the contrary, two studies of Kok *et al*[23] and Koch *et al*[19] demonstrated that the combination therapy significantly prolong OS compared to sorafenib monotherapy (381 d *vs* 204 d, HR = 0.74, 95%CI: 0.63-0.88, $P = 0.021$[23]; 12.8 mo *vs* 10.8 mo, 16.5 mo *vs* 8.4 mo, HR = 0.34, 95%CI: 0.23-0.53, $P < 0.001$[19]). In agreement with the majority of these studies, our meta-analysis also revealed a significantly longer OS in patients receiving TACE + sorafenib than in those receiving sorafenib monotherapy. Besides, the outcomes of PFS, TTP, and DCR were also significantly improved by the addition of TACE. Regarding safety, the incidence of any AE was increased due to the addition of TACE; however, no significant difference was found in grade ≥ 3 AEs. Specifically, the most common AEs were HFSR, diarrhoea, and hypertension for sorafenib, while abdominal pain for TACE[27]. Our meta-analysis indicated that the addition of TACE did not seem to increase toxicity associated with sorafenib. Taken together, the presented data support using sorafenib/TACE combination therapy for the treatment of advanced HCC. However, these positive findings still need further confirmation by more high-quality multi-centre RCTs with large samples and reliable design.

The findings of our meta-analysis were limited by the small number of included studies (range, 2–5 comparative studies). Especially, the majority of included studies were not randomized, assessor-blinded trials. Our work was also limited by the obvious heterogeneity across studies used in the meta-analysis for several outcomes, which might originate from the differences in clinical characteristics of patients of different studies, such as ECOG-PS, BCLC stage, and Child-

