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Liver sinusoidal endothelial cell: An important yet often overlooked player in the liver fibrosis

Jiaorong Qu1,*, Le Wang1,*, Yufei Li2, and Xiaojiaoyang Li1

1School of Life Sciences, Beijing University of Chinese Medicine; 2School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China

Liver sinusoidal endothelial cells (LSECs) are liver-specific endothelial cells with the highest permeability than other mammalian endothelial cells, characterized by the presence of fenestrae on their surface, the absence of diaphragms and the lack of basement membrane. Located at the interface between blood and other liver cell types, LSECs mediate the exchange of substances between the blood and the Disse space, playing a crucial role in maintaining substance circulation and homeostasis of multicellular communication. As the initial responders to chronic liver injury, the abnormal LSEC activation not only changes their own physicochemical properties but also interrupts their communication with hepatic stellate cells and hepatocytes, which collectively aggravates the process of liver fibrosis. In this review, we have comprehensively updated the various pathways by which LSECs were involved in the initiation and aggravation of liver fibrosis, including but not limited to cellular phenotypic change, the induction of capillarization, decreased permeability and regulation of intercellular communications. Additionally, the intervention effects and latest regulatory mechanisms of anti-fibrotic drugs involved in each aspect have been summarized and discussed systematically. As we studied deeper into unraveling the intricate role of LSECs in the pathophysiology of liver fibrosis, we unveil a promising horizon that pave the way for enhanced patient outcomes. (Clin Mol Hepatol 2024;30:303-325)

Keywords: Liver sinusoidal endothelial cells; Liver fibrosis; Capillarization; Fenestrae; Intercellular communication

INTRODUCTION

Liver fibrosis commonly occurs as a pathological reparative response to chronic liver diseases, manifesting as the excessive sedimentation or abnormal distribution of extracellular matrix (ECM) components, including collagen and glycoprotein in the space of Disse.1 The majority of patients with liver fibrosis in the early stage have no obvious weaknesses or fatigue and don’t usually present with symptoms; however, as the disease progresses, symptoms such as pain, anorexia, sensitivity to fried foods and abdominal discomfort may gradually emerge and worsen to cirrhosis or hepatobiliary cancer,1 posing a significant threat to the global liver health. The main causes and underlying condi-

*These authors contributed equally to this work.
tions associated with liver fibrosis include but are not limited to chronic hepatitis, long-term alcohol abuse, liver-overloaded lipids, accumulated bile acids, chemicals, metabolic disorders and genetic factors. As hepatic parenchymal cells of the liver, hepatocytes play a crucial role in the early stages of liver fibrosis by responding to liver damage, resulting in alterations in gene expression, immune responses, and hepatocyte apoptosis, making hepatocytes one of the focal cell types in the pathogenesis of liver fibrosis. The damaged hepatocytes caused by oxidative stress and endoplasmic reticulum (ER) stress secrete cytokines such as tumor necrosis factor-alpha (TNF-α), which recruit other immune cells and induce inflammatory responses. The cumulative impacts of hepatocyte injury and regeneration, along with inflammation and the release of profibrotic factors triggered by immune cells, generate a fibrogenic milieu that additionally drives the activation of quiescent hepatic stellate cells (HSCs) into myofibroblast-like HSCs, commonly referred to as activated HSCs. Activated HSCs subsequently secrete ECM components that contribute to the development of liver fibrosis and remodeling of the liver morphology, which involves the gradual replacement of the normal liver parenchyma by a fibrous scaffold composed of collagen fibers and other ECM proteins, ultimately leading to the compromised organ function, making activated HSCs another focal cell type responsible for the pathogenesis of liver fibrosis.

The overlooked impact of liver sinusoidal endothelial cells (LSECs) on liver fibrosis

Although the function of hepatocytes and HSCs in Disse space during the development of the liver fibrosis has been extensively explored, the advancements in therapeutic outcomes of liver fibrosis is unsatisfactory, which is possibly attributed to the restricted comprehension of LSECs. What makes LSECs potentially exert pivotal influence on the progression of liver fibrosis? Initially, the alterations in LSEC phenotypes and functions could actively participate in the development of liver fibrosis. Under physiological homeostasis, LSECs, as the predominant nonparenchymal cell type in the liver, not only establish a protective barrier in the hepatic sinusoid but also possess important physiological functions, such as material filtration, endocytosis, antigen presentation and leukocyte recruitment, making LSECs extensively engaged in maintaining the microenvironmental equilibrium. Throughout the progression of liver fibrosis, the function of LSECs subsequently affects and presents material communication barriers and reduces permeability, thereby influencing the filtering and clearing function of the liver. The fenestrae of LSECs refer to the micropores or windows located on their surface, which are essential for facilitating the exchange of substances between the blood and Disse space. Specifically, there is a reduction in the quantity and size of LSEC fenestrations; under certain profibrotic conditions, they may even vanish. Then, the reduction or loss of LSEC fenestrae depresses the efficiency of blood filtration in the hepatic sinusoids, suggesting that microorganisms, cell fragments and other harmful substances that resulted in liver injury and liver fibrosis in the blood may not be adequately eliminated, thus increasing the risk of liver damage ultimately. Moreover, LSECs also participate in antigen presentation through fenestrae, thereby regulating the immune response. Novel clinical research from Ishikawa et al. reported the expression level of the scavenger receptor FcγRIIb in LSECs contributed to the clearance of small immune complexes in hepatic sinusoids during liver fibrosis of nonalcoholic steatohepatitis (NASH). Besides, once fenestration of LSECs occurs, the immune regulatory function of LSECs is

Abbreviations:
A-FABP, adipocyte fatty acid binding protein; AKAP12, A-kinase anchoring protein 12; Ang2, angiotensin II type 1 receptor; BMP9, bone morphogenetic protein 9; BRG1, brahma-related gene 1; CCL2, C-C motif chemokine ligand 2; CO, carbon monoxide; CXCR, C-X-C chemokine receptor; DLL4, delta-like ligand 4; ECM, extracellular matrix; EndMT, endothelial-mesenchymal transition; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal-regulated kinase; DLL4, delta-like ligand 4; ECM, extracellular matrix; EndMT, endothelial-mesenchymal transition; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor receptor; GATA4, GATA binding protein 4; HGF, hepatocyte growth factor; Hh, hedgehog; HIF-1α, hypoxia inducible factor-1α; HMGB1, high-mobility group box 1; HSCs, hepatic stellate cells; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecules; KLIFS, Krüppel-like factor 5; LDH-A, lactate dehydrogenase-A; LECT2, leucocyte cell-derived chemotaxin 2; LSECs, liver sinusoidal endothelial cells; LXRs, liver X receptors; MAPK, mitogen-activated protein kinase; MHC class II-α; MHC class II-β; MHC class II-β2m; MHC class II-Q; NADPH, nicotinamide adenine dinucleotide phosphate; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor kappa B; NO, nitric oxide; NOX4, NADPH oxidase 4; NRP-1, Neuropilin-1; PDGF, platelet-derived growth factor; PDGF-B, platelet-derived growth factor receptor β; PI3K, phosphatidylinositol-3'-kinase; PLA2s, phospholipase A2s; PPARγ, peroxisome proliferator-activated receptor γ; PPARγ, peroxisome proliferator-activated receptor γ; PLTP, plasma lipidtransfer protein; PKC, protein kinase C; PPARα, peroxisome proliferator-activated receptor α; PRP, plasminogen; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; VCAM1, vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor; VEGF, vascular endothelial growth factor receptor; YAP, Yes-associated protein
impaired, which further increase the susceptibility to immune-related liver diseases. Simultaneously, there is an enhancement in intercellular connections and the formation of the basement membrane, collectively leading to a process termed sinusoidal capillarization. Then, the capillarized LSECs lose their characteristically high scavenging capabilities that safeguard efficient vasculature, acquiring vasoconstriction, pro-inflammatory, and prothrombotic functions.

The ignored communication among LSECs and other hepatic cells in liver fibrosis

Given the significant functional role of LSECs in both physiological and pathological conditions, apart from the intrinsic changes of LSECs, more attention have been put on another crucial aspect. The ability of LSECs to mediate intercellular communication and molecular transfer significantly contributes to modulating disease progression by transmitting signals to hepatocytes or HSCs. In particular, hepatocytes play a critical role in the progression of liver fibrosis owing to their self-repair capacity. However, the significance of LSECs in this reparative mechanism is considerable and remains inadequately explored. LSECs have been reported to maintain intercellular communication and regulate signal transduction, thereby ensuring the coordination and effectiveness of hepatocytes in the repair process and governing liver fibrosis and regeneration. Mechanistically, the regulation of LSECs for hepatocytes are accomplished through multiple ways such as maintaining hemodynamic stability, stimulation of vascular proliferation, regulating immune response and preserving intercellular communication. Specifically, LSECs not only uphold microenvironmental equilibrium, such as hemodynamic stability in the liver, to diminish inflammation and foster a more conducive milieu for hepatocellular self-repair, but also secrete growth factors and cytokines, such as hepatocyte growth factor (HGF) that could promote hepatocyte proliferation and repair to alleviate liver fibrosis. Also, the decline in LSEC fenestrae would affect the exchange of nutrients, hormones and other important substances between the blood and hepatocytes, resulting in metabolic disorders and impaired cell functions.

In addition to hepatocytes, as we previously mentioned, LSECs can also affect HSCs or modulate the molecular exchange with HSCs, collectively controlling the progression of liver fibrosis. Under normal physiological circumstances in the liver, differentiated LSECs maintains the quiescent state of HSCs, whereas persistent HSC activation contributes to the progression of liver fibrosis. Nonetheless, during liver injury, differentiated LSECs could inhibit HSC activation, promote their transformation into fibroblasts and could not effectively control the collagen deposition of HSCs either through indirect mechanisms such as paracrine secretion and mechanical signaling, or by direct contact with HSCs, which led to the overactivation of HSCs and ameliorated the progression of liver fibrosis. Certain gaseous signaling molecules, such as nitrogen monoxide (NO) that contributes to uphold the vasodilation of hepatic sinusoids and reduce vascular tension, can be released by LSECs to balance the activation of HSCs in the Disse space. Interestingly, fibronectin produced by LSECs can influence HSC phenotypes, thereby promoting their activation, which can further produce more ECM component to destroy the functional LSEC phenotypes in turn. Hence, in contrast to the prevalent yet superficial hepatocytes and HSCs, with the permeability and ability to mediate substance exchange, LSECs actively participates in the cellular activities of multiple cell types during the development of liver fibrosis.

THE AIM OF THIS STUDY AND INVOLVED METHODS

Recently, a booming number of researches have focused on the function of LSECs in liver fibrosis. This comprehensive review not only aims to summarize the unique role and specific mechanisms of LSECs but also sheds light on the communication between LSECs and other cells in the development of liver fibrosis. Furthermore, various newly discovered natural compounds or molecules that exhibit potential in targeting LSECs are summarized and discussed, thereby emphasizing the promising applications of LSEC-targeted therapeutic drugs in the treatment of liver fibrosis. Here, we performed a literature review using the search terms “liver sinusoidal endothelial cell” and “liver fibrosis” in the PubMed database, limiting the search to articles published within the past decade with an impact factor equal to or greater than 3, by which we have collected 95 represen-
THE LSEC DEDIFFERENTIATION PROCESS IN LIVER FIBROSIS

LSEC fenestrae changes in the liver fibrosis

During LSEC dedifferentiation, LSEC fenestrae gradually decrease or vanish, resulting in reduced cell permeability and the formation of a continuous basement membrane between adjacent cells, ultimately resembling the configuration of continuous capillaries. Therefore, the loss of LSEC fenestrae constitutes the principal event defining LSEC capillarization. It is reported that a natural compound, curcumol could alleviate the defenestration and downregulate the expression of basement membrane proteins including laminin and collagen type IV in leptin-induced LSECs, thereby attenuating the process of liver fibrosis. It was considered to be accomplished through the downregulation of urokinase plasminogen activator (uPA)/uPA receptor (uPAR) as well as the upregulation of matrix metallopeptidase 13 (MMP13) that responded for the treatment of curcumol. Additionally, riociguat, a soluble guanylate cyclase stimulator, sustained the differentiation of LSEC and reinstated fenestration in liver fibrosis tissues, elevating the liver sinusoid permeability. Furthermore, trimethylamine-N-oxide as gut microbial metabolite was found to increase LSEC fenestrations and reduced the basement membrane, thereby collectively preserving murine vascular function and mitigating the progression of liver fibrosis.

The cellular cytoskeleton includes microfilaments, microtubules, and intermediate filaments. Among them, the microfilament network formed by fibrous actin and microfilament-associated proteins on the cell membrane surface make the cell membrane strong and tough. Additionally, the microfilament network is highly involved in membrane deformation and maintaining cell shape, which are essential in the development of liver fibrosis. It is reported that dynamic remodeling of the cytoskeleton and redox homeostasis are highly involved in the formation of fenestrations in LSECs. According to the reports, it has been demonstrated that bone morphogenetic protein 9 (BMP9) regulated LSEC fenestration, making it a crucial paracrine regulator for maintaining liver homeostasis and hindering liver fibrosis. In cell culture, it has been observed that LSECs underwent dedifferentiation and exhibited fenestrae loss, simulating a process reminiscent of capillarization. Notably, the dynamic cytoskeleton microfilament network was reported to be highly participated in the process. LSEC fenestrae were restructured by administering cytochalasin D (Cyto D), an F-actin-depolymerizing agent, which predominantly dispelled the formation of large stress fibers in LSEC dedifferentiation, leading to fenestrae reformation and consequently mitigating liver fibrosis. Besides, the dynamic remodeling of the cellular cytoskeleton is intricately linked to cellular destiny, encompassing apoptosis, aging and oxidative states. Oxidative stress-induced injury mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) is also documented to exacerbate LSEC defenestration through premature senescence related to progesterin. Sirtuin 1 (SIRT1), an essential protector resisting oxidative stress and senescence, mediated deacetylation of p53, which preserved LSEC defenestration and ameliorated liver fibrosis by suppressing premature senescence induced via oxidative stress and maintaining cytoskeleton. In summary, we believe that targeting the dynamic remodeling process of the cytoskeleton might be a extremely promising research direction. Developing inhibitors that target the cytoskeletal microfilament network could potentially disrupt the mechanical LSEC integrity, thereby inhibiting the formation of fenestrations.

As liver fibrosis progresses, the porosity of LSECs gradually decreased, and their membrane permeability decreased. Vascular endothelial growth factor (VEGF) not only enhanced fibrogenesis but also facilitated liver tissue repair and fibrosis resolution by mediating sinusoidal permeability. As reported, VEGF was reported to facilitate the engraftment of bone marrow mesenchymal stem cells (BMSCs), increase hepatocellular regeneration and ameliorate hepatic function, which mechanistically attributed to the upregulation of VCAM1 and increased LSEC permeability.