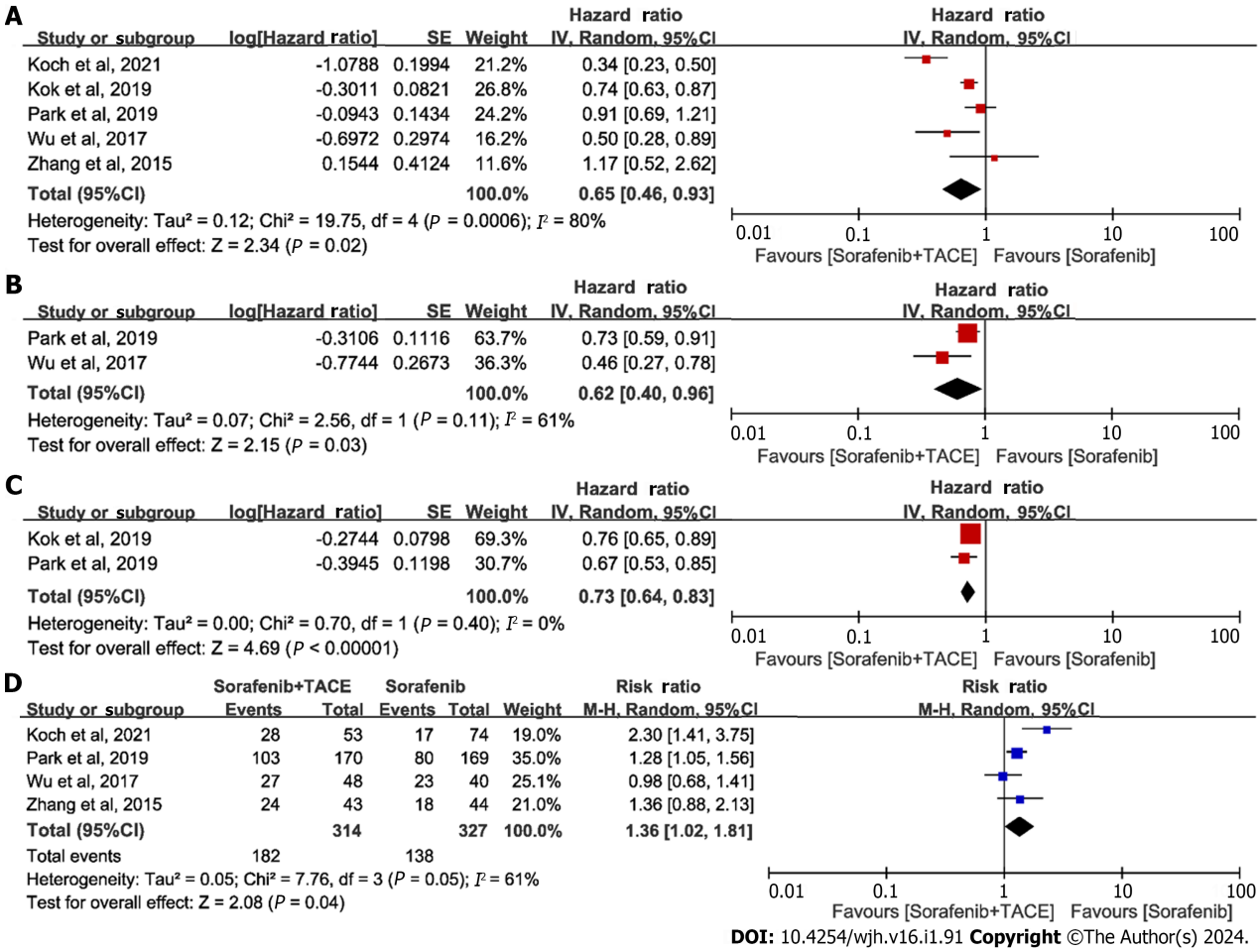

Figure 2 Meta-analysis of efficacy outcomes in patients with advanced hepatocellular carcinoma receiving sorafenib plus transarterial chemoembolization or sorafenib alone. A: Forest plot of overall survival; B: Forest plot of progression free survival; C: Forest of time to progression; D: Forest plot of disease control rate. The pooled results were calculated by using a random-effects model. 95%CI: 95% confidence interval; TACE: Transarterial chemoembolization.

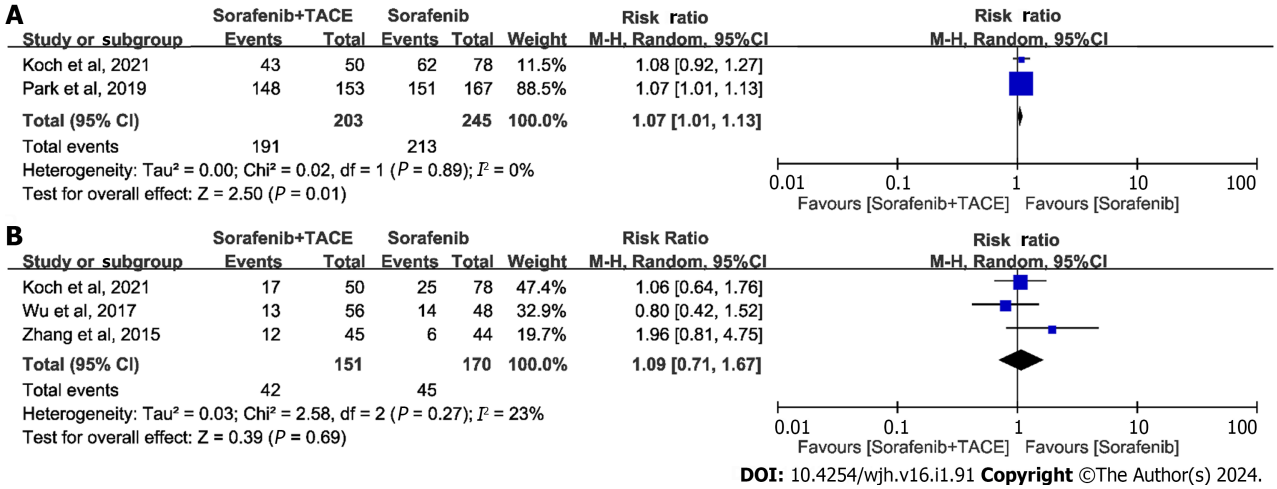

Figure 3 Meta-analysis of safety outcomes in patients with advanced hepatocellular carcinoma receiving sorafenib plus transarterial chemoembolization or sorafenib alone. A: Forest plot of any adverse event (AE); B: Forest plot of grade ≥ 3 AEs. 95%CI: 95% confidence interval; TACE: Transarterial chemoembolization.

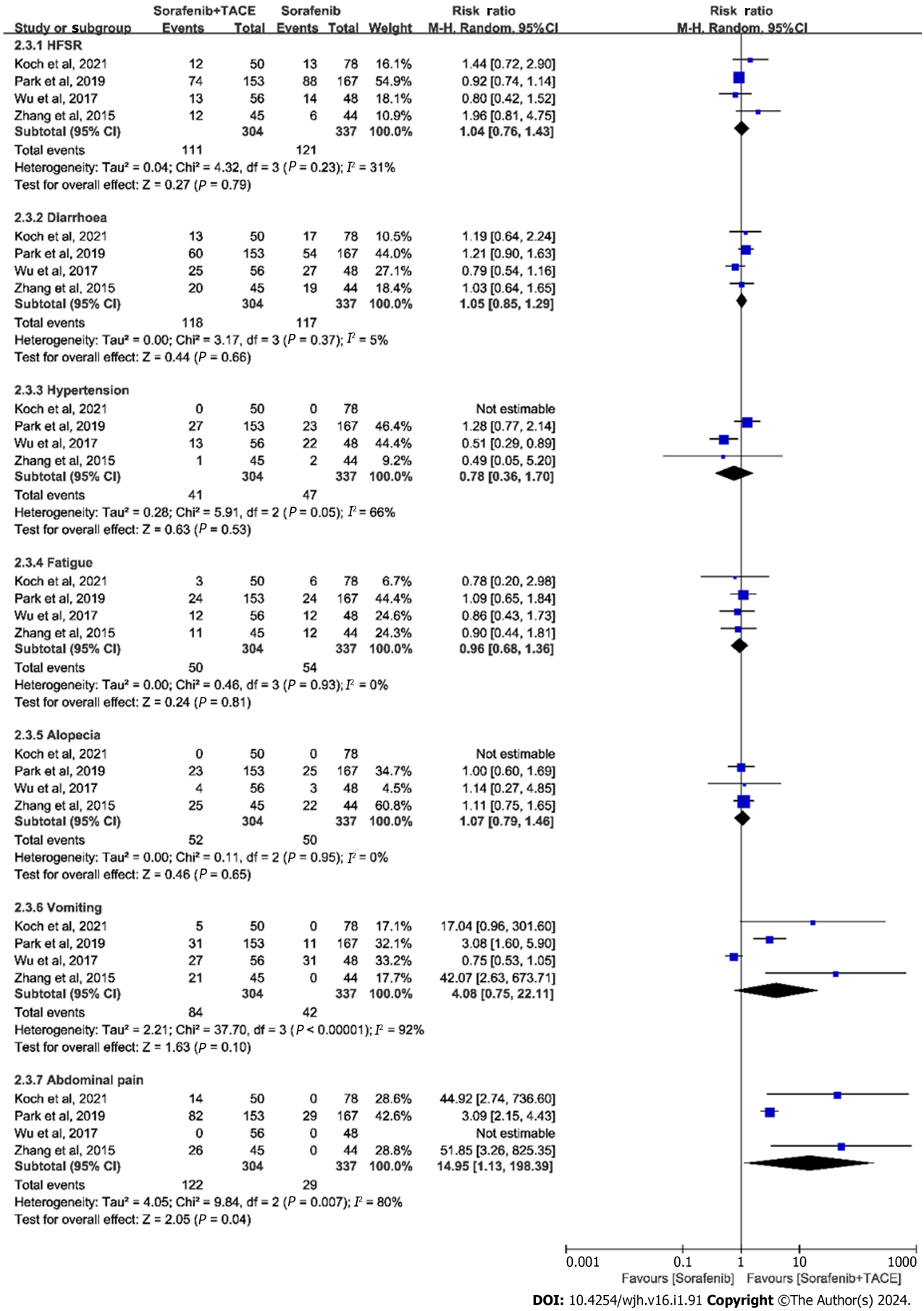

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Figure 4 Meta-analysis of incidence of typical AEs in patients with advanced hepatocellular carcinoma receiving sorafenib plus

transarterial chemoembolization or sorafenib alone. AE: Adverse event; HFSR: Hand-foot skin reaction; 95%CI: 95% confidence interval; TACE: Transarterial chemoembolization.

Pugh class. Finally, there were differences across included trials in the definition of tumour response. The phase III study defined tumour response using the RECIST 1.1 criteria[20], three studies used mRECIST criteria[19,21,22], and one study did not report the criteria used[23].

CONCLUSION

In summary, the combination of sorafenib with TACE has superior efficacy to sorafenib monotherapy, as evidenced by the prolonged OS, PFS, and TTP, as well as the increased DCR. The addition of TACE does not cause additional toxicity associated with sorafenib. Additional RCTs are required to further investigate the clinical benefit of this combination therapy in treatment of advanced HCC.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a rising global health problem which represents one of the leading causes of cancer-related mortality. Although remarkable advances in treatments have been achieved for HCC, the overall prognosis is still dismal in patients, especially those at advanced stage. Several trials have focused on combining sorafenib with other systemic therapies to augment its clinical benefit.

Research motivation

Recently, a number of comparative trials worldwide have been conducted to investigate whether sorafenib/transarterial chemoembolization (TACE) combination therapy could improve clinical outcomes in patients with advanced HCC, compared with sorafenib monotherapy. However, the obtained findings are conflicting.