LSECs mediate liver fibrosis via controlling capillarization or angiogenesis

LSECs exhibited specific characteristics, including the absence of basement membrane proteins and incomplete...
tight junctions. In the initial phase of liver fibrosis, LSEC capillarized and dedifferentiated to lose their protective properties, showing as losing fenestrae, the unique ultra-microstructure of LSECs, forming organized basement membrane and enhanced tight junctions.\(^{28}\) Meanwhile, LSECs can also stimulate neovascularization by secreting angiogenic factors, thereby fostering the fibrotic process. It is worth noting that LSEC capillarization precedes the activation of macrophages and HSCs in the process of liver fibrosis, making it a crucial process in the development of liver fibrosis. A previous clinical study conducted on patients with chronic hepatitis C confirmed that LSECs underwent only morphological alterations following hepatitis C virus infection, but in the initial stage of fibrosis, LSEC capillarization was observed, suggesting that LSEC vascularization may contribute to the development of liver fibrosis.\(^{29}\) Specifically, Maretti-Mira et al.\(^{30}\) suggested that the formation of capillarization is not attributed to the dedifferentiation of pre-existing LSECs in the liver, but rather to the incomplete LSEC differentiation derived from the bone marrow. Throughout the liver fibrosis process, these bone marrow-derived endothelial cells are recruited to the liver to repair damaged LSECs. However, in this process, they fail to completely differentiate into LSECs with typical fenestrations, leading to capillarization, which contradicts the previous theory that capillarization occurs in LSECs through the process of dedifferentiation, resulting in the loss of fenestrations. A recent study revealed that aging triggered LSEC capillarization, and the deficiency of endothelial C-kit could further disturb dynamic equilibrium in LSECs, along with enhancement of fibrosis and inflammation related factors expression including tissue inhibitor of metalloproteinases 1 (TIMP-1), collagen1, platelet-derived growth factor (PDGF)\(^{β}\), IL-6 and IL-1\(^{β}\), thus exacerbating inflammation and liver fibrosis in NASH.\(^{31}\)

Furthermore, in addition to LSEC capillarization, hepatic angiogenesis that defined as the formation of new blood vessels from pre-existing ones was also highly related with LSECs and contributed to the development of liver fibrosis.\(^{8}\) CD31, VEGF, CD34 and tyrosine kinase with immunoglobulin like and EGF like domains 1 (Tie1) are considered as the detectable markers of angiogenesis. It is reported that heparinoid compound sulodexide downregulated the expression of CD31, CD34 and laminin\(^{32}\) that have been proved to mediate LSEC capillarization, then further reversed the fibrotic progression of liver. Furthermore, CU06-1004, an endothelial dysfunction blocker, exerted the function of anti-fibrosis partially attributed to subduing hepatic sinusoidal capillarization through the downregulation of CD31 expression.\(^{33}\) VEGF is a publicly known factor that highly associated with the LSEC capillarization and particularly, hepatic angiogenesis. Levels of VEGF and angiopoietin-2 in the serum of mice with liver fibrosis showed notable elevation.\(^{34}\) Wang et al.\(^{35}\) supported that the regulation of LSEC angiogenesis via VEGF played an important role in the process of liver fibrosis. Neuropilin-1 (NRP-1) is a complex transmembrane receptor that binds to both VEGF and vascular endothelial growth factor receptor 2 (VEGFR2) and is abundantly expressed in LSECs. Typically, when NRP-1 is overexpressed in LSECs, VEGFR2 is up-regulated through the regulation of focal adhesion kinase and its corresponding kinase activity.\(^{36}\) In addition, NRP1 potentiated VEGFR2-related angiogenesis via the phosphatidylinositol 3’-kinase (PI3K)/AKT pathway. Additionally, several agents have been documented to influence LSEC angiogenesis via modulating the protein function of VEGF. Leptin-stimulated LSECs exhibited the features of capillarization, which resulted from the increased expression of endothelin-1, VEGF, laminin and type IV collagen.\(^{36}\) Otherwise, plumbagin was found to reverse the capillarization of hepatic sinusoids by downregulating the expression of these mRNA. Several studied have proved that tetramethylpyrazine (TMP), a natural anti-angiogenic ingredient originally isolated from Ligusticum wallichii, could ameliorate liver fibrosis both in vitro and in vivo. Zhao et al.\(^{37}\) reported that the anti-fibrotic effects of TMP were achieved by mitigating LSEC capillarization via downregulating pro-angiogenic growth factors and their receptors, including VEGF-A, VEGFR2, PDGF\(^{β}\) and platelet-derived growth factor receptor \(β\) (PDGF-R\(^{β}\)) along with the angiogenic pathways that responded to these factors.\(^{37}\) Additionally, the in vivo administration of levistilide A exhibited remarkable anti-fibrotic effects, which is evidenced by a reduction in collagen deposition and neovascularization.\(^{38}\) Notably, this study attributed this phenomenon of anti-angiogenesis to the effects of levistilide A on preventing sinusoid capillarization through the downregulation of genes involved in the VEGF signaling pathway, including CD31, VEGF, and VEGFR2. Furthermore, aside from small molecular agents, biomacromolecules could also regulate VEGF signaling. Hu-
man recombinant endostatin, endostar, has been demonstrated to decrease the expression of VEGFR1 and VEGFR2, resulting in reduced LSEC capillarization. In addition, the anti-angiogenic effects of clinical drugs that regulated VEGF-related signaling have been widely researched, demonstrating remarkable efficacy in anti-fibrosis. Vatalanib, a clinical VEGFR inhibitor, was identified to inhibit the mRNA expression of transforming growth factor (TGF-β), VEGFR1 and VEGFR2 and then altering the LSEC phenotypes, which decreased sinusoidal capillarization and ameliorated liver fibrosis. Another clinical drug, lenvatinib could inhibit hepatic neovascularization and the expression of proangiogenic factors, including VEGF1, VEGF2, and VEGF-A, which collectively suppressed angiogenesis and ameliorated liver fibrosis. Besides, administration of carvedilol hindered the development of liver fibrosis by depressing the expression of angiogenic factors such as VEGF and angiopeptin-2, leading to the suppression of sinusoidal capillarization and the changes in LSEC phenotypes. Moreover, olmesartan treatment not only lessened the levels of VEGF, angiotensin II and PDGF, but also decreased the expression of their receptors such as VEGFR1, VEGFR2, and angiotensin II type 1 receptor (AT1R) in liver fibrotic mice, thus blocking the angiogenesis of LSEC phenotype. Furthermore, olmesartan inhibited hepatic sinusoidal remodeling.

In addition to VEGF, hypoxia inducible factor-h (HIF-1α) is another common transcription factor that mediates LSEC capillarization and angiogenesis, thus participating in the regulation of liver fibrosis. It was reported that miR-322/424 upregulated the expression of HIF-1α protein in LSECs by binding to the mRNA of Cullin 2, leading to its degradation and consequently exacerbating pathological angiogenesis. In addition, numerous investigations have demonstrated that traditional Chinese medicine and natural compounds regulated LSEC capillarization and angiogenesis to ameliorate liver fibrosis by regulating HIF-1α and its associated pathways. Chinese medicinal formulation Fu-zhenghuayu ameliorated LSEC capillarization, hepatic angiogenesis and angiogenesis-associated genes, such as CD31, VEGF, VEGFR2, p-ERK and HIF-1α, and ultimately inhibiting hepatic fibrosis. Meanwhile, Xuefuzhuyu decoction was also reported to attenuate liver fibrosis via anti-angiogenesis, which might be achieved by the lower VEGF level in LSECs induced by the suppressed HIF-1α expression. In addition, carthami flos extract (CFE), originating from edible herb carthami flos that was traditionally functioned from edible herb carthami flos that was traditionally functioned in ameliorating blood circulation and modulating angiogenesis, was shown to not only suppress the platelet-derived growth factor receptor beta (PDGFRβ)/ERK/HIF-1α and VEGFR2/AKT/eNOS pathway, but also alleviate LSEC capillarization by modulating the expression of CD31, CD34 and vWF in liver fibrosis. Notably, Zhang et al. reported that hypoxia the expression of VEGF-A and angiopoietin 2 (Ang2) in LSECs, which was reversed by oroxylin A, a natural active ingredient derived from Scutellariae radix. Mechanistically, oroxylin A downregulated the nuclear translocation of yes-associated protein (YAP), and subsequently suppressed the expression of target genes such as HIF-1α, VEGF-A and Ang2, ultimately leading to antiangiogenic effects. The hedgehog (Hh) pathway was the upstream signaling of HIF-1α and regarded as a conserved morphogenic signaling, which could regulate LSEC capillarization and angiogenesis in a HIF-1α dependent or independent way. Hh signaling not only directly activated the transcription of HIF-1α, but also mediatelly influenced HIF-1α by activating PROX1 transcription, which subsequently control the protein stabilization of HIF-1α, thus accelerating LSEC angiogenesis. Notably, a natural compound, curcumol inhibited LSEC angiogenesis by inhibiting Hh signaling and the downstream HIF-1α expression. Subsequent research revealed that the increase in the expression of Hh signaling markers, including sonic hedgehog, patched-1, smoothened (SMO) and glioblastoma 1 (GLI1), which are stimulated by VEGF-A, was attenuated at both the mRNA and protein levels following the in vitro administration of TMP, indicating that disruption of Hh signaling plays a pivotal role in improving sinusoidal angiogenesis and inhibit LSEC capillarization via TMP. Another study reported that liver X receptors (LXRs), a subset of the nuclear receptors, governed classical Hh signaling and maintained LSEC differentiated phenotypes, resulting in the suppression of sinusoidal capillarization and angiogenesis.

Overactivation of Notch signaling promotes HSC activation, expedites collagen synthesis and cell proliferation, thereby accelerating the advancement of liver fibrosis. Recent studies have reported that proteins associated with the Notch signaling are highly involved in LSEC angiogenesis. In addition, a-kinase anchoring protein 12 (AKAP12),
a scaffold protein, could inhibit LSEC angiogenesis, as interrupting the formation of basement membrane and suppressing endothelin-1 expression, leading to the fibrosis resolution.51 Interestingly, the latest research findings indicate that delta-like ligand 4 (DLL4), a ligand related to the Notch pathway, plays a crucial role in preserving hepatic sinusoidal homeostasis and is principally expressed in LSECs. Overexpression of DLL4 in LSECs has been shown to enhance hepatic sinusoidal capillarization and sinusoidal tension, thereby exacerbating liver fibrogenesis, which might be regulated by activating Notch signaling.52 Gu et al.53 discovered that miR-30c directly targeted DLL4 and subdued the downstream Notch pathway, leading to the suppression of proliferation, migration and angiogenesis ability of LSECs, which further regulated pathological angiogenesis in vivo and improved liver fibrogenesis. Therefore, it is suggested that DLL4 and other proteins related to Notch pathway may serve as a pivotal factor in angiogenesis and represent potential targets for therapeutic intervention in this process. Another research reported that leukocyte cell-derived chemotaxin 2 (LECT2), a functional ligand for Tie1 (a specific orphan receptor), exhibited anti-angiogenic properties both in vitro and in vivo by inducing Tie1 dephosphorylation, suppressing endothelial cell migration and neovascularization.54 In addition, LECT2 exhibited multiple effects on hepatic vascular remodeling, inhibiting portal angiogenesis and promoting sinusoidal capillarization, consequently intensifying liver fibrogenesis.

Several studies have reported that when blood flows, shear stress is generated, which can be sensed and responded to by molecules on the cell surface and initial the signal transduction, resulting in the changes of behavior and function of LSECs, such as the control of nucleocytoplasmic translocation of transcription factors. Fluid shear stress can activate the expression of krüppel-like factors (KLFs), and facilitate their nuclear translocation, which could mediate LSEC angiogenesis, subsequently impacting the process of hepatic fibrosis.55 A large number of articles have reported that the natural compound curcumol has demonstrated significant therapeutic effects on fibrosis through mediating the KLFs-related signaling. Gao et al.56 demonstrated that, Krüppel-like factor 5 (KLF5) activation led to the upregulation of angiogenesis markers including CD31 and CD34, which was notably reversed by treatment with curcumol, further decreasing the production of mitochondrial reactive oxygen species (ROS), increasing the antioxidative stress response, and selectively suppressing extracellular-signal-regulated kinase (ERK) phosphorylation in a liver fibrosis mouse model. Another study found that curcumol restrained KLF5 protein and mRNA expression by suppressing autophagy and inducing p62 accumulation in LSECs in vitro, which further inhibited pathological angiogenesis of LSECs during liver fibrosis progression.57 According to a recent study, KLF5 could influence the angiogenic characteristics of LSECs by modulating the glycolytic process, and regulating the expression of lactate dehydrogenase-A (LDH-A) by transcriptionally combining with its promoter. Meanwhile, LDH-A was found to possess a non-enzymatic role within the nucleus, where it interacts with KLF5 to form a transcriptional complex, further potentiating KLF5 activity and resulting in a positive feedback loop.58 Additionally, this KLF5/LDH-A feedback loop could be disrupted following the administration of curcumol that downregulated glycolytic enzymes induced by KLF5 overexpression in mRNA levels and decreased the intimate connection of KLF5 and LDH-A, thus collectively inhibiting the angiogenic properties of LSECs and attenuating liver fibrosis. During liver fibrosis, simvastatin promoted the up-regulation of KLF2 and eNOS expression in LSECs.59 Interestingly, in response to the abnormal fluid shear stress happened in liver fibrosis, KLFs were activated, exacerbating the inflammatory phenotype of LSECs, thereby representing potential targets for inhibiting the progression of liver fibrosis. The summarized pathways that we mentioned above were shown in Figure 1.

Other phenotype changes or transition of LSECs in liver fibrosis

In addition to alterations in LSEC morphology, such as defenestration, LSECs, as one of the most vulnerable liver cell types after liver injury, not only exhibit multiple phenotypic changes, including characteristic changes or transitions to an inflammatory phenotype but also undergo a series of changes to acquire a mesenchymal phenotype, collectively resulting in loss of the ability to maintain liver homeostasis. Brougham-Cook et al.50 conducted numerous in vitro experiments to explore alterations of LSEC phenotypes in diverse liver fibrosis microenvironments. They found that microenvironmental factors such as stiff-
ness, ECM and soluble factors profoundly influenced the expression of LSEC phenotype markers, including Lyve-1, VE-cadherin, and CD31, which symbolized the changes of LSEC phenotypes. Following lanifibranor treatment, an agonist of pan-peroxisome proliferator-activated receptor (pan-PPAR), improvements in fenestration and alleviation of capillarization in LSECs were observed, along with a simultaneous decrease in the expression of VCAM1, intercellular adhesion molecule 1 (ICAM1), and E-selectin in LSECs, along with a simultaneous decrease in the expression of VCAM1, intercellular adhesion molecule 1 (ICAM1), and E-selectin in LSECs, which indicated an improvement in LSEC phenotype and a reduction in pro-inflammatory phenotype.61 In summary, lanifibranor reduced hepatic vascular resistance and improved microvascular functions and the LSEC phenotypes, thereby alleviating liver inflammation and leading to the regression of liver fibrosis.

However, as we have mentioned, the LSEC phenotype is frequently variable and can undergo dynamic transitions, including processes like endothelial-mesenchymal transition (EndMT). When LSECs undergo this process, they lose specific endothelial cell functions and phenotypes, while acquiring the characteristics of mesenchymal cells. Ruan et al.62 discovered that during the process of liver fibrosis, capillarized LSECs underwent partial EndMT, resulting in the redundant production of ECM components such as collagen and other fibrosis-related proteins produced by LSECs, which further accumulated in the liver sinusoids, thus promoting liver fibrosis. In this process, LSECs experienced a continuous phenotypic alteration, transitioning from normal cell dedifferentiation to capillarization and subsequently to EndMT. Hammoutene et al.63 reported that the impairment of autophagy in metabolic dysfunction-associated steatotic liver disease (MASLD) could lead to the promotion of EndMT in LSECs, as well as induce cellular inflammation and apoptosis, ultimately facilitating the formation of liver fibrosis. Notably, the underlying mechanisms of this process have gradually been revealed. Notably, the transcriptional regulator megakaryocytic leukemia 1 (MKL1) and signal transducer and activator of transcription 3 (STAT3) collaborate to stimulate the transcription of Twist1, thereby amplifying TGF-β induced EndMT in

Figure 1. Mechanisms of LSEC angiogenesis and the related pharmacological intervention in the process of liver fibrosis. VEGF and HIF-1α were the two most crucial pathways in the process of LSEC angiogenesis. Treatments such as lenvatinib, levistilide A, olmesartan, TMP and FZHY could inhibit the VEGF pathway, exerting anti-angiogenic effects. Oroxylin A, CFE, curcumol and TMP could reverse the activation of angiogenesis caused by the HIF-1α pathway. Angiogenesis mediated by DLL4/Notch pathway was suppressed by miR-30c. AKAP12 inhibited EDN1-mediated angiogenesis. Olmesartan inhibited PDGF-mediated angiogenesis. Levistilide A, CU06-1004, and sulodexide could reduce LSEC angiogenesis by inhibiting the expression of CD31 or CD34. LSEC, liver sinusoidal endothelial cell; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia inducible factor-1α; TMP, tetramethylpyrazine; DLL4, delta-like ligand 4; PDGF, platelet-derived growth factor.
LSECs and worsening liver fibrosis. It has been reported that brahma-related gene 1 (BRG1) could regulate the production of ROS and EndMT in endothelial cells both in vivo and in vitro, which depended on the production of NADPH oxidase 4 (NOX4), suggesting that targeting BRG1-NOX4 axis might inhibit the development of liver fibrosis via blocking EndMT process. A recent study has demonstrated that elevated serum level of high-mobility group box 1 (HMGB1) could induce EndMT in LSECs. This EndMT process led to heightened ECM production, ultimately resulting in a diminished capacity of LSECs to inhibit HSC activation during liver fibrosis. Moreover, the administration of the anti-fibrosis natural compound silymarin was reported to reduce serum HMGB1 levels and suppress EndMT via promoting the expression of Egr1 that could alleviate liver fibrosis by inhibiting HMGB1. Similarly, the natural polyphenolic compound chlorogenic acid (CGA) not only reduced the release of HMGB1 in LSECs but also decreased ECM deposition in the liver sinusoids and HMGB1-induced ECM production, thus alleviating liver fibrosis in NASH.

**REMODELING OF INTRACELLULAR PATHWAYS FOR LSEC HOMEOSTASIS BALANCE**

The vascular secretion signals of LSECs regulate liver fibrosis

Vascular secretory signals, which were predominantly generated by the majority of endothelial cells, play a crucial role in regulating vascular remodeling, inflammatory responses, and fibrotic processes. Dysregulation of these genes can exacerbate liver damage and lead to functional impairments, underscoring their significance in the progression of liver fibrosis. Similar to endothelial cells elsewhere, LSECs also produce the different vascular secretion signals during acute and chronic liver injuries, giving rise to two completely different pathological processes: liver regeneration or fibrosis. With acute liver injury, LSECs upregulated the expression of CXCR7 and activated the transcription factor ID1, subsequently promoting liver regenerative vascular niche through the secretion of pro-regenerative vascular factors. However, with chronic liver injury, the activation of fibroblast growth factor receptor 1 (FGFR1) induced upregulation of CXCR4 expression in LSECs, which further suppressed the pro-regenerative CXCR7-ID1 pathway and accelerated liver fibrosis, indicating the dominance of the FGFR1-CXCR4 vascular secretion pathway. Serving as essential ligands for chemotactic receptors, numerous chemokines participate in both vascular secretion and the liver fibrosis process of LSECs. During early CXCL1-mediated liver fibrosis, glycolysis enhanced the vascular secretion processes involving CXCL1 in LSECs, which was attributed to glycolysis promoting CXCL1 expression via the movement of NF-kB and its interaction with actin polymerization, thereby promoting the progression of liver fibrosis. In the liver, GATA4 functioned as a developmentally-induced transcription factor intricately linked to liver development and regeneration. Depletion of GATA4 in liver triggered HSC activation and accelerated the process of liver fibrosis. According to a recent study, GATA4 deficiency in LSECs could induce sinusoidal capillarization in liver fibrosis, leading to the re-expression of fibrogenic vascular secretory factors including PDGFβ, SPARC1L, ESM1, and IGFBP5. The endothelial transcriptional regulator MYC, which is mediated by GATA4, was activated and functioned as a downstream driver of liver fibrosis along with the vascular secretory growth factors PDGFβ and PDGFRβ. In summary, the GATA4/MYC/PDGFRβ/PDGFRβ axis associated with vascular secretory signaling could regulate liver fibrosis, making it a potential therapeutic target. In addition to transcriptional regulation, epigenetic modification was identified as another pathway through which these exogenous substances stimulate LSECs. As a transcriptional coactivator, P300 could modify histone proteins and promote gene activation, which was reported to accelerate the process of liver fibrosis. Besides, P300 could upregulate the expression of C-C motif chemokine ligand 2 (CCL2) via interacting with nuclear factor kappa B (NF-kB) and bromodomain containing 4 (BRD4) in LSECs after liver injury, thus stimulating LSECs to release the vascular secretion signals associated with liver diseases and exacerbating liver fibrosis.