Research objectives

To investigate the potential synergies and safety of sorafenib plus TACE *vs* sorafenib alone for treating advanced HCC.

Research methods

This meta-analysis involved a large sample size to evaluate whether sorafenib plus TACE provides clinical benefit *vs* sorafenib monotherapy in patients with advanced HCC, in terms of overall survival (OS), progression free survival (PFS), time to progression (TTP), disease control rate (DCR), and adverse events (AEs).

Research results

It was found that patients treated with sorafenib plus TACE had better prognoses in terms of prolonged OS, PFS, and TTP, as well as increased DCR. Besides, the incidence of any AE was increased due to the addition of TACE; however, there was no significant effect on grade ≥ 3 AEs.

Research conclusions

The combination of sorafenib with TACE has superior efficacy to sorafenib monotherapy, with an acceptable safety profile.

Research perspectives

The addition of TACE to sorafenib is clinically feasible and safe in patients with advanced HCC. The positive findings of the present study might be beneficial to the management of advanced HCC. Additional randomized controlled studies are still necessary to further validating these clinical benefits.

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FOOTNOTES

Co-first authors: Hong-Jie Yang and Bin Ye.

Author contributions: Yang HJ and Ye B were responsible for data acquisition, analysis, and interpretation and drafted the article; Liao JX and Lei L were responsible for data analysis and interpretation and revised the article; Chen K was responsible for conception and design of the study and critical revision of the article; all authors issued final approval for the version to be submitted. Yang HJ and Ye B contributed equally to this work as co-first authors. The reasons for designating Yang HJ and Ye B as co-first authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-first authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-first authors best reflects this diversity. Third, Yang HJ and Ye B contributed efforts of equal substance throughout the research process. In summary, we believe that designating Yang HJ and Ye B as co-first authors is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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CASE REPORT

Pylephlebitis-induced acute liver failure: A case report and review of literature

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Abstract

BACKGROUND

Pylephlebitis is an extremely rare form of septic thrombophlebitis involving the portal vein, carrying high rates of morbidity and mortality.

CASE SUMMARY

We present a case of a 42-year-old male with no past medical history who presented with acute onset of abdominal pain and altered mental status with laboratory tests demonstrating new-onset acute liver failure. Pylephlebitis was determined to be the underlying etiology due to subsequent workup revealing polymicrobial gram-negative anaerobic bacteremia and complete thrombosis of the main and left portal veins. To our knowledge, this is the first documented case of acute liver failure as a potential life-threatening complication of pylephlebitis.

CONCLUSION

Our case highlights the importance of considering pylephlebitis in the broad differential for abdominal pain, especially if there are co-existing risk factors for hypercoagulability. We also demonstrate that fulminant hepatic failure in these patients can potentially be reversible with the immediate initiation of antibiotics and anticoagulation.

Key Words: Portal vein thrombosis; Septic thrombophlebitis; Gram negative anaerobic bacteremia; Pylephlebitis; Acute liver failure; Case report

Core Tip: Septic thrombosis of the portal vein, also known as pylephlebitis, is difficult to diagnose as it often presents with non-specific symptoms including fever and abdominal pain. As a result, a high clinical suspicion for pylephlebitis is warranted since this condition is life-threatening without treatment. We aim to highlight acute liver failure as a possible life-threatening sequela of pylephlebitis. Furthermore, we demonstrate that prompt initiation of antibiotics and possible anticoagulation can result in complete resolution of fulminant hepatic failure.

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INTRODUCTION

Acute liver failure (ALF) is a life-threatening form of severe hepatocyte necrosis that results in impaired synthetic function and encephalopathy in individuals without preexisting liver disease[1]. Abrupt damage to the liver parenchyma can result in a triad of clinical findings including rapid elevation of aminotransferase enzymes levels, altered mental status, and impaired coagulation[2]. ALF typically manifests within a few days of an acute insult, has a disease course of less than 26 wk, and can be distinguished from acute on chronic decompensated liver failure by the absence of previous liver disease[3,4]. Approximately 3000 annual cases of ALF occur in the United States each year with the most common cause being acetaminophen toxicity[5]. Other reported etiologies include viral hepatitis, drug-induced liver injury, ischemia, autoimmune hepatitis, and Budd-Chiari syndrome[6]. To date, few case reports have demonstrated portal vein thrombosis as a cause of new-onset hepatic dysfunction[7-9]. Even more rare is septic thrombophlebitis of the portal vein, known as pylephlebitis, resulting in ALF. To our knowledge, we present the first reported case of pylephlebitis resulting in fulminant hepatic failure in a young patient which was successfully reversed with prompt initiation of antibiotics and anticoagulation.