A noteworthy star signaling: the NO pathway of LSECs regulates liver fibrosis

Publicly acknowledged, gas signaling molecules play important roles in various physiological and pathological processes, encompassing vascular regulation, cellular apop-
tosis, and inflammatory responses, among others. NO, as a second messenger molecule with high free radical activity, plays a crucial role in the maintenance of vascular tone and the regulation of blood pressure, which was produced by eNOS (endothelial nitric oxide synthase) in endothelial cells. Reportedly, activation of Notch in endothelial cells altered the vascular secretion function of LSECs and inhibited the eNOS-sGC pathway to downregulate Wnt2a and Wnt9b that are publicly known as hepatocyte mitogens to promote hepatocellular regeneration, subsequently accelerating LSEC dedifferentiation and exacerbating liver fibrosis. Shao et al. reported that BRG1 suppressed eNOS activity and reduced the bioavailability of NO in LSECs by regulating the vascular secretion signaling of LSECs, contributing to the worsening of liver fibrosis. Similarly, Jiang et al. reported that the infection with schistosoma japonicum resulted in decreased differentiation and increased LSEC dedifferentiation, along with reduced NO secretion and enhanced TGF-β secretion. Meanwhile, several chemical and natural compounds have shown great anti-fibrosis effects via modulating the NO signaling. Human placental extract (HPE) has been reported to enhance the vitality of LSECs through increased eNOS expression, while also improving serum AST and ALT levels, indicating the amelioration of liver fibrosis in NASH. After the administration of emricasan, the LSEC phenotypes changed, such as the restoration of endothelial fenestration and increased bioavailability of NO, which further led to the activation of eNOS and cGMP enhancement, ultimately alleviating liver fibrosis. In addition, a natural polyphenolic flavonoid resveratrol possesses the potential to enhance hepatic vasorelaxation and endothelial dysfunction and increase NO bioavailability in LSECs, while decrease thromboxane A2 production, downregulate the expression of TGF-β and NF-κB and reduce desmin and α-SMA protein expression, ultimately attenuating liver fibrosis.

NO and ROS are balanced under physiological circumstances, a critical aspect for maintaining homeostasis in LSECs. ROS can scavenge NO by superoxide (O2•−), resulting in the reduced NO bioavailability, thus promoting the development of liver fibrosis. After liver injury, there was a gradual transition from a pro-regenerative phenotype to a pro-fibrotic phenotype in LSECs, which influenced the process of liver fibrosis through the ERK1/2-AKT axis. Mechanistically, ERK1/2 shifted the equilibrium between NO and ROS towards NO, thus maintaining LSEC homeostasis and promoting regeneration. On the other hand, AKT could tilt the balance towards ROS, resulting in LSEC dysfunction and promoting liver fibrosis. Zheng et al. provided additional evidence that curcumol regulates hepatic angiogenesis by hindering the upstream signaling pathway of NO and subsequently modulating NO expression. They also found that curcumol could downregulate the VEGF/AKT/eNOS pathway, leading to the enhancing fenestration of sinusoidal endothelial cells and attenuating liver angiogenesis. Additionally, another mechanism by which NO modulates the ROS process during liver fibrosis may be involved in the induction of autophagy. Autophagy in endothelial cells contributed to preserving the dynamic equilibrium of LSECs by enhancing the bioavailability of NO and eliminating accumulated ROS, which maintained LSEC phenotype, reduced oxidative stress, and ultimately reversed liver fibrosis. However, this phenomenon was predominantly observed during the initial phases of liver injury, but in late-stage chronic damage, autophagy was insufficient to reverse fibrosis. Previous studies have reported conflicting roles of autophagy in liver fibrosis. The autophagic activity in LSECs also exhibits opposing effects on the progression of liver fibrosis. Autophagic degradation of Caveolin-1 (Cav-1) and remodeling of F-actin were found to promote the LSEC defenestration and the development of CCL4-induced liver fibrosis by suppressing the NO-dependent PI3K-Akt-mTOR pathway. Crucially, whether promoting or inhibiting autophagy, both exert their effects on liver fibrosis by intervening the downstream NO signaling pathways. Similar with NO, carbon monoxide (CO) act as antagonists of endothelin-1 by mediating relaxation of sinusoidal vessels, but the function of CO in LSEC and the intercellular communication involving LSECs for liver fibrosis still not well-studied, which was worthy for further investigation.
INTERCELLULAR COMMUNICATION INVOLVING LSECs AND ITS IMPLICATIONS IN LIVER FIBROSIS

LSECs indirectly mediate liver fibrosis via hepatocytes

The reciprocal interaction between hepatocytes and LSECs is pivotal for maintaining normal liver physiological function and structure. LSECs, on one hand, safeguard hepatocytes by regulating the permeability of the sinusoidal capillaries, while on the other hand, they activated hepatocyte proliferation through inhibiting the expression of hepatocyte growth inhibitor TGF-β and promoting the secretion of growth factors. It was reported that growth factors, including but not limited to BMP2 and BMP6, were secreted by LSECs in a manner of paracrine to hepatocytes, regulating hepcidin-dependent iron homeostasis and fibrotic processes. TAZ-knockout in LSECs could decrease NO production and interrupt the normal regeneration process after partial hepatectomy, which subsequently lead to aberrant transport of cellular signaling for hepatocytes, ultimately promoting the development of liver fibrosis. Substance P, a neuropeptide expressed in numerous tissues, have been shown to ameliorate tumor necrosis factor (TNF-α) induced endothelial dysfunction, in numerous and promote LSECs to release HGF, which promoted hepatocellular regeneration in a paracrine manner.

It has been reported that during hepatocellular damage process, LSECs exhibited reduced scavenger function, a phenomenon induced by the inflammatory factors and other cytokines released by hepatocytes. Reportedly, CD147 as glycoprotein was found to ameliorate the progression of liver fibrosis through mutual communication between hepatocytes and LSECs. Mechanistically, the overexpression of CD147 promoted AKT phosphorylation and activated the PI3K/AKT pathway, which stimulated hepatocytes to produce VEGF-A that induced the expression of VEGFR-2 in LSECs, thus stimulating angiogenesis and exacerbating liver fibrosis. In addition, in the context of liver injury, damaged hepatocytes secreted semaphorin 3E, which triggered the contraction of LSECs and orchestrates liver sinusoidal regeneration, thus facilitating wound healing. Simultaneously, during the reconstruction of sinusoid structures, semaphorin 3E overexpression in denatured hepatocytes could activate HSCs in LSEC-mediated manner, resulting in the progression of liver fibrosis process. Given the critical function of HMGB1 in preserving LSEC phenotypes and its recognized capacity to influence cell fate, we also detected the effects of HMGB1 on ages LSECs and liver damage. Our study reported that oxidative hepatocytes highly expressed and released HMGB1 into the liver microenvironment, which were further taken up and involved in LSEC fenestration and vascularization, but luckily, was inhibited by acteoside, a small molecular from Rehmanniae Radix Praeparata. Thus, it is worthwhile to explore whether and how the HMGB1 secreted by hepatocytes intervened in the process of liver fibrosis by modulating LSEC functions.

LSECs indirectly mediate liver fibrosis via HSCs

During liver injury, the capacity of LSECs to prevent HSC activation is compromised or diminished, attributable to the LSEC dedifferentiation and LSEC capillarization. Multiple signaling pathways altered in LSECs could impact HSC activation, primarily through the paracrine mediation of secretory factors. Reportedly, LSECs-derived adipocyte fatty acid binding protein (A-FABP) exacerbated liver fibrosis in mice by potentiating LSEC capillarization and activating HSCs, which was mechanistically attributed to the stimulation of the Hh signaling and the compromised inhibitory effect of LSECs on HSC activation. A-FABP originating from LSECs also influenced HSCs in a paracrine manner, which enhanced the transactivation of TGF-β by regulating c-Jun N-terminal kinase (JNK)/c-Jun signaling in HSCs, subsequently aggravating liver fibrosis. Additionally, plasma kallikrein (PLK) within LSECs has been shown to promote the activation of TGF-β, leading to the activation of HSCs and promoting liver fibrosis. According to a recent report, downregulation of HIF-1α or CXCR4 alleviated the LSEC dedifferentiation and suppressed HSC activation, which can partially be attributed to the reduced release of PDGF-BB when LSEC dedifferentiation was inhibited, leading to the downregulation of PDGFR-β in HSCs. While elevated expression of CXCR4 resulted in increased PDGFB-BB expression in LSECs. PDGF-BB bound to its receptor PDGFR-β on LSECs, inhibiting the expression of CXCR7 and promoting LSEC dedifferentiation, which subsequently leads to HSC activation. In general, the HIF-1α/CXCR4/
PDGF-BB/CXCR7 axis could regulate the LSEC dedifferentiation, thereby mediating HSC activation and liver fibrosis. For transcriptional regulation, silencing a transcription factor associated with LSECs, Zeb2 suppressed the expression of secretory factors including Gdf15, Igf1, and Ltf, in LSECs, which functioned as ligands that could activated the target genes in HSCs, thus mitigating the progression of liver fibrosis. Furthermore, a novel cellular communication mechanism between LSECs and HSCs was discovered, which was regulated by long non-coding RNA (lncRNA). Chen et al. discovered that lncRNA Airn can sustain LSEC differentiation both in vitro and in vivo, subsequently suppressing HSC activation and stimulating hepatocyte proliferation by upregulating the expression of Wnt2a and HGF through the paracrine pathway of LSECs, thus improving liver fibrosis. The mechanism can be summarized as the interaction between Airn and EZH2, which maintained LSEC differentiation via the KLF2-eNOS-sGC pathway, thus maintaining HSC quiescence and boosting hepatocyte proliferation.

Besides, mechanical signal transduction and vesicle transport are also involved in the transport of substances between LSECs and HSCs. Liu et al. established fibrotic microniches (FμNs) that consisted of two-dimensional cultured LSECs and HSCs covered with three-dimensional collagen hydrogel to explore the regulatory of LSECs in HSC activation. Mechanistically, in the initial stage of liver fibrosis, LSEC angiogenesis is mechanistically linked to

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**Figure 2.** Communication among LSECs, HSCs and hepatocytes around the space of Disse during liver fibrosis. The expression of Zeb2 and eNOS in LSECs maintained HSC quiescence. Arin interacted with EZH2 to maintain HSC quiescence and hepatocyte proliferation through releasing WNT2A and HGF in paracrine manners. When stimulated by CD147, hepatocytes promoted LSEC capillarization through the PI3K/AKT/VEGF-A/VEGFR2 axis. PDGF-BB stimulated by CXCR4, TGF-β stimulated by S1PR2 or PLK, JAM-A and A-FABP and were released from LSECs in paracrine manners and then activated HSCs. Exosomal SPHK1 from LSECs activated HSCs through vesicle transport. VCAM1 derived from LSECs stimulates HSC activation through Hippo and YAP1 pathways. Notch and BRG1 inhibit eNOS, reducing the bioavailability of NO and losing the ability to suppress HSCs. A-FABP, adipocyte fatty acid binding protein; AKT, AKT serine/threonine kinase; BRG1, brahma-related gene 1; CXCR4, C-X-C chemokine receptor 4; eNOS, endothelial nitric oxide synthase; EZH2, enhancer of zeste homolog 2; HGF, hepatocyte growth factor; HSC, hepatic stellate cell; IVA-PLA2, group IVA phospholipase A2; JAM-A, junctional adhesion molecule A; LSEC, liver sinusoidal endothelial cell; NO, nitric oxide; PDGF-BB, platelet-derived growth factor-BB; PDGFR-β, platelet-derived growth factor receptor β; PI3K, phosphoinositide 3-kinases; PLK, polo-like kinase; S1PR2, sphingosine-1-phosphate receptor 2; SPHK1, sphingosine kinase 1; TGF-β, transforming growth factor beta; VCAM1, vascular cell adhesion molecule 1; VEGFA, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor 2; WNT2A, WNT family member 2a; YAP1, yes-associated protein 1.
the condensation of collagen fibrils. These compacted collagen fibers then act as mechanical transducers, transmitting signals to HSCs through the discoidin domain receptor 2 (DDR2)-JAK2/P13K/AKT-myocardin pathway, ultimately promoting the development of liver fibrosis. Chen et al.\textsuperscript{97} conducted research examining the influence of autophagy on LSECs during hyperaldosteronism. They found that aldosterone-induced autophagy promoted the degradation of multivesicular bodies in LSECs, resulting in a reduction in the quantity and quality of extracellular vesicles transferred from LSECs to HSCs, which were responsible for maintaining the quiescence of HSCs. Consequently, HSCs were activated, culminating in the progression of liver fibrosis in the context of hyperaldosteronism.\textsuperscript{97} Liao et al.\textsuperscript{98} reported that the overexpression of Stpr2 in LSECs regulated the phosphorylation of YAP, leading to the activation the expression of TGF-β and excessive production of excessive ECM, which ultimately enhancing HSC activation and exacerbating liver fibrosis. VCAM1 derived from LSECs also mediated the activation of HSCs and promoted the development of liver fibrosis by activating the YAP1 pathway in HSCs.\textsuperscript{99} Although there is no report indicating that the SIRP2 could be packaged in exosomes and transported from LSECs to HSCs at present, it has been demonstrated that SPHK1, which shares a similar function with SIRP2 as regulating the generation of SIP, has been confirmed to be transported within exosomes.\textsuperscript{100} Additionally, exosomal sphingosine kinase 1 (SphK1) derived from LSECs has been identified as crucial in facilitating HSC migration, activation, and AKT phosphorylation, which further sustained HSC activation. The in vitro experiments demonstrated that the phenolic compound, salidroside, could alleviate this pathological process by inhibiting AKT phosphorylation, thereby reducing liver fibrosis.\textsuperscript{100} Several agents have been reported to exhibit antifibrotic effects on communication between LSECs and HSCs. A prior investigation demonstrated that Scutellariae radix downregulated the expression of monocyte chemotactic protein 1 (MCP-1) to inhibit lipopolysaccharide-induced activation of LSECs, thereby suppressing HSC migration and ultimately alleviating liver fibrosis.

**Figure 3.** The interaction among LSECs with other cells. LSECs promote the transformation of macrophages into KCs through the binding of DLL4 to Notch receptor in macrophage. LSECs regulate the recruitment of KCs by secreting MMPs and TIMPs. The ligands such as ICAM-1 and VCAM-1 in KCs bind with the integrins on KCs and participate in the direct adhesion process between KCs and LSECs. TGFβ, DLL4, and LXR ligand in LSECs can interact with receptors such as TGFβR, Notch, and LXR in KCs, then maintaining the identity of KCs. Moreover, LSECs can secrete pro-inflammatory mediators to activate KCs. Th1 and Th2 cells promote or inhibit angiogenesis in LSECs through the Rho-ROCK-myosin pathway. LSEC, liver sinusoidal endothelial cell; DLL4, delta-like ligand 4; VCAM1, vascular cell adhesion protein 1; TGF, transforming growth factor.
fibrosis. On the other hand, LSECs possess the ability to activate HSCs through direct contact. Several studies have reported the involvement of junctional adhesion molecules (JAM) in the interactions between LSECs and HSCs in liver fibrosis. Brozat et al. demonstrated that JAM-A played a key role in maintaining the structural integrity of LSECs and preventing capillarization, which might keep the quiescence of HSCs through LSECs-derived paracrine mechanism, thus attenuating hepatic fibrogenesis. In addition, the expression of classical JAM including JAM-B and JAM-C, elevated throughout the progression of liver fibrosis. After activation, HSCs produced JAM-C, which allows activated HSCs as pericytes to connect with JAM-B-expressing LSECs, facilitating the interaction between the two cell types. Simultaneously, JAM-B and JAM-C exhibited heterophilic interactions, thereby modulating the interplay between LSECs and HSCs, ultimately controlling liver fibrosis. A recent study showed that IVA phospholipase A2 (IVA-PLA2), a key isozyme of PLA2s in mammals, expressed in LSECs may promote sinusoidal capillarization in the liver, further leading to HSC activation and ensuing collagen deposition, which ultimately accelerated the process of liver fibrosis.

LSECs interact with other cells in liver fibrosis

In the preceding section, we discussed the effect of LSECs on hepatocytes and HSCs (Fig. 2), then we will delve into how could other cell types mediate liver fibrosis by influencing LSECs (Fig. 3). T helper cells and Kupffer cells (KCs) play crucial roles in the initiation and aggravation of liver fibrosis via modulating immune responses, inflammatory reactions and the release of cytokines. Zhong et al. reported auxiliary T helper (Th) 1 and Th2 cells interacted with LSECs in vivo though different adhesion molecule to exert contrary effects on the process of liver fibrosis. The interaction between Th1 cells and LSECs could potentially expedite the reduction of LSEC fenestrae via the Rho-ROCK-myosin pathway, promoted LSEC angiogenesis, and ultimately exacerbating liver fibrosis, while the interaction between Th2 cells and LSECs exhibited the opposite tendency. During the process of liver fibrosis, LSECs could function as antigen-presenting cells that could recruit KCs by controlling the secretion of different matrix metalloproteases (MMPs) and tissue inhibitors of MMPs (TIMPs), which have been summarized in the previous review. Recent studies have delved deeper into unraveling the mechanisms underlying the communication between LSECs and other cells in liver fibrosis.
### Table 1. Agents regulating liver fibrosis through LSECs

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<td>ROS</td>
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<td>CCl4-induced fibrotic mice</td>
<td>LSECs</td>
<td>Inhibit angiogenesis</td>
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<td>Inhibit angiogenesis</td>
<td>KLF5</td>
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<td>Leptin-induced LSECs</td>
<td>LSECs</td>
<td>Attenuate angiogenesis</td>
<td>VEGF</td>
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<td>Cyto D</td>
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<td>LSECs</td>
<td>Reform fenestrae</td>
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<td>Levistilide A</td>
<td>CCl4-induced fibrotic rats</td>
<td>LSECs, HSCs</td>
<td>Inhibit angiogenesis</td>
<td>VEGF</td>
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<td>Plumbagin</td>
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<td>Reverse capillarization</td>
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<td>Resveratrol</td>
<td>CCl4-induced cirrhotic rats</td>
<td>LSECs, HSCs</td>
<td>Improve hepatic endothelial dysfunction</td>
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<td>TMAO</td>
<td>Western diet or CCl4-induced NASH mice</td>
<td>LSECs</td>
<td>Increase fenestrations</td>
<td>ATP1B1</td>
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<td><strong>Biomacromolecule</strong></td>
<td><strong>HPE</strong></td>
<td><strong>MCD-induced NASH mice</strong></td>
<td><strong>LSECs</strong></td>
<td><strong>Suppress LSEC damage</strong></td>
<td><strong>ATP1B1</strong></td>
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KCs. It is well known that KCs are tissue-resident macrophages located in hepatic sinusoidal cavities, making them a representative cell type of the liver. Sakai et al.\textsuperscript{106} found that the Notch ligand DLL4 in LSECs was highly expressed and connected with the corresponding receptors Notch1 or Notch2 in bone marrow (BM) progenitor cells to promote a transition of the gene expression profile toward that of KCs. Moreover, the ligands including the TGF-β family ligands, BMP2, DLL4 and endogenous LXR from LSECs were found to be highly expressed and connected with their corresponding receptors such as TGFβR, Notch1, Notch2, LXR in KCs, which was required for the maintenance of Kupffer cell identity.\textsuperscript{106} Likewise, the levels of integrins such as α4β1, αLβ2, αMβ2, and αXβ2 in KCs were increased while the expression of integrin ligands including Icam-1 and Vcam-1 that have the binding properties with these integrins were also increased, indicating that the integrins and integrin ligands were possibly participant in the direct adhesion process between KCs and LSECs.\textsuperscript{107} Furthermore, in severe liver injuries, it has been shown through various studies that LSECs release proinflammatory cytokines, including TNF-α, IL-6, IL-1, and CCL2, thereby activating KCs. In turn, when LSECs was cocultured with Kupffer cell, LSECs also exhibited pathological phenotype changes even in the suitable well-maintained elasticity of matrix,\textsuperscript{108} which indicated that activated KCs impair the normal LSEC functions. Additionally, another study has reached similar conclusions that LSEC dedifferentiation has been observed in an inflammatory microenvironment created by KCs, suggesting that the co-culture of LSECs and KCs is likely not conducive to maintenance of LSECs.\textsuperscript{109}

**FUTURE EXPECTATION**

This review provides a detailed description of the alterations that occurs in LSECs during the process of liver fibrosis and how these changes subsequently mediate liver fibrosis through distant pathways (Fig. 4). At the same time, an overview was provided on the therapeutic benefits of diverse clinical drug, small molecular agent, biomacromolecule, Chinese medicinal formulae and Chinese medicinal herb in addressing liver fibrosis by targeting LSECs (Table 1). Consequently, LSECs, which have received limited at-
Among various fenestrated endothelial cells, glomerular endothelial cells (GEnC) exhibit the closest resemblance to LSECs in terms of structural and functional characteristics. The fenestration of GEnC is the key to glomerular filtration barrier, which is similar to that of LSECs as hepatic sinusoid filtration barrier. Given the significant impact of enhancing GEnC fenestrae on the prognosis of chronic kidney disease, it is plausible that the regulation of LSEC fenestrae could similarly contribute to improving liver fibrosis. Mechanistically, it is reported that Eps15 homology domain-containing protein 3 (EHD3) could regulate GEnC fenestrae through endocytosis recirculation of VEGF or interacting with cytoskeleton. Notably, the cytoskeleton remodeling is also closely related to the formation of LSEC fenestration. Zapotoczny et al. have shown that the destabilization of the actin-spectrin scaffold induced by damide and iodoacetic acid (IAA) in LSECs was highly involved in the formation of fenestrations, indicating that spectrin could potentially serve as a therapeutic target for modulating LSEC permeability. Based on these findings, it is crucial to conduct further studies to clarify the pivotal role of the cytoskeleton in LSEC fenestration. Such investigations hold promise as an effective strategy for ameliorating liver fibrosis by governing the LSEC permeability.

We have summarized in this review that when LSECs undergoes EndMT, it currently facilitates the development of liver fibrosis, which is attributed by the loss of original cellular phenotypes and characteristics features in LSECs as well as promoting ECM deposition, one of the main characteristics of liver fibrosis, collectively resulting in abnormal changes in liver structure and function to aggravate liver fibrosis. Yet, limited information was available regarding the instigator of fibrosis associated with EndMT. During the process of liver fibrosis, there is a significant increase in the production of ROS, which exceeds the clearance capacity of cells and leads to an increase in oxidative stress within cells. The surplus ROS in LSECs fosters the EndMT of LSECs, which might be considered as an essential factor to control the EndMT process.

The complex vascular networks in liver play divergent roles in the process of hepatic fibrosis. The increase number of portal vessels alleviates liver fibrosis, while the augmentation of hepatic sinusoidal capillaries and central vessels can aggravate hepatic fibrosis. At present, a considerable number of studies have reported the efficacy of anti-angiogenic drugs in the treatment of hepatic fibrosis, yet the results have shown variability and inconsistency.
among different investigations. One contributing factor to the complicated efficacy may be the underestimation of the microvascular network induced by LSECs. Hence, a more holistic approach to liver fibrosis treatment requires the incorporation of various signaling pathways that regulate endothelial function. This review summarizes the therapeutic effects of different pharmacological interventions on liver fibrosis by inhibiting the expression of angiogenesis-related factors, protecting or restoring LSEC fenestrae, maintaining the LSEC differentiation phenotypes, regulating LSEC vascular secretory signals and affecting communication between LSECs and HSCs. While these agents have shown promising effectiveness in cellular and animal studies, they have not yet progressed to the clinical research phase and still cannot achieve accurate targeting of LSECs. Therefore, exploring therapeutic remedies or administration routes that specifically target LSECs may become a new approach for anti-fibrosis therapy strategy in the future. It is worth noting that the combination administration of adeno-associated viral vector serotype 9 (AAV9)-LECT2-short hairpin RNA (shRNA) specifically targeting LSECs, along with bevacizumab targeting angiogenesis, showed a positive antifibrotic effect and fewer side effects. This indicates that inhibiting the microvascular network initiated by LSECs and other intrahepatic vascular networks concurrently could lead to increased efficacy. Similarly, since hyaluronic acid (HA) could only be phagocytosed by LSECs, it was reported that HA-coupled liposomes nanoparticle could deliver interferon regulatory factor 1 into LSECs in vivo, representing a novel and specific delivery approach. Notably, oncoprotein-induced transcript 3 (Oit3) was considered as a promising hallmark gene for targeting LSEC. Additionally, a liposomal system could deliver compound to specific cells via modified the liposome with specific peptide. Thus, we suggest design a Oit3-targeted peptide combined with liposomal system could achieve the delivery of compound to LSECs, providing a novel direction for the intervention of liver fibrosis.

In addition, another reason for the inefficacy of antiangiogenic drugs may be that regulating a single type of vascular endothelial cell is not enough to fully regulate multiple vascular networks for the treatment of liver fibrosis. Xu et al. reported a new viewpoint concerning capillarization and angiogenesis. Usually, when discussing hepatic angiogenesis, estimating sinusoidal capillarization solely based on counting positive vascular markers (such as CD31) may lead to an inaccurate assessment of capillary formation, as it inappropriately includes portal vein angiogenesis. This means that the inaccurate inference of ‘increased angiogenesis’ might result from combining the counting of positive CD31 markers from portal vessels and sinusoidal capillaries. It is worth mentioning that they did not define sinusoid capillarization as angiogenesis but vascular remodeling, while capillarization and angiogenesis should be regarded as two relatively independent functional processes.

Authors’ contribution

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Conflicts of Interest
The authors declare that there are no conflicts of interest.

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LSEC: An overlooked player in liver fibrosis


Unmet needs in the post-direct-acting antivirals era: The risk and molecular mechanisms of hepatocellular carcinoma after hepatitis C virus eradication

Chung-Feng Huang1,2,3,4,*, Manar Hijaze Awad5,*, Meital Gal-Tanamy5, and Ming-Lung Yu1,2,3,6

1Hepatobiliary Division, Department of Internal Medicine, Kaohsiung Medical University Hospital; 2College of Medicine and Center for Liquid Biopsy and Cohort Research, Kaohsiung Medical University, Kaohsiung, Taiwan; 3Faculty of Internal Medicine and Hepatitis Research Center, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; 4Ph.D. Program in Translational Medicine, College of Medicine, Kaohsiung Medical University and Academia Sinica, Kaohsiung, Taiwan; 5Molecular Virology Lab, The Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel; 6School of Medicine and Doctoral Program of Clinical and Experimental Medicine, College of Medicine and Center of Excellence for Metabolic Associated Fatty Liver Disease, National Sun Yat-sen University, Kaohsiung, Taiwan

Hepatitis C virus (HCV) infection is one of the major etiologies of hepatocellular carcinoma (HCC) with approximately 30% of HCC being due to HCV infection worldwide. HCV eradication by antivirals greatly reduces the risk of HCC; nevertheless, HCC remains to occur in chronic hepatitis C (CHC) patients who have achieved a sustained virological response (SVR). The proportion of post-SVR HCC among newly diagnosed HCC patients is increasing in the direct-acting antiviral (DAA) era and might be due to preexisting inflammatory and fibrotic liver backgrounds, immune dysregulation between host and virus interactions, as well as host epigenetic scars, genetic predispositions and alternations. By means of applying surrogate markers and adopting risk stratification, HCC surveillance should be consistently performed in high-risk populations. In this review, we discuss the possible molecular mechanism, risk factors, and HCC surveillance strategy for HCC development after HCV eradication in CHC patients. (Clin Mol Hepatol 2024;30:326-344)

Keywords: HCV; HCC; SVR; Genetic; Epigenetic; Surveillance

Corresponding author: Ming-Lung Yu
Hepatobiliary Division, Department of Internal Medicine, Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan
Tel: +886-7-3121101 ext.7475, Fax: +886-7-3234553, E-mail: fish6069@gmail.com
https://orcid.org/0000-0001-8145-1900

Meital Gal-Tanamy
Molecular Virology Lab, The Azrieli Faculty of Medicine, Bar-Ilan University, Henrietta Szold 8, Zefat, 1311502, Israel
Tel: +972522959848, Fax: +972522959848, E-mail: Meital.Tanamy@biu.ac.il
https://orcid.org/0000-0001-7831-6847

*Equal contribution.
INTRODUCTION

An estimated 60 million people are infected with hepatitis C virus (HCV). Chronic hepatitis C (CHC) is a global public health threat since 10–20% will develop liver complications including decompensated cirrhosis and hepatocellular carcinoma (HCC). HCC is the fifth most common cancer and the second most common cause of cancer death with approximately 30% of HCC being due to HCV infection. The endorsement of the World Health Assembly and declaration of viral hepatitis elimination by 2030 set by the World Health Organization have facilitated HCV management in recent years. With the great innovation of direct-acting antivirals (DAAs), nearly 10 million CHC patients were treated with DAAs between 2015 and 2019. It is anticipated that a significantly increased number of CHC patients will be in post-curative status in the following decades, and how to deal with such post-sustained virological response (SVR) CHC patients is of great importance.

HCV eradication by antivirals greatly reduces the risk of HCC with up to 70% of HCC risk being reduced either by interferon-based regimens or DAAs. Nevertheless, HCC is still found in CHC patients who have achieved SVR. The annual incidence of HCC after HCV eradication ranges from 0.6% to 4.9%, of which the risk did not differ between those who received interferon-based regimens or DAAs after adjusting potential confounders. However, unlike in the interferon era when more elderly patients with advanced liver disease were relatively contraindicated for antivirals, more CHC patients who possess the two HCC risk factors have been cured by DAAs. As a consequence, the proportion of post-SVR HCC among newly diagnosed HCC patients is increasing in the DAA era; for example, in comparison to HCV-viremic HCC and non-HCV HCC, the percentage of post-SVR HCC increased from 3% between 2009 and 2012 to 16% between 2017 and 2019 in a Japanese study. Taken collectively, it is critical to address the topic of post-SVR HCC due to the increasing health burden year-by-year. In this review, we discuss the possible molecular mechanism, risk factors, and HCC surveillance strategy for HCC development after HCV eradication.

SCREENING TARGETS AND HCC SURVEILLANCE STRATEGIES

The recommendation for the HCC surveillance target population is based on cost-effectiveness analysis. Screening the population with an annual incidence of 1.5% or greater has been generally acceptable in the past. A recent study has shown the incremental cost-effectiveness ratio would be >50,000 USD per quality-adjusted life-year, a traditional willingness-to-pay threshold, if the incidence is less than 1.32%. Another study took the costs of surveillance harm into consideration by simulating 1 million patients with compensated cirrhosis. Biannual surveillance with ultrasonography plus alpha-fetoprotein (AFP) would be cost-effective for an HCC incidence rate >0.4% provided by surveillance adherence >19.5% if the willingness-to-pay threshold was set at USD 100,000.11 It should be noted that the cost-effectiveness analyses were based on the Markov model, but a prospective interventional study is lacking; furthermore, the analyses did not consider benefits or costs of emerging HCC treatment modalities as well. Importantly, the willingness-to-pay threshold is largely dependent on the support of the healthcare system, which tends to vary among regions. To this end, it is difficult to make a conclusion about defining the best candidates for post-SVR surveillance if the decision merely relies on cost-effective judgments.

All international societies agree that cirrhotic patients should receive HCC surveillance, although different recommendations are being suggested according to different regional consensus (Table 1). The Asian Pacific Association for the Study of the Liver (APASL) recommends screening all SVR patients with sonography and tumor markers, including AFP, protein induced by vitamin K absence or antagonist-II (PIVKA-II) and lectin-reactive AFP (AFP-L3). For patients with fibrotic stage 0–2, surveillance should be performed every 6 months for the first two years.

Abbreviations:
AST, aspartate aminotransferase; AST, alanine aminotransferase; AFP, alpha-fetoprotein; CHC, chronic hepatitis C; DAA, direct-acting antiviral; FIB-4, fibrosis-4 index; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; MICA, MHC class I chain-related A; SNP, single nucleotide polymorphism
then annually. For patients with fibrotic stages 3–4, surveillance should be performed every 6 months. The European Association for the Study of the Liver (EASL) recommends screening patients with fibrotic stages 3–4 every 6 months. EASL does not recommend using tumor markers as the screening tool due to potential false positivity. By contrast, the recent American Association for the Study of Liver Diseases guideline suggests screening cirrhotic patients every 6 months by sonography in addition to the tumor marker, AFP. The Taiwan Association for the Study of the Liver (TASL) has more stringent recommendations, which suggest screening for fibrotic stage 0–1 (F0–1) with HCC risk factors or fibrotic stage 2 every 6–12 months and fibrotic stage 3–4 every 3–6 months.\(^{6,12-15}\)

A controversy exists about whether patients with F3 could be discharged\(^ {10}\) or should receive regular post-SVR HCC surveillance since fibrosis regression after HCV eradication would also decrease HCC risk.\(^ {14}\) A meta-analysis showed that the incidence of HCC after HCV eradication by DAAs was 2.99 per 100 person-years and 0.47 per 100 person-years in cirrhotic and non-cirrhotic patients, respectively. For patients with F3, the incidence of HCC was 0.63 per 100 person-years. Based on the relatively low incidence of HCC, the authors concluded that screening HCC for F3 patients was not warranted.\(^ {17}\) Notably, the definition of each fibrotic stage was not universal across studies and misclassification would exist. APASL did not mention the definition of each fibrotic stage, and only EASL defined F3 by histology or liver stiffness measurement (transient elastography 10–13 kPa, Aixplorer 9–13 kPa or Acoustic Radiation Force Impulse 1.6–2.17 m/s).\(^ {18}\) Accordingly, the management of SVR patients with advanced fibrosis should be individualized in terms of local healthcare policies.