CASE PRESENTATION

Chief complaints

A 42-year-old male with no past medical history presented with a three-day history of right upper quadrant abdominal pain and altered mental status.

History of present illness

Three-day history of right upper quadrant abdominal pain and altered mental status.

History of past illness

Non-contributory.

Personal and family history

Non-contributory.

Physical examination

In the emergency department, he was visibly jaundiced. Vitals were significant for fever of 38.5° C or 101.4° F, tachycardia at 135 beats-per-minute, and hypotension with blood pressure of 88/48 mmHg.

Laboratory examinations

Initial laboratory tests were normal except for leukocytosis of 22.2 $\times 10^3/\mu L$ (Ref: 4.5-11.0 $\times 10^3/\mu L$) and elevated lactic acid at 4.1 mmol/L (Ref: 0.5-2.0 mmol/L).

Imaging examinations

Due to concern of sepsis, broad-spectrum antibiotics were started, along with fluid resuscitation. However, a computed tomography (CT) scan of the abdomen and pelvis with intravenous (IV) contrast did not reveal any signs of infection or evidence of cirrhosis. Additional investigations for a source of infection including chest X-ray, urinalysis, right upper quadrant ultrasound, magnetic resonance cholangiopancreatography, and transthoracic echocardiogram failed to reveal an infective source.

FINAL DIAGNOSIS

Two days after admission, blood cultures grew Fusobacterium necrophorum and Bacteroides ovatus. The blood culture susceptibility report demonstrated sensitivity to ampicillin/sulbactam with the antibiotic regimen narrowed appropriately. Ultimately, a vascular abdominal ultrasound was ordered, demonstrating complete thrombosis of both the main and left portal veins (Figure 1, respectively). The diagnosis of spontaneous pylephlebitis was made.

TREATMENT

A therapeutic dose heparin drip was started. Repeat blood cultures two days later showed resolution of the bacteremia. An investigation for the patient's risk factors for hypercoagulability revealed an extensive smoking history of 20-pack years and obesity with a body mass index of 31.2 kg/m^2, in addition to the sepsis-induced inflammatory state. A peripherally inserted central catheter was placed and the patient was discharged on a six-week total course of IV ampicillin/sulbactam 3 g every six hours and also transitioned to a six-month total course of oral rivaroxaban 20 mg daily.

OUTCOME AND FOLLOW-UP

The patient was scheduled for outpatient follow-up in the clinic one week later with repeat blood work at that time demonstrating complete resolution of the liver impairment and discharged from the hospital after an nine day hospital course.

DISCUSSION

Thrombophlebitis is characterized by a venous inflammation accompanied by venous thrombosis[10]. When an endovascular thrombus occurs in the setting of concurrent infection, it is referred to as septic thrombophlebitis[11]. The specific term pylephlebitis is used to describe an extremely rare form of septic thrombophlebitis of the portal vein with an estimated annual incidence of 0.37-2.7 cases per 100000[12]. Pylephlebitis typically occurs in response to an abdominal inflammatory process that results in uncontrolled infection in the regions adjacent or draining into the portal venous system, most often caused by Gram-negative anaerobic bacteria[13]. This is similar to Lemierre's syndrome, caused by the same organism as seen in our case, Fusobacterium necrophorum, in which the bacterium extends to the parapharyngeal space causing septic thrombophlebitis of the internal jugular vein[14]. In this same vein, pylephlebitis shares similar pathophysiologic concepts as Lemierre's syndrome and can be attributed to vascular changes from gram-negative anaerobic bacteria infiltration[14].

Multiple case reports have described various intra-abdominal inflammatory conditions such as diverticulitis, appendicitis, and pancreatitis developing into septic portal vein thrombosis due to direct invasion from an adjacent nonvascular infection[15-18]. Diagnosis is often challenging as common presentations include non-specific symptoms such as generalized abdominal pain and fever[19]. A CT scan with oral and IV contrast is the imaging modality of choice as it can detect both the portal vein thrombosis and intra-abdominal infection.

In the absence of a source of inflammation or infection like our case, another proposed mechanism involves an obstructive clot that promotes bacterial colonization in a manner similar to that of ascending cholangitis[20]. In cases of pylephlebitis where no intra-abdominal infection can be identified, an investigation as to the pathogenesis of the underlying acute thrombosis of the portal vein is warranted. A majority of cases of portal vein thrombosis occur in patients with cirrhosis or malignancy due to the inherent hypercoagulable nature of these conditions[21]. However, a small subset of patients may develop thrombosis in this unusual vascular territory as a result of other prothrombogenic conditions including inherited and acquired thrombophilias[22]. After these conditions are ruled out, it is reasonable to attribute the portal vein clot to a multifactorial etiology if various pro-thromboembolic risk factors are present such as ongoing inflammation, extensive smoking history, and obesity as in our patient[23].