For patients with mild fibrosis, those with comorbidities or ongoing risk behaviors (alcohol use, diabetes mellitus [DM], or obesity) shall be kept for HCC surveillance.\(^ {12}\) DM is well-recognized as the oncogenic factor of HCC through the mechanisms of the hyperinsulinemia-related PI3K/AKT/mTOR-signaling pathway, oxidative stress and chronic inflammation.\(^ {19}\) Pre-DM status has even been reported to carry a higher risk of HCC than normoglycemic status in SVR patients with mild fibrosis.\(^ {20}\) Meanwhile, metabolic syndrome (MS) has been shown to increase HCC risk in SVR patients with advanced fibrosis. Patients with MS, in particular those with DM, had a 3.03-fold higher risk of HCC compared to those without MS.\(^ {21}\) As increased body weight and hepatic steatosis have been reported after HCV eradication,\(^ {22}\) the concurrence of steatotic liver disease should be incorporated with HCV infection with respect to holistic care in the post-SVR era\(^ {23}\) since it might be an in-

<table>
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<tr>
<th>Society</th>
<th>APASL</th>
<th>EASL</th>
<th>AASLD</th>
<th>TASL</th>
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<tr>
<td>Target population</td>
<td>All patients (C2)</td>
<td>F3, F4 (A1)</td>
<td>F4 (B2)</td>
<td>All patients (B1)</td>
</tr>
<tr>
<td>Screening for patients with mild fibrosis with comorbidities</td>
<td>Yes (A1)</td>
<td>Yes (A1)</td>
<td>No (B2)</td>
<td>Yes (B1)</td>
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<tr>
<td>Screen Interval</td>
<td>F0-2: every 6 months for 2 years, then every 12 months</td>
<td>Every 6 months indefinitely (A1)</td>
<td>Every 6 months indefinitely (A1)</td>
<td>F0-1 with HCC risk factors* and F2: every 6–12 months (B1)</td>
</tr>
<tr>
<td>Modality</td>
<td>Sonography+ tumor markers (AFP, PIVKA-II, AFP-L3) (A1)</td>
<td>Sonography (B1)</td>
<td>Sonography with AFP (B1)</td>
<td>N/A</td>
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APASL, The Asian Pacific Association for the Study of the Liver; EASL, The European Association for the Study of the Liver; AASLD, American Association for the Study of Liver Diseases; TASL, Taiwan Association for the Study of the Liver; F0-2, fibrotic stage 0-2; F3, fibrotic stage 3; F4, fibrotic stage 4; cirrhosis. AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II; N/A, not available; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ALT, alanine aminotransferase.

*Past HCC history, HBV/HCV dual infection, older age, male gender, presence of dysplastic nodule, alcohol consumption, diabetes mellitus, low albumin, low platelet count, high AFP post-treatment, high ALT post-treatment, and high g-GT level pre- and post-treatment. Evidence grading denotes evidence quality (A: high, B: moderate, C: low) and recommendation (1: strong, 2: weak).
dependent risk factor for HCC after HCV eradication.\textsuperscript{24,25}

**HOW LONG SHOULD POST-SVR HCC SURVEILLANCE BE MAINTAINED?**

A study using a microsimulation model suggested that rather than lifelong monitoring, screening for post-SVR HCC is cost-effective up to age 70 in those with cirrhosis and up to age 60 in those with stable advanced fibrosis.\textsuperscript{26} The aforementioned meta-analysis indicated a pooled HCC incidence after SVR in patients with cirrhosis was very high (2.99/100 person-years) but would decline as time went by after HCV eradication.\textsuperscript{27} For example, the incidence of HCC was 6.17% for studies with a follow-up period less than 1 year and decreased to 1.83% for studies with a follow-up period greater than 3 years. Notably, HCC risk remains and persists up to decades after HCV eradication.\textsuperscript{27,28} All three regional guidelines suggest post-SVR HCC surveillance should be maintained indefinitely for the recommended target populations.\textsuperscript{6,12,13}

**RISK FACTORS AND PREDICTORS OF POST-SVR HCC**

Plenty of risk factors or surrogate markers have been identified to predict post-SVR HCC.\textsuperscript{15} As mentioned earlier, liver cirrhosis per se is the major risk factor predictive of post-SVR HCC. Liver fibrosis would augment after HCV eradication, and post-treatment liver fibrotic change could be more accurate than the pre-treatment status in predicting HCC.\textsuperscript{16} Of the non-cirrhotic patients, several surrogate markers/predictors have been reported, which could be briefly divided into fibrosis-related (age, platelet count, aspartate aminotransferase [AST] to platelet ratio index [APRI], fibrosis-4 index [FIB-4], AST/alanine aminotransferase [ALT] ratio, albumin) or non-fibrosis-related (DM, HCV genotype 3, AFP and gamma-glutamyl transferase level).\textsuperscript{20,27-33}

Developing an HCC prediction model by weighing and combining individual risk factors may help to promote risk stratification (Table 2). A web-based, model-guided strategy has been developed to facilitate HCC screening, of which age, platelet count, AST/ALT ratio, and albumin level

**Table 2.** Selected HCC prediction model after achieving sustained virological response

<table>
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<th>Regimen</th>
<th>Parameter</th>
<th>Accuracy/ discrimination power</th>
<th>Note</th>
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<tr>
<td>DAA</td>
<td>Age, LSM, alcohol consumption, albumin</td>
<td>AUC: 0.91 in the gene-score and 0.85 in the nomogram</td>
<td>Decision-tree-based algorithms based on genetic alterations and clinical profile</td>
</tr>
<tr>
<td>DAA</td>
<td>Baseline LSM, 1-year delta-LSM and albumin</td>
<td>Bootstrapped AUC 0.67–0.80</td>
<td>Stratify HCC risk in patients with compensated advanced chronic liver disease</td>
</tr>
<tr>
<td>DAA</td>
<td>Age, LSM, alcohol consumption, albumin and AFP</td>
<td>Harrell’s C: 0.77</td>
<td>Predict patients with very low risk of HCC to avoid unnecessary surveillance</td>
</tr>
<tr>
<td>IFN; DAA</td>
<td>FIB-4 and gene score including post-treatment TAS1R3, FOSL1 and ABCA3</td>
<td>AUC: 0.91 in the gene-score and 0.85 in the nomogram</td>
<td>Decision-tree-based algorithms based on genetic alterations and clinical profile</td>
</tr>
<tr>
<td>IFN; IFN plus DAA; DAA</td>
<td>Major determinant: age, platelet count, AST/ALT ratio and albumin Index, hemoglobin and AFP</td>
<td>Gonen and Heller’s κ-statistic: 0.70 – 0.77</td>
<td>excellent correlation in patients with cirrhosis/SVR; no correlation in patients with cirrhosis/no SVR</td>
</tr>
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IFN, interferon; DAA, direct-acting antiviral; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LSM, liver stiffness measurement; AFP, alpha-fetoprotein; AUC, area under the curve; FIB-4, fibrosis-4 index.
were the four major determinants. A dynamic transient elastography-based model has also been created to identify very low-risk patients, which helps to avoid unnecessary surveillance. An algorithm that combines age, liver stiffness measurement, alcohol consumption, albumin, and AFP in SVR patients with advanced liver chronic disease successfully stratified HCC risk, while an artificial intelligence-based prediction model using the recurrent neural network of age, sex, race, HCV genotype, and 24 laboratory tests has proven to be more accurate than using the traditional regression model. A decision-tree algorithm combining gene score (TAS1R3, FOSL1, and ABCA3) and FIB-4 has also been created to predict post-SVR HCC. Nevertheless, regarding all these contributions to the field, the “black box” of artificial intelligence-based study outcomes awaits further validation in clinical practice.

HCC RECURRENT AFTER ACHIEVING SVR

Unlike CHC patients who received interferon-based therapy, an increased HCC recurrence risk was postulated in the early era of DAAs. A large-scale study did not suggest a higher HCC recurrence rate in DAA-treated patients compared to those untreated. Following this, pooled analysis also did not reveal a higher HCC recurrence risk. Owing to the heterogeneity of patient characteristics and varying follow-up periods among published papers, Sapeña et al. conducted an individual patient data meta-analysis from 21 studies and found that the HCC recurrence rate did not differ between DAA-exposed (14.75 per 100 person-years) and DAA-unexposed (23.21 per 100 person-years) patients after a median follow-up period of 15 months. The result highlights the fact that the recurrence rate remained high even after HCV eradication, and CHC patients with curative HCC should receive close follow-up after achieving SVR. APASL recommended following SVR patients with HCC history every 4 months.

GENETIC POLYMORPHISM AND SOMATIC MUTATION SIGNATURES POST SVR

HCC is a multifactorial disease that is the result of genetic and epigenetic alterations, followed by the process of selection. Recent extensive sequencing of liver cancer samples identified genomic signatures and driver genes associated with HCC. However, the genomic profile of liver genomes after SVR is currently poorly defined. A lower frequency of mutated ARID genes in HCV-SVR as compared to HCV-positive tumors was found; mutations in this gene are specifically induced in HCV-related HCC. In contrast, mutations in the KEAP1 and PREX2 genes were more frequently identified in HCV-SVR samples as compared to HCV-positive samples, and were previously reported in HCC. We have summarized the mechanisms of post-SVR HCC in Table 3. These mutations result in resistance to oxidative stress while inducing metabolic transformation of cancer cells, and therefore, could be valid therapeutic and prognostic markers for HCC post-curation.

Recent findings show significant differences between HCV-related HCC following treatment with DAAs vs. IFN. In SVR patients, TP53 mutations as well as genomic abnormalities were significantly more frequent in DAA-treated patients as compared to IFN-treated patients, suggesting that mutations in TP53 result in genomic instability after SVR by DAAs and might serve as prognostic markers for post-cured HCC specifically after DAAs treatment. In contrast, in IFN-treated patients, a higher activation of the PI3K/AKT/mTOR pathway was observed and was associated with tumor aggressiveness and invasive phenotypes.

In addition to somatic mutations, several recent genome-wide association studies (GWAS) have shown high prevalence of alleles in specific loci in HCV-related HCC cases, which bear potential as markers for predisposition for HCC, also after SVR. For example, the 5’ flanking region of major histocompatibility complex (MHC) class I chain-related A (MICA) (6p21.33) in the MHC of class I region and single nucleotide polymorphisms (SNPs) in the HLA-DQB1 locus are associated with HCC. In SVR patients, a variant of toll-like-like 1 (tl1) gene on chromosome 4 (rs17047200), associated with the TGFβ signaling pathway, was suggested as a marker of increased risk for HCC. Recently, a genetic risk score associated with hepatic steatosis, including patatin-like phospholipase domain-containing protein 3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2), membrane-bound O-acyltransferase domain-containing 7 (MBOAT7), and glucokinase regulator (GCKR), was reported to be related to HCC development in cirrhotic patients. Consequently, hepatic fat might be a
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<th>Reference</th>
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**Table 3. Continued**
prognostic marker for HCC development in patients cured by DAAs and a target for chemoprevention. Polymorphism in interferon-λ3 (formerly known as interleukin-28B [IL28B]) was also found to be associated with increased risk for HCC pre- and post-SVR.57

To summarize, genetic predictors for HCC, either genetic variants or somatic mutations in cirrhotic patients, could enable stratification of cured patients for personalized HCC monitoring. Since gene expression profiles encompass both the epigenetic landscape and genomic aberrations, more multi-omics studies are required to elucidate the relationship between the two and their relative contributions to cancer progression.

**EPIGENETICS AND GENE EXPRESSION SCAR POST SVR**

Epigenetics is the study of heritable events occurring in the genome that determine chromatin structure but not in the DNA sequence, including post-translation histone modifications (PTMs), DNA methylation and RNA-based mechanisms, and affects transcription programs.58,59 These modifications may shift between active and silent states, resulting in activation or repression of gene expression.60 They depend on the activity of specific enzymes such as histone acetyltransferase (HAT), Gcn5/PCAF and p300/ CBP, histone deacetylase (HDACs), and histone methyltransferase enzymes (HMTs).61,62 Viruses can impose epigenetic changes that alter host transcription programs, thereby promoting their own propagation, and may contribute to cancer occurrence. We and others have recently shown that the altered epigenetic state associated with HCV infection persists, to some extent, even after cure following DAAs and IFN-based treatments.63-67 This observation is consistent in various HCV infection models, including immortalized human liver and hepatoma cells, a human liver chimeric mouse model, and post-SVR human liver samples. In cell culture, HCV-induced changes in active chromatin markers H3K4Me3 and H3K9Ac and silent chromatin marker H3K9Me3 were associated with altered expression of genes involved in cancer-related pathways. A positive correlation was observed between chromatin modification and gene expression in HCV-infected cells, both before and after virus eradication.
by DAA. This persistent epigenetic imprint was recapitu-
lated in pre- and post-SVR human liver samples, even 
years after virus eradication by DAA. Interestingly, our 
data indicate more reversion of both RNA and epigeneti-
expression scars.63,64,67 The nature of the immune response induced by HCV infec-
tion determines the outcome of infection, i.e., whether it 
resolves or progresses to chronic infection, and contributes 
to HCC development. Since complete viral elimination by 
DAA is possible, HCV infection is a unique model to study 
the effect of infection and its eradication on immune re-
sponses and clinical outcomes. Due to the residual risk of 
liver diseases and HCC after cure with DAA, it is impor-
tant to understand whether the HCV-induced alterations in 
the immune response return to normal after viral eradica-
tion. Indeed, recent studies have reported that the altered 
characteristics and functions of various immune cells in 
chronic HCV infections persist to some extent as an immu-
nological imprint after cure with DAA; accordingly, the 
elimination of HCV by DAAs and its influence on the im-
mune response could affect the development of hepatocar-
cinogenesis.

Overall, the innate immune response following HCV cure 
is only partially restored. A decrease in ISGs expression 
and type I IFN response in peripheral blood mononuclear 
cells in the liver was observed after DAAs treatment, there-
fore resulting in a weaker antitumor-immune state and con-
tributing to hepatocarcinogenesis.72-75 In patients with acute 
or chronic infection following virus elimination by DAAs 
treatment,76,77 and spontaneously resolved HCV infections,78 cytokines levels were decreased but still not re-
turned to normal range. Yet, most of these studies were 
conducted within several months after treatment, and lon-
ger follow-up studies are still required.

NK cells play an important role in the innate anti-HCV im-
mune response,79 but are damaged in chronic HCV infec-
tions.80,81 Following HCV eradication by DAAs, some of the 
damaged phenotypes and functions of NK cells were re-
versed,82,83 while some functions persisted, such as de-
creased intra-individual NK cell diversity.84 NK cells also 
bear antitumor activity, and their frequency is associated 
with HCC recurrence-free survival.86 Downregulation of NK 
group 2D (NKG2D), which is important for NK cell antitu-
tumor activity, has been reported in association with HCC occurrence and recurrence post-DAA therapy. In addition to NK cells, unique innate-like T-cells such as γδ T-cells and mucosa-associated invariant T (MAIT) cells were found to be impaired pre- and post-cure of chronic HCV infections and may underlie pathologies and post-cure HCC development.

An imprinted adaptive immune response was also observed after HCV cure. Effective CD8+ and CD4+ T cell responses were associated with HCV clearance, while weak and exhausted responses were associated with chronic infection. Persistent induction of T cells in chronic infection leads to T-cell exhaustion, which was only partially restored after cure with DAA- and IFN-based treatments. Specifically, mitochondrial function and transcription programs of exhausted HCV-specific CD8+ T-cells were not fully recovered following cure and were associated with a distinct epigenetic signature and post-cure HCC development. Furthermore, CD4+ T cells remained impaired in chronic HCV infections after cure with DAAs.

The levels of circulating regulatory T cells (T regs) remain persistently high long after HCV cure with DAAs and increase with HCC progression, suggesting that high T regs levels after DAA treatment may be related to post-cure HCC. T regs count correlates with myeloid-derived suppressor cell counts, which are increased in chronic HCV patients and remain high following cure with DAAs.

To summarize, although some functions of the innate and adaptive immune responses associated with HCV infections are normalized after HCV cure with DAs, many persist after cure. These may lead to a pro-cancerous environment that may contribute to post-cure HCC. Understanding the molecular mechanisms that impact these immunological scars may set foundations for their prevention or reversal, though, for example, epigenetic drugs that revert the epigenetic scar in immune cells.

PROTEOMIC AND METABOLIC PROGNOSTIC MARKERS POST-SVR

The changes in the levels of cytokines and chemokines after achieving SVR have been proposed as predictors of HCC. Lu et al. showed that downregulation of members of the TNF superfamily, including TNF-α and TNF-like weak inducer of apoptosis (TWEAK), increased the risk of HCC development. A strong prediction model for post-SVR HCC treated with either DAA or IFN-included FIB-4, hemoglobin A1c, and levels of TNF-α and TWEAK. An increase in TNF-α levels is associated with increased hepatic inflammation and HCC risk, suggesting that its pretreatment concentration predicts post-SVR HCC risk and an association between its persistently high expression after SVR and the development of HCC. The sharp decline in TNF-α after cure may impair immune surveillance and inhibit antitumor response. Moreover, a correlation between high pretreatment serum levels of 12 immune mediators and post-treatment HCC development was identified, as well as high levels of IL-13 and IL-4.