Complications of pylephlebitis are scarcely documented in the literature but small bowel infarction, hepatic abscesses, and septic pulmonary emboli have been reported[24]. To our knowledge, there have been no documented cases of new-onset ALF as a sequela of pylephlebitis to date. As a result, the mechanism of ALF in pylephlebitis is poorly understood. The potential pathogenesis may first involve the natural progression of pylephlebitis which typically first involves thrombophlebitis of the smaller mesenteric veins with subsequent migration of the thrombosis to the larger portal veins [25]. It is possible that this thromboembolic event may rapidly occur in the same manner as pulmonary embolism, preventing adequate hemodynamic compensatory responses. It is also possible that liver failure may originate from an alternative mechanism of diffuse microemboli in the smaller hepatic vessels similar to those postulated and seen in patients with coronavirus disease 2019[26]. With approximately 75% of hepatic blood flow coming from the portal venous system, acute suppurative thrombosis can cause significant damage to hepatocytes and allow progression to ALF when there is immediate complete occlusion of one or more portal veins[27]. Unlike cases of portal vein thrombosis in cirrhotic patients where the obstructive clot forms slowly in the portal venous system, the rapid nature of pylephlebitis may not allow for normal hepatic artery vasodilation and the development of venous collaterals which typically takes 3-5 wk to