Circulating protein biomarkers of HCC, such as AFP, have also been suggested as prognostic markers. More specifically, higher pre- and post-treatment levels of AFP were associated with HCC development. However, the accuracy, in particular the sensitivity of AFP, is an issue. Other circulating protein biomarkers including wisteria floribunda agglutinin-positive Mac-2-binding protein, serum sphingolipids, VEGF, and angiopoietin-2 also associated with post-curative HCV-related HCC occurrence. A large cohort study found that the MICA A allele and high serum MICA (sMICA) levels correlated with HCC development, but only in cirrhotic non-SVR patients. A follow-up study found its levels to be lower and to gradually decline in non-HCC compared to HCC patients, while higher sMICA levels gradually increased in post-SVR HCC, but only in MICA GG, and not A allele, carriers.

Circulating microRNA (miRNA) profiles have also been suggested as biomarkers for HCC development. MIR-3197 was identified as a potential prognostic marker for HCC risk during DAA treatment. Circulating miRNA levels of members of the Let-7 family were associated with fibrosis progression, were downregulated in HCV infection and lower in patients who developed HCC after SVR compared to those without HCC. This may be related to the antitumor activity of Let-7, which downregulates chronic inflammation.
MODULATION OF RISK GENE SIGNATURE WITH TARGETING AGENTS FOR HCC POST SVR

Identification of potentially reversible HCV-related alterations in epigenetics and gene expression might contribute to efforts to reduce risk of HCC post-SVR. The discovery of druggable targets requires the elucidation of molecular mechanisms that drive these altered signatures. Recently, specific molecular pathways were identified as inducers of the epigenetic state dysregulated by HCV and as potential targets for HCC chemoprevention. Nakagawa et al. studied...
ied the pan-etiology PLS gene, which predicts the risk for HCC both before and after SVR. They identified the pro-fibrosis lysophosphatidic acid pathway as a potential chemoprevention target. Inhibition of this pathway by inhibitors AM063 and AM095 resulted in reversal of the altered expression of gene signature after SVR and fibrosis attenuation and prevented HCC development in animal and in vitro models. Another potential druggable target is the EGFR, which is a cofactor for HCV entry into cells and is also activated by HCV and contributes to HCC development. The EGFR inhibitor erlotinib induced reversion of the PLS genes altered expression and prevented progression of cirrhosis and HCC in animal models. We found that erlotinib reversed gene expression and epigenetic signatures after HCV cure with DAAAs. In addition, the unfolded protein response (UPR) that is activated by HCV has been reported to contribute to HCV-induced epigenetic and transcriptional alterations; treatment with the UPR inhibitor BAPTA partially reversed this effect.

Targeting epigenetic enzymes as a genome-wide approach was recently demonstrated to efficiently reverse the epigenetic and gene expression signatures associated with HCV infection. We showed that the HAT p300/CBP inhibitor C646 reversed the persistent changes in H3K9Ac induced by HCV and the associated gene expression signature. A panel of inhibitors targeting epigenetic enzymes, including HATs, bromodomain-containing proteins 3/4 (BRD3/4), mixed-lineage leukemia protein/WD repeat domain 5 complexes and HDACs, reversed the altered expression of the PLS genes in an HCV-infected cell culture model. In addition, the authors observed common gene expression patterns between the HCC etiologies HCV and NASH, and the reversion of this signature following treatment with the BRD4 inhibitor as well as inhibition of cancer progression and liver inflammation in a NASH mouse model. Further studies are urgently needed to identify additional druggable targets for prevention of liver disease and HCC development both pre- and post-SVR.

**CHEMOPREVENTION OF POST-SVR HCC**

Cigarette smoking is demonstrated as the risk of liver fibrogenesis and hepatocarcinogenesis, and smoking cessation may decrease the risk of HCC. Whether quitting smoking reduces post-SVR HCC in CHC patients remains to be explored. Albeit DM is a risk factor for HCC, metformin use seemed to play a protective role in CHC-related HCC. Tsai et al. enrolled 7,249 Taiwanese CHC patients who achieved SVR, and the 5-year cumulative HCC incidence was 10.9% in diabetic non-metformin users and 2.6% in diabetic metformin users, compared to 3.0% in individuals without DM. Diabetic patients without metformin use had a 2.83-fold risk of HCC compared to non-diabetic patients, whereas the risk of HCC in diabetic patients who used metformin had reduced and had similar risk of HCC as with non-diabetic patients. Recent meta-analysis and pooled data has showed that statin and aspirin might reduce HCC risk as a whole, and while the role of aspirin in preventing post-SVR HCC in CHC is elusive. A Taiwanese cohort has demonstrated the chemopreventive effect of statin in reducing HCC risk in SVR patients.

**IMPACT OF ANTIVIRAL THERAPY ON HCV VIREMIC HCC PATIENTS**

Earlier reports have indicated an inferior SVR rate in HCC patients who received DAAAs. A meta-analysis including 49 studies has shown a lower SVR rate of 73.1% in ac-
tive HCC patients compared to that of 92.6% in inactive patients and of 93.3% in non-HCC patients. Notably, much of the data came from the reports using suboptimal regimens in the early DAA era. With the current standard of care regimens, sofosbuvir/velpatasvir and glecaprevir/pibrentasvir used in two nationwide studies from Taiwan have validated similar treatment efficacy among patients with/without inactive or active HCC. HCC is no longer an unfavorable factor associated with treatment failure with the application of more potent DAAs. Imperatively, long-term survival would be better for the viremic HCC patients receiving subsequent HCV eradication compared to those who remain persistently viremic.

A postulation is that HCC patients might benefit from viral eradication in terms of more preserved liver function, potentially allowing for salvage anti-cancer treatments once the patients encounter primary HCC treatment failure. The result suggests that it is better-late-than-never to treat HCV. CHC patients with active HCC should be treated with DAAs aggressively unless a short life expectancy due to HCC is anticipated. Lastly, HCV eradication significantly reduce hepatic vein pressure gradient. Recompensation occurs in a substantial proportion of decompensated patients. The benefit of HCC risk reduction in decompensated patients during the recovery of liver function reserve is controversial. The conduction of a prospective treated-versus-untreated controlled trial is unethical and impractical. Recently a meta-analysis including 4 retrospective studies have demonstrated a marginal benefit of 26% HCC risk reductions (95% confidence interval 0.52, 1.00; P=0.05) in DAA treated decompensated patients compared to untreated control.

In conclusion, post-SVR HCC remains as occurring in a subset of CHC patients due to preexisting inflammatory and fibrotic liver background, immune dysregulation as well as host epigenetic scar, genetic predispositions and alternations (Fig. 1). There are remaining unmet needs in post-SVR HCC surveillance and management (Table 4). By means of applying surrogate markers and adopting risk stratification, HCC surveillance should be consistently performed in high-risk populations.

Authors’ contributions
Conception and design: Ming-Lung Yu. Manuscript drafting and critical revision: Chung-Feng Huang, Manar Hijaze Awad, Meital Gal-Tanamy and Min-Lung Yu. Approval of the final version of the manuscript: Ming-Lung Yu and Meital Gal-Tanamy.

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Conflicts of Interest

Ming-Lung Yu: Research support (grant) from AbbVie, BMS, Gilead, Merck and Roche diagnostics. Consultant of AbbVie, BMS, Gilead, Roche and Roche diagnostics. Speaker of AbbVie, BMS, Eisai, Gilead, Roche and Roche diagnostics.

Chung-Feng Huang: Speaker for AbbVie, BMS, Gilead, Merck, and Roche.

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899.


Sorafenib vs. Lenvatinib in advanced hepatocellular carcinoma after atezolizumab/bevacizumab failure: A real-world study

Young Eun Chon¹,*, Dong Yun Kim², Mi Na Kim², Beom Kyung Kim², Seung Up Kim², Jun Yong Park², Sang Hoon Ahn³, Yeonjung Ha¹, Joo Ho Lee¹, Kwan Sik Lee¹, Beodeul Kang³, Jung Sun Kim³, Hong Jae Chon³, and Do Young Kim²

¹Department of Gastroenterology, CHA Bundang Medical Center, CHA University, Seongnam; ²Department of Internal Medicine, Yonsei University College of Medicine, Seoul; ³Department of Medical Oncology, CHA Bundang Medical Center, CHA University, Seongnam, Korea

Study Highlights

• This study compared treatment and survival outcomes of lenvatinib and sorafenib as second-line treatments for advanced HCC following ATE+BEV failure.

• Although overall survival was comparable between the two treatments after adjustment, lenvatinib treatment demonstrated significantly better PFS than sorafenib, both before and after adjusting for patient characteristics.

• The findings enhance our understanding of effective treatment strategies for advanced HCC, suggesting the potential role of lenvatinib for improving PFS.

• These insights are valuable for optimizing therapy in advanced HCC patients who do not respond to first line ATE+BEV treatment, thereby helping to bridge the gap between the current clinical guidelines and the real-world treatment for advanced HCC.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the second leading cause of cancer deaths in South Korea and worldwide. In contrast to patients with early-stage or intermediate-stage HCC, who may have many treatment options for HCC such as surgery, radiofrequency ablation, transplantation, or transarterial chemoembolization, patients with advanced HCC typically only derive survival benefits from systemic therapies. Unfortunately, more than 50% of patients with HCC are first diagnosed at advanced stages, and these patients...
are more likely to undergo systemic therapies. Sorafenib, an oral tyrosine kinase inhibitor (TKI), has been used as a standard systemic HCC treatment since 2007. However, another potent TKI lenvatinib was introduced in 2018 and has played a leading role in improving the clinical outcomes of advanced HCC. In 2020, an immunotherapy-based combination regimen of atezolizumab (ATE, an immune checkpoint inhibitor) and bevacizumab (BEV, a targeted therapy agent) provided a survival benefit over sorafenib in overall survival (OS) at 12 months (67.2% vs. 54.6%, \( P < 0.001 \)) and progression-free survival (PFS, 6.8 vs. 4.3 months, \( P < 0.001 \)) in patients with advanced HCC. Currently, the landscape of systemic treatment for unresectable HCC has changed to the use of ATE+BEV as the initial treatment unless there are contraindications.

However, the optimal second-line and subsequent treatments for patients with advanced HCC who have progressed on first-line ATE+BEV treatment have not been clearly defined in the national and international guidelines for HCC. This may be because the molecular expression of HCC is so diverse that there are no established biomarkers to guide subsequent treatment, and survival and clinical outcomes have not been established in real-world settings.

Only a few clinical studies have demonstrated the OS and PFS of patients who underwent various second-line therapies such as lenvatinib or sorafenib after ATE+BEV failure. However, there was an insufficient number of patients in these studies, and baseline characteristics were not adjusted for an exact comparison. In the present study, we compared the clinical effectiveness of sorafenib and lenvatinib as a second-line treatment after failure of ATE+BEV in unadjusted and matched patient cohorts with advanced HCC in a real-world setting.

**MATERIALS AND METHODS**

**Patients and definitions**

This retrospective study included patients who started second-line systemic treatment with sorafenib or lenvatinib after HCC progressed on ATE+BEV between August 2019 and December 2022 at one of two university hospitals (Severance Hospital and CHA Bundang Medical Center). All study patients had been diagnosed with unresectable HCC, either histologically or clinically, following HCC guidelines.

Recorded data included age, sex, performance status and serum concentrations of tumor markers of alpha-fetoprotein (AFP) and protein induced by vitamin K antagonist-II (PIVKA-II). Hepatitis B virus (HBV) infection was defined as hepatitis B surface antigen seropositivity for more than 6 months, and hepatitis C virus (HCV) infection was defined as seropositivity for anti-HCV antibody. HBV and/or HCV infection were classified as viral etiology, whereas alcohol, non-alcoholic steatohepatitis, and other chronic liver diseases were classified as non-viral etiologies. The hepatic functional reserve was assessed with the Child-Pugh score. Tumor characteristics assessed in all patients included Barcelona Clinic Liver Cancer (BCLC) stage; tumor number; tumor size; and the presence of lymph node metastasis, extrahepatic metastasis, macrovascular invasion, and extrahepatic lesions. The number of previous ATE+BEV treatment cycles was also recorded. The study protocol was reviewed and approved by the Institutional Review Boards of Severance Hospital (IRB No. #4-2023-1273) and CHA Bundang Medical Center (IRB No. #2021-07-071), which waived the requirement for informed patient consent due to the retrospective nature of the analyses. The study was performed in accordance with the ethical standards of the latest amended Declaration of Helsinki.

**Treatment regimens**

All study patients had been treated previously with a combination of ATE (1,200 mg) and BEV (15 mg/kg), administered intravenously every 3 weeks. After confirmation of tumor progression on ATE+BEV treatment, subsequent systemic therapy was administered at the discretion of the attending specialists. The dose and interval of treatment regimen adhered to standard protocols. Patients received oral lenvatinib (12 mg/day for bodyweight ≥60 kg or 8 mg/day for bodyweight <60 kg) or sorafenib 400 mg twice-daily for 4 weeks in a cycle. Treatment was continued until disease progression, unacceptable toxicity, or death. Dosage was adjusted considering each patient’s tolerability.
Assessment of clinical outcomes

Patients received oral lenvatinib or sorafenib for 4 weeks per cycle, with treatment response and safety evaluated every 8 to 12 weeks (i.e., after every two to three treatment cycles). The radiological response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 based on the results of liver dynamic computed tomography or magnetic resonance imaging (if appropriate). The objective response rate (ORR) was defined as the proportion of patients who achieved a complete response (CR) or partial response (PR), and the disease control rate (DCR) was defined as the proportion of patients who achieved CR, PR, or stable disease (SD). Treatment-related adverse events (AEs) were assessed according to the Common Terminology Criteria for Adverse Events version 5.0. OS was defined as the interval from the initiation of second-line treatment to death or final follow-up, and PFS was defined as the interval from the initiation of second-line treatment to the date of disease progression or death from any cause, whichever occurred first.

Statistical analysis

Variables were expressed as mean ± standard deviation, median (interquartile range [IQR]), and number (%). Differences between continuous variables were assessed by Student’s t-tests or Mann–Whitney U-tests, whereas differences between categorical variables were assessed by chi-square tests or Fisher’s exact tests. Survival curves were generated by the Kaplan–Meier method and differences between treatment groups were assessed by log-rank tests. Factors independently predictive of OS and PFS were assessed using a multivariable Cox proportional hazards regression model. The possible effects of selection bias and potential confounders between the two groups were reduced by 1:1 propensity score (PS) matching. Statistical analyses were performed using SAS software (ver. 9.4; SAS Institute, Cary, NC, USA) and R software (version 4.3.1; http://cran.r-project.org/, accessed 16 June 2023). Two-sided P-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics of the study population

Figure 1 shows a diagram of the study population. A total of 194 patients who received ATE+BEV as first-line sys-
Cytomimetic therapy and subsequently discontinued treatment between August 2019 and December 2022 at one of two university hospitals (Severance Hospital and CHA Bundang Medical Center) was considered eligible. During ATE+BEV treatment, 34 patients died, and 8 patients experienced compromised liver function and/or clinical deterioration. Thus, excluding these 42 patients, 152 patients discontinued ATE+BEV due to disease progression. After excluding 16 more patients who experienced compromised liver function and/or clinical deterioration after cessation of ATE+BEV, a total of 136 patients received second-line treatment after progression on first-line ATE+BEV treatment. Of these patients, 86 (63.2%) received sorafenib, 40 (29.4%) received lenvatinib, 8 (5.9%) received regorafenib, and 2 (1.5%) underwent 5-FU based infusion chemotherapy. Finally, the present study enrolled 126 patients, 40 (31.7%) who received lenvatinib, and 86 (68.3%) who received sorafenib after ATE+BEV failure.

The baseline characteristics of the patients at the start of second-line treatment are depicted in Table 1. The median patient age was 63 years (IQR, 55–70 years), and 111 (88.1%) patients were male. HCC was of viral etiology in 92 (73.0%) patients, 109 (86.6%) patients were classified as having BCLC stage C tumors, and 91 (72.2%) as having Child-Pugh A liver function. The median number of previous ATE+BEV treatment cycles was 4 (IQR, 3–6). The proportion of patients with Child-Pugh A liver function was significantly higher (92.5% vs. 62.8%, \( P<0.001 \)) and the median number of previous ATE+BEV treatment cycles was significantly higher in the lenvatinib group compared to the sorafenib group (6 [IQR, 4–10] vs. 3 [IQR, 2–6], \( P<0.001 \)). There were no other significant differences in baseline characteristics between the 2 groups.

To reduce the effects of confounding variables and selection bias, PS matching was conducted using the following factors: age, sex, tumor size, tumor number, extrahepatic metastasis, lymph node metastasis, and Child-Pugh class. PS matching on a 1:1 ratio resulted in 36 pairs of patients with balanced baseline characteristics. However, the median number of ATE+BEV treatment cycles was significantly higher in the lenvatinib group than in the sorafenib group (Table 1).
### Table 1. Continued

| Variables | Before matching | | | | | After propensity score matching | | | | |
|-----------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|           | Total (n=126)  | Lenvatinib (n=40) | Sorafenib (n=86) | **P-value** | Lenvatinib (n=36) | Sorafenib (n=36) | **P-value** | | | | |
| Child-Pugh Class | | | | | | | | | | | |
| A         | 91 (72.2) | 37 (92.5) | 54 (62.8) | 0.001 | 33 (91.7) | 32 (88.9) | 0.564 |
| B         | 35 (27.8) | 3 (7.5) | 32 (37.2) | | 3 (8.3) | 4 (11.1) |
| Child-Pugh score | | | | | | | | | | | |
| 5         | 65 (51.6) | 29 (72.5) | 36 (41.9) | 0.001 | 25 (69.4) | 26 (72.2) | 0.799 |
| 6         | 26 (20.6) | 8 (20.0) | 18 (20.9) | | 8 (22.2) | 6 (16.7) |
| 7         | 23 (18.3) | 3 (7.5) | 20 (23.3) | | 3 (8.3) | 4 (11.1) |
| 8         | 12 (9.5) | 0 | 12 (14.0) | | 0 | 0 |
| Number of intrahepatic tumors | | | | | | | | | | | |
| Single    | 43 (34.1) | 16 (40.0) | 27 (31.4) | 0.343 | 13 (36.1) | 15 (41.7) | 0.617 |
| Multiple  | 83 (65.9) | 24 (60.0) | 59 (68.6) | | 23 (63.9) | 21 (58.3) |
| Maximal size of intrahepatic tumor, cm | 4.5 (2–8.5) | 4.9 (2.4–9.6) | 4.5 (1.8–7.8) | 0.411 | 3.45 (2.05–9.05) | 4.25 (1.75–7.50) | 0.875 |
| Extrahepatic metastasis | 87 (69) | 29 (72.5) | 58 (67.4) | 0.690 | 27 (75) | 29 (80.6) | 0.778 |
| Lymph node metastasis | 48 (38.1) | 20 (50.0) | 28 (32.6) | 0.061 | 19 (52.8) | 13 (36.1) | 0.157 |
| Macrovascular invasion | 58 (46.0) | 17 (52.1) | 41 (47.7) | 0.588 | 14 (38.9) | 16 (44.4) | 0.637 |
| AFP, ng/mL | 406 (19–5,967) | 181 (12–8,307) | 546 (25–4,783) | 0.514 | 123 (12–2,836) | 727 (25–5,119) | 0.338 |
| PIVKA-II, mAU/mL | 1,344 (203–14,120) | 1,626 (168–7,106) | 1,328 (273–4,307) | 0.793 | 1,909 (168–7,106) | 1,425 (193–9,963) | 0.385 |
| Previous atezolizumab plus bevacizumab treatment cycles, number | 4 (3–6) | 6 (4–10) | 3 (2–6) | 0.001 | 6 (4–10) | 3 (2–4) | 0.007 |

Values are presented as median (interquartile range), or number (%).

Matching variables: age, sex, tumor size, tumor number, extrahepatic metastasis or lymph node metastasis, Child-Pugh class.

ECOG PS, Eastern Cooperative Oncology Group performance status; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein; PIVKA-II, prothrombin-induced by vitamin K absence or antagonist-II.
## Table 2. Clinical responses of second-line treatment

<table>
<thead>
<tr>
<th>Clinical responses</th>
<th>RECIST 1.1</th>
<th>mRECIST</th>
<th>P-value</th>
<th>RECIST 1.1</th>
<th>mRECIST</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unadjusted cohort</strong></td>
<td>Lenvatinib (n=40)</td>
<td>Sorafenib (n=86)</td>
<td>P-value</td>
<td>Lenvatinib (n=40)</td>
<td>Sorafenib (n=86)</td>
<td>P-value</td>
</tr>
<tr>
<td>Best overall response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete response</td>
<td>0</td>
<td>0</td>
<td>0.719</td>
<td>0</td>
<td>0</td>
<td>0.153</td>
</tr>
<tr>
<td>Partial response</td>
<td>3 (7.5)</td>
<td>5 (5.8)</td>
<td>&lt;0.001</td>
<td>6 (15.0)</td>
<td>6 (7.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stable disease</td>
<td>24 (60.0)</td>
<td>16 (18.6)</td>
<td>0.004</td>
<td>22 (55.0)</td>
<td>15 (17.4)</td>
<td>0.002</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>11 (27.5)</td>
<td>47 (54.7)</td>
<td>&lt;0.001</td>
<td>10 (25.0)</td>
<td>47 (54.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Could not be evaluated</td>
<td>2 (5.0)</td>
<td>18 (20.9)</td>
<td>0.022</td>
<td>2 (5.0)</td>
<td>18 (20.9)</td>
<td>0.022</td>
</tr>
<tr>
<td>Objective response rate</td>
<td>3 (7.5)</td>
<td>5 (5.8)</td>
<td>0.153</td>
<td>6 (15.0)</td>
<td>6 (7.0)</td>
<td>0.153</td>
</tr>
<tr>
<td>Disease control rate</td>
<td>27 (67.5)</td>
<td>21 (24.4)</td>
<td>&lt;0.001</td>
<td>28 (70.0)</td>
<td>21 (24.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Propensity score-matched cohort</strong></td>
<td>Lenvatinib (n=36)</td>
<td>Sorafenib (n=36)</td>
<td>P-value</td>
<td>Lenvatinib (n=36)</td>
<td>Sorafenib (n=36)</td>
<td>P-value</td>
</tr>
<tr>
<td>Best overall response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete response</td>
<td>0</td>
<td>0</td>
<td>0.127</td>
<td>0</td>
<td>0</td>
<td>0.127</td>
</tr>
<tr>
<td>Partial response</td>
<td>2 (5.6)</td>
<td>3 (8.3)</td>
<td>&lt;0.001</td>
<td>5 (13.9)</td>
<td>4 (11.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stable disease</td>
<td>22 (61.1)</td>
<td>5 (13.9)</td>
<td>0.004</td>
<td>20 (55.6)</td>
<td>4 (11.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>10 (27.8)</td>
<td>22 (61.1)</td>
<td>&lt;0.001</td>
<td>9 (25.0)</td>
<td>22 (61.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Could not be evaluated</td>
<td>2 (5.6)</td>
<td>6 (16.7)</td>
<td>0.134</td>
<td>2 (5.6)</td>
<td>6 (16.7)</td>
<td>0.134</td>
</tr>
<tr>
<td>Objective response rate</td>
<td>2 (5.6)</td>
<td>3 (8.3)</td>
<td>0.127</td>
<td>5 (13.9)</td>
<td>4 (11.1)</td>
<td>0.127</td>
</tr>
<tr>
<td>Disease control rate</td>
<td>24 (66.7)</td>
<td>8 (22.2)</td>
<td>&lt;0.001</td>
<td>25 (69.4)</td>
<td>8 (22.2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as number (%) or % as appropriate.

RECIST, Response Evaluation Criteria in Solid Tumors.
Clinical responses of second-line treatment

Responses to second-line treatment according to RECIST 1.1 and mRECIST criteria are presented in Table 2. None of the patients in either group achieved CR, whereas 3 (7.5%) patients in the lenvatinib group and 5 (5.8%) in the sorafenib group achieved PR. ORR was similar between the lenvatinib and sorafenib groups (7.5% vs. 5.8%, \(P=0.719\)). As SD was significantly higher in the lenvatinib group (60.0% vs. 18.6%, \(P<0.001\)), DCR was significantly higher in the lenvatinib than in the sorafenib group (67.5% vs. 24.4%; \(P<0.001\)). Treatment responses in the PS-matched cohort were similar to those in the unadjusted cohort, showing significantly higher DCR in the lenvatinib group compared to the sorafenib group (66.7% vs. 22.2%, \(P<0.001\), Table 2). Assessment of treatment responses according to mRECIST criteria yielded comparable results, with ORRs not differing significantly between the two treatment groups and DCR being significantly higher in the lenvatinib group (\(P<0.001\)).

Survival outcomes of second-line treatment

In the unadjusted cohort, 81 (64.3%) patients died after a median follow-up of 5.5 months (IQR, 3.5–9.3 months), and 107 (83.6%) patients experienced disease progression after a median follow-up of 2.1 months (IQR, 1.4–3.5 months). Evaluation of the unadjusted cohort showed significantly longer median OS in the lenvatinib group than in the sorafenib group (10.3 [IQR, 6.8–N/A] vs. 5.6 [IQR, 4.7–

![Figure 2. Kaplan–Meier analysis of survival outcomes in overall patients treated with lenvatinib or sorafenib. (A) Overall survival (OS); (B) Progression-free survival (PFS).](https://doi.org/10.3350/cmh.2023.0553)

Table 3. Survival outcomes of second-line treatment

<table>
<thead>
<tr>
<th>Survival outcomes</th>
<th>Lenvatinib</th>
<th>Sorafenib</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted cohort</td>
<td>n=40</td>
<td>n=86</td>
<td></td>
</tr>
<tr>
<td>Overall survival, months (95% CI)</td>
<td>10.3 (6.8–NA)</td>
<td>5.6 (4.7–9.0)</td>
<td>0.019</td>
</tr>
<tr>
<td>Progression-free survival, months (95% CI)</td>
<td>3.5 (3.0–4.2)</td>
<td>1.8 (1.6–2.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>PS-matched cohort</td>
<td>n=36</td>
<td>n=36</td>
<td></td>
</tr>
<tr>
<td>Overall survival, month (95% CI)</td>
<td>10.3 (6.1–NA)</td>
<td>7.5 (4.7–11.3)</td>
<td>0.353</td>
</tr>
<tr>
<td>Progression-free survival, months (95% CI)</td>
<td>3.5 (3.0–4.2)</td>
<td>1.8 (1.6–2.4)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Values are presented as median (interquartile range).

Objective response was assessed with RECIST 1.1.

CI, confidence interval; PS, propensity score; RECIST 1.1, Response Evaluation Criteria in Solid Tumors 1.1.
9.0] months, \( P = 0.019 \); Fig. 2A, Table 3). Median PFS was also significantly longer in the lenvatinib than in the sorafenib group (3.5 [IQR, 3.0–4.2] vs. 1.8 [IQR, 1.6–2.3] months, \( P = 0.001 \); Fig. 2B, Table 3). In the PS-matched cohort, 44 (61.1%) patients died after a median follow-up of 8.0 months (IQR, 5.7–10.9 months), and 61 (84.7%) patients experienced disease progression after a median follow-up of 2.8 months (IQR, 1.9–3.5 months).

Following PS matching, however, the median OS did not differ between the two groups (lenvatinib vs. sorafenib, 10.3 [IQR, 6.1–N/A] vs. 7.5 [IQR, 4.7–11.3] months, \( P = 0.353 \); Fig. 3A, Table 3). PFS showed a similar trend to the unadjusted cohort and was superior PFS in the lenvatinib group even after PS matching (lenvatinib vs. sorafenib, 3.5 [IQR, 3.0–4.2] vs. 1.8 [IQR, 1.6–2.4] months, \( P = 0.003 \); Fig. 3B, Table 3).

Predictors for survival outcomes

Table 4 depicts the predictors for survival outcomes. During the median follow-up of 5.5 (IQR, 3.47–9.33) months, 81 (64.3%) patients died. Univariable Cox regression analyses showed ECOG performance score 2, Child-Pugh class B, tumor size ≥10 cm, PIVKA-II concentration ≥1,000 mAU/mL, sorafenib treatment, and number of previous ATE+BEV treatment cycles (<3) as predictors for death. Subsequent multivariable analyses revealed that Child-Pugh class B (adjusted hazard ratio [aHR]=2.472; 95% confidence interval [CI], 1.433–4.266; \( P = 0.001 \)), and PIVKA-II concentration ≥1,000 mAU/mL (aHR=1.710; 95% CI, 1.015–2.883; \( P = 0.044 \)) were independently predictive of death.

Over a median follow-up of 2.07 (IQR, 1.37–3.47) months, 107 (83.6%) patients showed disease progression. Univariable Cox-regression analyses selected ECOG performance score 2, Child-Pugh class B, extrahepatic metastasis, sorafenib treatment, and number of previous ATE+BEV treatment cycles (<3) as significant predictors for disease progression. Subsequent multivariable analyses showed extrahepatic metastasis (aHR=1.991; 95% CI, 1.265–3.132; \( P = 0.002 \)) and sorafenib treatment (aHR=1.852; 95% CI, 1.142–3.003; \( P = 0.012 \)) to be independent predictors for disease progression.

Survival outcomes according to third-line treatment

Eighteen (40%) patients in the lenvatinib group and 44 (51.2%) patients in the sorafenib group received third-line treatment (\( P = 0.519 \)). The third-line treatment options included cabozantinib, ipilimumab plus nivolumab, nivolumab, regorafenib, and sorafenib (Supplementary Table 1). In the lenvatinib group, the median OS did not differ between

Figure 3. Kaplan–Meier analysis of survival outcomes in PS-matched patients treated with lenvatinib or sorafenib. (A) Overall survival (OS); (B) Progression-free survival (PFS).

Table 4. Predictors for survival outcomes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Death</th>
<th>Disease progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariable</td>
<td>Multivariable</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Age, years, ≥63</td>
<td>0.135</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td>ECOG PS, 2 (vs. 0/1)</td>
<td>0.001</td>
<td>0.697</td>
</tr>
<tr>
<td>Etiology, viral (vs. non-viral)</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td>BCLC stage, C (vs. B)</td>
<td>0.566</td>
<td></td>
</tr>
<tr>
<td>Child-Pugh Class, B (vs. A)</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Tumor numbers, ≥2</td>
<td>0.052</td>
<td></td>
</tr>
<tr>
<td>Maximal tumor size, ≥10 cm</td>
<td>0.039</td>
<td>0.275</td>
</tr>
<tr>
<td>Extrahepatic metastasis</td>
<td>0.259</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td>Macrovascular invasion</td>
<td>0.331</td>
<td></td>
</tr>
<tr>
<td>AFP, ng/mL, &gt;200</td>
<td>0.147</td>
<td></td>
</tr>
<tr>
<td>PIVKA-II, mAU/mL, &gt;1,000</td>
<td>0.018</td>
<td>0.044</td>
</tr>
<tr>
<td>Sorafenib (vs. lenvatinib)</td>
<td>0.021</td>
<td>0.565</td>
</tr>
<tr>
<td>Previous atezolizumab plus bevacizumab treatment cycles, n&lt;3</td>
<td>&lt;0.001</td>
<td>0.084</td>
</tr>
</tbody>
</table>

ECOG PS, European Cooperative Oncology Group performance status; BCLC, Barcelona clinic liver cancer; AFP, alpha-fetoprotein; PIVKA-II, prothrombin-induced by vitamin K absence or antagonist-II; CI, confidence interval; HR, hazard ratio.
patients who did and did not receive third-line treatment in both the unmatched ($P=0.870$, Supplementary Fig. 1A) and PS-matched ($P=0.940$, Supplementary Fig. 1B) cohorts. However, median OS was significantly affected by the type of third-line treatment in both the unmatched ($P<0.001$, Supplementary Fig. 2A) and PS-matched ($P=0.002$, Supplementary Fig. 2B) cohorts. Most of the patients (15/18, 83.3%) in the lenvatinib group received sorafenib as third-line treatment, with few or none receiving other third-line treatments.

Evaluation of the sorafenib group showed that OS was significantly longer among patients in the unmatched cohort who did than did not receive third-line treatment ($P=0.009$, Supplementary Fig. 1C), although this trend was not observed in the PS-matched cohort ($P=0.440$, Supplementary Fig. 1D). Median OS was not affected by the type of third-line treatment in both the unmatched ($P=0.920$, Supplementary Fig. 2C) and PS-matched ($P=0.330$, Supplementary Fig. 2D) cohorts.

Survival outcomes according to number of previous ATE+BEV treatment cycles

There was no difference in survival outcomes between the lenvatinib group and sorafenib group according to the number of previous ATE+BEV treatment cycles ($\leq3$ vs. $>3$) in both the unmatched and PS-matched cohorts (Supplementary Figs. 3, 4 and Supplementary Table 2).

Safety profiles

Treatment-related AEs are shown in Table 5. Treatment-related AEs of any grade occurred in 32 patients (80.0%) of the lenvatinib group and 75 patients (87.2%) of the sorafenib group ($P=0.282$). Grade 3 or 4 AEs occurred in 35.0% and 38.4% of patients in the lenvatinib and sorafenib groups, respectively ($P=0.145$). There were no deaths caused directly by grade 5 AEs. Most AEs were manageable, and the percentage of AEs leading to the discontinuation of treatment did not differ between the two groups (lenvatinib vs. sorafenib, 12 [30.0%] vs. 16 [18.6%] patients; $P=0.152$).

The most common AEs of any grade in the lenvatinib group were proteinuria (57.5%), AST elevation (50.0%), and thrombocytopenia (50.0%), and while those in the sorafenib group were total bilirubin elevation (54.7%), AST elevation (52.3%), and ALT elevation (25.6%). The most common grade 3 or 4 AEs in the lenvatinib group were proteinuria (30.0%), AST elevation (10.0%), and gastrointestinal bleeding (7.5%), and those in the sorafenib group were rash (4.7%), hand-foot syndrome (HFS, 4.7%), and AST elevation (2.3%).