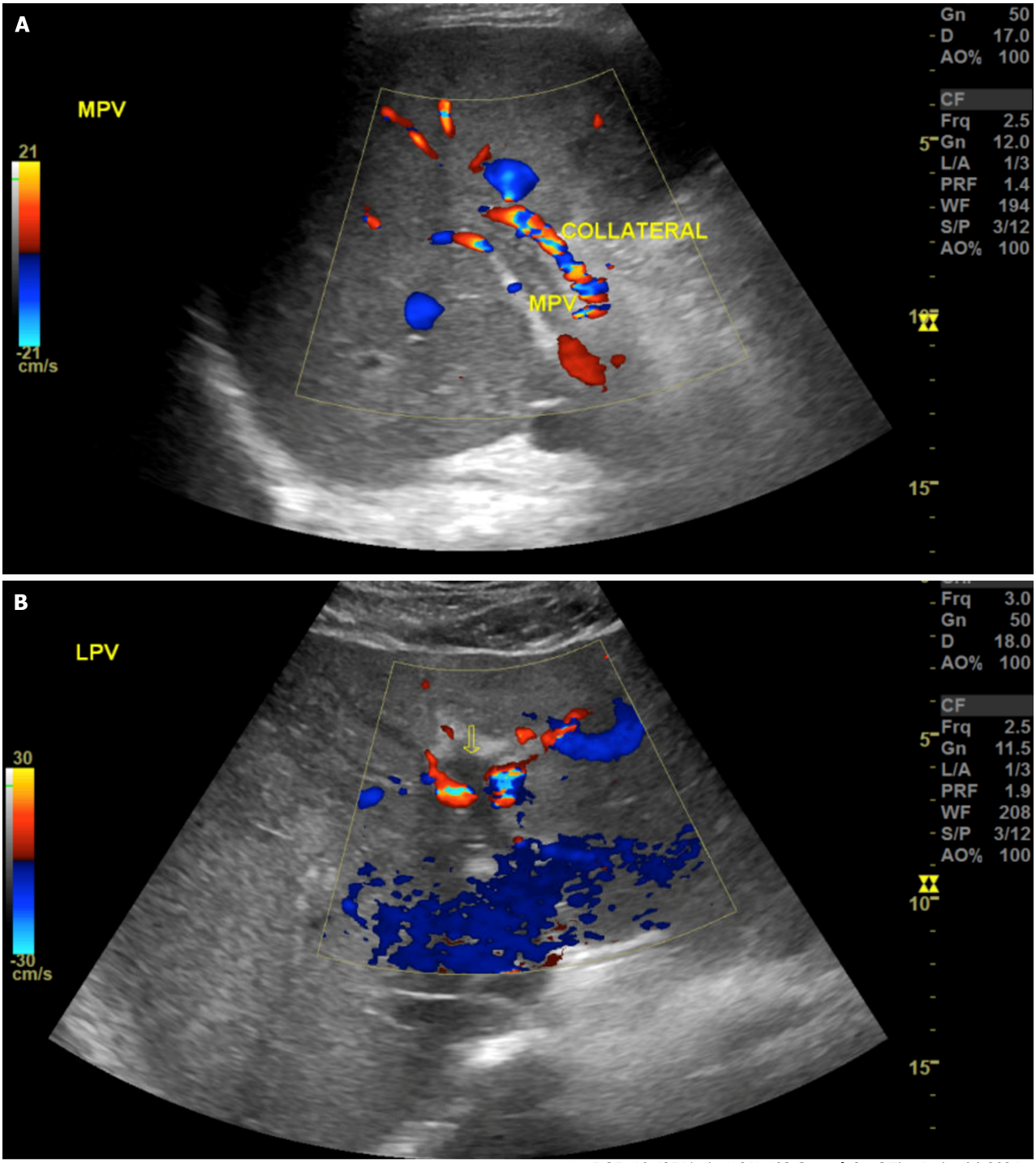

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Figure 1 Abdominal vascular ultrasound. A: With complete thrombosis of the main portal vein; B: With complete thrombosis of the left portal vein.

fully develop[28].

Recognizing ALF as a complication of septic thrombophlebitis is of clinical importance as this condition carries significant morbidity and a mortality rate of 30-50%[29]. Antibiotics are the primary form of therapy with parenteral antibiotics recommended, followed by a transition to an oral regimen for a total duration of 4-6 wk[30]. While Rivaroxaban was utilized in our case with success, the role of anticoagulation is an area of current controversy[13,31]. Emerging research, however, points to improved patient outcomes with the use of anticoagulation. To highlight this point, one retrospective review by Naymagon *et al*[32] reviewed 67 patients with pylephlebitis and found that the use of anticoagulation significantly improved the rate of portal vein thrombosis resolution. Additional studies with larger patient populations are needed to further confirm these findings, along with a special focus on which specific type of anticoagulation has the greatest efficacy. Despite the life-threatening nature of septic portal vein thrombophlebitis, it is often overlooked in the differential diagnosis of new-onset abdominal pain and fever. Awareness of pylephlebitis is important as swift recognition and initiation of broad-spectrum antibiotics in conjunction with possible anticoagulation is paramount to reducing mortality[33]. Ultimately, our case aims to highlight acute liver failure as an extremely rare

presentation of septic portal vein thrombosis and demonstrates that this disease process is reversible with prompt intervention.

CONCLUSION

Septic thrombosis of the portal vein, also known as pylephlebitis, is difficult to diagnose as it often presents with non-specific symptoms including fever and abdominal pain. A high degree of clinical suspicion for pylephlebitis is warranted since this condition carries high morbidity and mortality without treatment. It is important to recognize acute liver failure as a possible life-threatening sequela of pylephlebitis. Antibiotics should be administered immediately, along with consideration for anticoagulation, as it can potentially lead to complete resolution of fulminant hepatic failure in these patients.

FOOTNOTES

Author contributions: Hapshy V wrote and edited the manuscript in addition to contributing to the literature review; Imburgio S wrote and edited the manuscript in addition to contributing to the literature review; Sanekommu H wrote and edited the manuscript; Nightingale B wrote and edited the manuscript; Hossain MA and Taj S edited and supervised the manuscript; Patel S edited and supervised the manuscript.

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LETTER TO THE EDITOR

Review on article of effects of tenofovir alafenamide and entecavir in chronic hepatitis B virus patients

Yu-Tong Sun, Qian-Qian Chen

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Abstract

This letter comments on the article which reported that tenofovir alafenamide may increase blood lipid levels compared with entecavir in patients with chronic hepatitis B published on *World J Hepatol* 2023 August 27. We review the related research content, topic selection, methodology, conclusions, strengths and weaknesses of this article. And evaluate it in relation to other published relevant articles.

Key Words: Tenofovir alafenamide; Entecavir; Serum lipid levels; Hepatitis B virus

Core Tip: With the significant increase in the incidence of nonalcoholic fatty liver disease (NAFLD) in China, the number of patients with co-morbid chronic hepatitis B (CHB) and NAFLD has gradually increased. This letter comments on a published study which showned that CHB patients treated with tenofovir alafenamide (TAF) had higher levels of total cholesterol than CHB patients treated with entecavir; however, TAF-induced dyslipidemia did not increase the incidence of NAFLD. We comment on the article.

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TO THE EDITOR

The prevalence of nonalcoholic fatty liver disease (NAFLD) in China has increased significantly in recent decades, giving rise to co-morbid chronic hepatitis B (CHB) and NAFLD in some patients. Many patients with hepatitis B virus (HBV) infection require long-term antiviral drugs such as tenofovir alafenamide (TAF) and entecavir (ETV), which are recommended as first-line agents in the guideline of HBV treatment. It has been shown that TAF has a lipid-enhancing effect in patients with human immunodeficiency virus (HIV) infection. However, A comparison of the effects of TAF and ETV on lipid patients with HBV has not yet been investigated.

The aim of this letter is to discuss the effects of TAF on blood lipid levels and the risk of developing NAFLD in CHB patients, and to compare changes in lipid levels before and after antiviral therapy with TAF or ETV.

We have read with interest the article published on *World J Hepatol* by Lai *et al*[1]. In this study, 336 patients with CHB treated with ETV or TAF were enrolled. The baseline data of patients with CHB and the clinical characteristics, lipids, and metabolic factors before and approximately 1 year after TAF or ETV treatment were statistically analyzed using SPSS 23.0. In addition, the effects of ETV and TAF on high-density lipoprotein, low-density lipoprotein, triglycerides, and total cholesterol (TCHO) were evaluated using a propensity score-matched model.

Post-treatment TCHO levels were significantly higher in the TAF group than in the ETV group. In the propensity score-matched model, TCHO levels were significantly higher than baseline levels in patients in the TAF treatment group, whereas there was no difference in the ETV group. Using logistic regression analysis, body mass index (BMI), gender and other levels were significantly related to TCHO levels. But 1 year of TAF treatment did not increase the incidence of NAFLD. Therefore, in this study TCHO was higher in patients treated with TAF than in patients with CHB who received ETV, but there was no increase in the incidence of NAFLD due to TAF-induced dyslipidemia.

This is a comprehensive study. TAF has been used as a first-line treatment for HIV and CHB infected patients, and can increase blood lipid levels in HIV patients[2]. In addition to comparing the baseline data and clinical features of patients treated with TAF and ETV, this study compared changes in lipid profiles and determined whether NAFLD increased before and after TAF or ETV therapy. The impact and extent of TAF achieving elevated lipid levels compared with ETV, and the correlation between BMI, gender, hypertension, baseline TCHO, CK-MB levels and elevated TCHO levels were assessed.

The research topic is new. In the context of the increasing prevalence of NAFLD and the 84 million people with HBV infection in China, in addition to existing studies showing that TAF can increase lipid levels in patients with HIV, patients treated with TAF had a greater reduction in their lipid profile than those treated with ETV[3,4]. Limited data are available in terms of the effect of TAF on metabolism-related complications in patients with CHB and the effect of ETV on lipids has not yet been reported in post-marketing studies. Therefore, it is important to investigate whether TAF raises lipid levels and increases the prevalence of NAFLD in patients with CHB compared with ETV.

The study is scientifically sound in methodology. Patients with insufficient years of drug use, interference due to other antiviral drugs or comorbidities associated with other liver-related diseases or heavy alcohol intake were excluded, and 336 CHB patients taking TAF or ETV in a single center were enrolled. They were divided into the group receiving TAF and the group receiving ETV. Pre-treatment lipid profiles and repeat lipid assessments were performed 1 year after the initiation of antiviral therapy. Baseline information and data related to clinical characteristics, metabolic levels, and lipids were collected from the enrolled patients before antiviral therapy and after 1 year of treatment. Statistical analysis was performed using SPSS 23.0. Normally distributed continuous variables were expressed as mean ± SD, Student's *t*-tests were performed to assess whether the differences in the treatment groups were statistically significant. Categorical variables were described using frequencies and proportions. This article utilized suitable statistical techniques to examine variables within the in-group and components. Differences in each lipid profile component between pre- and post-treatment were calculated, and the data was analyzed using propensity matching score and logistic regression analysis.

The findings of this study are innovative. It was found that CHB patients treated with TAF had higher elevations in TCHO than those treated with ETV and that metabolic factors were associated with elevated TCHO levels. There have studies found that metabolic factors can reduce the danger of hepatocellular carcinoma in patients with HBV[5]. With few studies on the subject, these findings provide guidance for future treatment of patients with CHB combined with NAFLD.

The study has the following shortcomings: The study period was short; whether TAF and ETV can increase the prevalence of NAFLD in patients with CHB was not effectively verified; this study was a single-center retrospective study, which is prone to retrospective bias and selection bias. Therefore, a large-sample multicenter prospective trial is necessary to verify these findings.

FOOTNOTES

Author contributions: Sun YT drafted the article; Chen QQ made critical revisions related to important intellectual content of the manuscript.

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