HFS was significantly more frequent in the sorafenib group than in the lenvatinib group (19.8% vs. 5.0%, $P=0.031$). In contrast, rates of hypertension, thrombocytopenia, anemia, anorexia, proteinuria, and hypothyroidism (all $P<0.05$) were significantly higher in the lenvatinib group than in the sorafenib group.

DISCUSSION

One of the major strengths of this study was its comparison of treatment outcomes and survival in matched cohorts of patients with HCC who received second-line lenvatinib or sorafenib who experienced progression on ATE+BEV treatment. This is the first study to compare treatment outcomes and survival of second-line lenvatinib and sorafenib in matched HCC patient cohorts who experienced progression after ATE+BEV treatment. PFS was significantly superior in the lenvatinib group in both unadjusted and matched cohorts. However, OS seemed to be longer in the lenvatinib group in the original cohort but was not statistically different after PS matching between the 2 treatment groups.

The main survival outcomes of our study are in line with a previous study by Yoo et al. In that study, the median PFS was significantly higher in the lenvatinib group compared to the sorafenib group (6.1 vs. 2.5 months, $P=0.004$), whereas the median OS was not significantly different between the 2 groups (16.6 vs. 11.2 months, $P=0.347$). The results of our study reinforce the previous results by reproducing the same trend even after adjusting for baseline characteristics by PS matching. A recent global study by Persano et al. showed significantly better OS and PFS in the lenvatinib group compared to the sorafenib group (hazard ratio for OS, 0.45; 95% CI, 0.24–0.83; reference sorafenib group). The superior PFS may partially attributed to the inherent potential of lenvatinib which slows disease progression and prolongs PFS. When lenvatinib was used
### Table 5. Adverse events

<table>
<thead>
<tr>
<th>Types of adverse events</th>
<th>Total (n=126)</th>
<th>Lenvatinib (n=40)</th>
<th>Sorafenib (n=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Any grade</td>
<td>Grade 3 or 4</td>
<td>Any grade</td>
</tr>
<tr>
<td>All</td>
<td>107 (84.9)</td>
<td>47 (37.3)</td>
<td>32 (80.0)</td>
</tr>
<tr>
<td>AST elevation</td>
<td>65 (51.6)</td>
<td>6 (4.8)</td>
<td>20 (50.0)</td>
</tr>
<tr>
<td>Total bilirubin elevation</td>
<td>63 (50.0)</td>
<td>4 (3.2)</td>
<td>16 (40.0)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>39 (31.0)</td>
<td>1 (0.8)</td>
<td>20 (50.0)</td>
</tr>
<tr>
<td>ALT elevation</td>
<td>37 (29.4)</td>
<td>3 (2.4)</td>
<td>15 (37.5)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>36 (28.6)</td>
<td>1 (0.8)</td>
<td>16 (40.0)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>31 (24.6)</td>
<td>2 (1.6)</td>
<td>16 (40.0)</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>26 (20.6)</td>
<td>13 (10.3)</td>
<td>23 (57.5)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>22 (17.5)</td>
<td>2 (1.6)</td>
<td>6 (15.0)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>22 (17.5)</td>
<td>0</td>
<td>17 (42.5)</td>
</tr>
<tr>
<td>Nausea</td>
<td>21 (16.7)</td>
<td>1 (0.8)</td>
<td>10 (25.0)</td>
</tr>
<tr>
<td>Anemia</td>
<td>18 (14.3)</td>
<td>0</td>
<td>10 (25.0)</td>
</tr>
<tr>
<td>Rash</td>
<td>17 (13.5)</td>
<td>5 (4.0)</td>
<td>4 (10.0)</td>
</tr>
<tr>
<td>Hand-Foot Syndrome</td>
<td>17 (13.5)</td>
<td>4 (3.2)</td>
<td>2 (5.0)</td>
</tr>
<tr>
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<td>15 (11.9)</td>
<td>0</td>
<td>14 (35.0)</td>
</tr>
<tr>
<td>Pruritus</td>
<td>10 (7.9)</td>
<td>1 (0.8)</td>
<td>4 (10.0)</td>
</tr>
<tr>
<td>Oral Mucositis</td>
<td>10 (7.9)</td>
<td>0</td>
<td>6 (15.0)</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>9 (7.1)</td>
<td>0</td>
<td>4 (10.0)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>7 (5.6)</td>
<td>1 (0.8)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
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<td>5 (4.0)</td>
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<td>Constipation</td>
<td>4 (3.2)</td>
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<tr>
<td>Headache</td>
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<td>0</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>Chest pain</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>2 (1.6)</td>
<td>0</td>
<td>2 (5.0)</td>
</tr>
</tbody>
</table>

Values are presented as number (%).

AST, aspartate aminotransferase; ALT, alanine aminotransferase.
as a first-line systemic treatment in the REFLECT trial, it showed significantly better PFS than sorafenib. In our previous study comparing the efficacy between first-line ATE+BEV and lenvatinib, lenvatinib showed non-inferior PFS compared to ATE+BEV. In a Japanese single-arm study showing oncologic outcomes of lenvatinib after ATE+BEV failure, the median OS and PFS were comparable to those of Yoo et al. (OS, 15.7 months in Japanese study vs. 16.6 months in Yoo et al.; PFS, 4.4 vs. 6.1 months). Another Japanese study reported that the median OS and PFS of patients treated with lenvatinib after ATE+BEV failure were 13.5 months and 4.0 months, respectively, similar to previous findings. Median OS and PFS were slightly shorter, at 12.8 months and 3.7 months, respectively, in a US study that included mostly Caucasian patients. In our multivariate analyses of predictors for survival outcome, lenvatinib was a significant predictor for better PFS. These collective findings support the superiority of lenvatinib over sorafenib on PFS after ATE+BEV treatment failure. However, as several other clinical trials (regorafenib, cabozantinib) are investigating the efficacy of second-line TKIs with different targets in patients with HCC after ATE+BEV failure, a comparison of results between these drugs is anticipated.

The relatively low ORR of lenvatinib in second-line treatment may contribute to the comparable OS between the 2 groups. According to the REFLECT trial, which compared oncologic and survival outcomes between lenvatinib and sorafenib as a first-line treatment in patients with HCC, the ORR of lenvatinib was much higher than that of sorafenib when assessed with mRECIST (40% vs. 13%) and RECIST 1.1 (19% vs. 7%). However, the ORR of the second-line treatments in our study was 7.5% in the lenvatinib group and 5.8% in the sorafenib group. In PS-matched analysis, the ORR was reversed, and sorafenib showed a numerically better rate over lenvatinib (5.6% vs. 8.3%) but without statistical significance. Further studies are needed to determine whether the ORR of lenvatinib may be reduced by prior exposure to an anti-angiogenic agent such as BEV.

Another peculiar finding of our study is that survival outcomes of both drugs were inferior to those of Yoo et al. (lenvatinib OS, 16.6 months; sorafenib OS, 11.2 months) and those of Persano et al. (lenvatinib OS, 17.0 months; sorafenib OS, 14.2 months). These differences could have occurred because our study population included different baseline characteristics and larger numbers of Child-Pugh class B patients with poor liver function and ECOG 2 patients with poor performance. These findings are in agreement with results showing that PFS and OS were longer in patients with Child-Pugh Class A than Class B liver function who were treated with lenvatinib after ATE+BEV failure. Since study by Yoo et al. only included patients who received ATE+BEV in clinical trials, patients in that study are more selected ones compared to those from real-world study. The proportion of patients (89.5%, 136/152) who received second-line treatment after disease progression instead of conservative care after disease progression on ATE+BEV was higher in our study than in other studies. This implies that more patients with marginal liver function or poor general condition after first-line treatment underwent second-line treatment.

In this study, the absence of extrahepatic metastasis at the start of second-line treatment and receipt of lenvatinib treatment were significant predictors for better PFS, and low PIVKA-II level and good liver function were associated with superior OS. Aside from known predictors such as liver function or tumor extent, there is great interest in the choice of individual-based secondary drug selection for effects on clinical outcomes. There is an ongoing concern about whether to re-challenge with immune treatment or to use TKI after ATE+BEV failure. For patients who have achieved a durable response to ATE+BEV for a long time, some suggest that another immunotherapy-based regimen would be beneficial than switching to a TKI. In our subgroups analyses, neither third-line treatment or previous numbers of ATE+BEV cycle differentiate the OS of the patient. Since the analysis related to third-line treatment was conducted on a limited sample size, further verification in a larger cohort is necessary to confirm these findings. In addition, future studies should focus on finding biomarkers to determine whether to continue immunotherapy or to switch to targeted therapy.

This study had several limitations. First, the study design was retrospective, which may have introduced selection bias and confounders. We adopted various statistical methods to overcome these limitations by performing PS matching and multivariate regression analyses to adjust for different baseline characteristics. Second, we only included Asian patients from 2 centers, and it was difficult to conduct a multifaceted analysis; thus, the conclusion may not
be generalized to patients of other races and countries. Third, as ATE+BEV was introduced in Korea in May 2020, the follow-up duration for second-line treatment may be insufficient to draw firm conclusion. Despite these limitations, this is the first study to suggest the oncologic outcomes of patients who underwent second-line lenvatinib or sorafenib treatment after ATE+BEV treatment failure, with adjustments of baseline characteristics by PS matching.

In conclusion, lenvatinib showed favorable PFS with similar OS compared to sorafenib as a second-line therapy for unresectable HCC after ATE+BEV failure in a well-matched cohort and real-world setting. Future studies with a larger sample size and longer follow-up are needed to confirm this finding and to optimize second-line treatment in such patients.

Authors’ contribution
Conception: Young Eun Chon, Dong Yun Kim, Hong Jae Chon, and Do Young Kim; study design: Young Eun Chon, Dong Yun Kim, Hong Jae Chon, and Do Young Kim; participation in patient management and data collection: Young Eun Chon, Dong Yun Kim, Hong Jae Chon, and Do Young Kim; contribution to data acquisition: Young Eun Chon, Dong Yun Kim, Mi Na Kim, Beom Kyung Kim, Jun Yong Park, Yeonjung Ha, Joo Ho Lee, and Kwan Sik Lee, writing the paper: Young Eun Chon, Dong Yun Kim, Hong Jae Chon, and Do Young Kim; statistical analysis: Young Eun Chon, Dong Yun Kim, Hong Jae Chon, and Do Young Kim. All authors have reviewed the paper and approved the final version.

Conflicts of Interest
HJ Chon has received honoraria from Eisai, Roche, Bayer, ONO, MSD, BMS, Celgene, Sanofi, Servier, AstraZeneca, Sillajen, Menarini, GreenCross Cell, Boryung Pharmaceuticals, and Dong-A ST, and has received research grants from Roche, Dong-A ST, and Boryung Pharmaceuticals. The other authors have no potential conflicts of interest to disclose.

SUPPLEMENTARY MATERIAL
Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

CORRECTION
This article was corrected on July 17, 2024 due to a change in the author name.

REFERENCES


Multiomics profiling of buffy coat and plasma unveils etiology-specific signatures in hepatocellular carcinoma

Jiwon Hong¹,²*, Jung Woo Eun¹*, Geum Ok Baek¹, Jae Youn Cheong³, Seryoung Park¹, Soon Sun Kim³, Hyo Jung Cho³, and Su Bin Lim¹,²

¹Department of Biochemistry & Molecular Biology, Ajou University School of Medicine; ²Department of Biomedical Sciences, Graduate School of Ajou University; ³Department of Gastroenterology, Ajou University School of Medicine, Suwon, Korea

Study Highlights

- HBV-HCC and NV-HCC display distinct gene expression profiles compared to non-HCC at the buffy coat and plasma level.
- Validation by publicly available RNA-seq datasets and qRT-PCR confirms the reliability of the suggested etiology-specific HCC biomarkers.
- Single-cell RNA-seq analysis further confirms etiology-specific differences in DEG expression across distinct immune cell types found in blood.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer mortality worldwide.1 HCC etiologies are classified into viral infection and nonviral causes.2 Hepatitis B virus (HBV) is the main viral risk factor for HCC; nonviral causes include alcohol consumption and nonalcoholic fatty liver disease.2 Most HCC patients are diagnosed at an advanced stage, for which curative treatments are limited.3 Thus, proper HCC surveillance is important for early detection of HCC, which can lead to improved outcomes. Diagnostic methods such as biopsy and imaging techniques combined with serum alpha-fetoprotein (AFP) level assessment have been frequently used in clinical practice but have several limitations, including low sensitivity and false positivity.4,5 Additionally, HCC is characterized by vast intertumoral heterogeneity induced by diverse etiologies and intratumoral heterogeneity owing to the tumor micro-

Background/Aims: Hepatocellular carcinoma (HCC) is a leading cause of cancer mortality worldwide. Despite identification of several biomarkers for HCC diagnosis, challenges such as low sensitivity and intratumoral heterogeneity have impeded early detection, highlighting the need for etiology-specific blood biomarkers.

Methods: We generated whole-transcriptome sequencing (WTS) and targeted proteome data from buffy coat and plasma samples from HCC patients. By integrating etiological information on viral infection, we investigated the etiology-specific gene expression landscape at the blood level. Validation of differentially expressed genes (DEGs) was performed using publicly available RNA-seq datasets and qRT–PCR with AUC analyses.

Results: Differential expression analyses with multiomics data revealed distinct gene expression profiles between HBV-associated HCC and nonviral HCC, indicating the presence of etiology-specific blood biomarkers. The identified DEGs were validated across multiple independent datasets, underscoring their utility as biomarkers. Additionally, single-cell RNA-seq analysis of HCC confirmed differences in DEG expression across distinct immune cell types.

Conclusions: Our buffy coat WTS data and plasma proteome data may serve as reliable sources for identifying etiology-specific blood biomarkers of HCC and might contribute to discovery of therapeutic targets for HCC across different etiologies. (Clin Mol Hepatol 2024;30:360-374)

Keywords: Hepatocellular carcinoma; Blood buffy coat; Plasma; Transcriptomics; Proteomics
environment. These limitations have emphasized the need for blood-based biomarkers to improve early detection of HCC, with a cell-free DNA signature and the combination of protein induced by vitamin K absence II (PIVKA-II) and AFP as potential biomarkers.

Gradient centrifugation separates blood into erythrocytes, the buffy coat, and plasma. The buffy coat is enriched in platelets and white blood cells, including peripheral blood mononuclear cells (PBMCs). PBMCs and plasma have been widely used for identification of blood-based biomarkers via transcriptomic and proteomic profiling, including several blood-based HCC biomarkers. However, many limitations remain to be overcome, such as HCC heterogeneity caused by different etiologies. Moreover, several studies have focused on transcriptome profiling using buffy coats to uncover altered gene signatures under disease conditions, including breast cancer and COVID-19. Buffy coat analysis enables understanding whole-cell responses in peripheral blood against specific diseases, overcoming the limitation of exclusion of several cell types, such as granulocytes and circulating tumor cells, among PBMCs. However, buffy coat profiling of HCC blood-based biomarkers, particularly those related to HCC etiology, has not yet been conducted.

Hence, we aimed to identify etiology-specific blood biomarkers of HCC utilizing whole-transcriptome sequencing (WTS) and targeted proteome data from buffy coat and plasma samples obtained from HCC patients with diverse etiologies. We classified HCC patients into HBV-associated HCC (HBV-HCC) and nonviral-associated HCC (NV-HCC) groups according to etiology information. Compared to those of non-HCC samples, we investigated the etiology-specific gene expression landscape of HCC at the blood level via validation with publicly available RNA-seq datasets and qRT-PCR data from our buffy coat samples. We expect that our established multiomics dataset will serve as a reliable and reproducible source for identifying etiology-specific blood biomarkers of HCC and help in discovering potential therapeutic targets for HCC across different etiologies.

MATERIALS AND METHODS

Patient enrollment

Blood samples and data were obtained from the Biobank of Ajou University Hospital, Suwon, South Korea, between January 2017 and August 2023. All experiments in this study were conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of Ajou University Hospital, Suwon, South Korea (AJOUIRB-EXP-2020-007). The requirement for informed consent was waived.

Blood sample preparation

Peripheral blood was collected from each individual directly into EDTA-containing tubes. The blood was centrifuged at 2,000xg for 5 minutes at 4°C, and the plasma or buffy coat was retained and aliquoted. Total RNA was isolated from buffy coats (300 μL) using TRIzol-LS (Invitrogen, Waltham, MA, USA) and purified using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The RNA concentration and integrity were measured by an Agilent 2100 bioanalyzer (G2939A; Agilent Technologies, Santa Clara, CA, USA).

Whole transcriptome sequencing of buffy coat samples

The total RNA concentration was calculated by Quant-IT Ribogreen (Invitrogen, #R11490). The integrity of the total RNA was assessed with a TapeStation RNA ScreenTape (Agilent, #5067-5576). Only high-quality RNA preparations with RIN greater than 7.0, were used for library construction. A library was independently prepared with an Illumina TruSeq Stranded Total RNA Library Prep Globin Kit (Illumina, San Diego, CA, USA, #20020613). Next, rRNA was removed using a Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat Globin) (Illumina). The cleaved RNA fragments were converted to first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen, #18064014) and random primers, followed by second-strand cDNA synthesis using DNA Polymerase I, RNase H and dUTP. The products were then purified and enriched via PCR to create the final
cDNA library. The libraries were quantified using KAPA Library Quantification kits according to qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS, #KK4854) and qualified using TapeStation D1000 ScreenTape (Agilent, #5067-5582). Indexed libraries were then subjected to an Illumina NovaSeq (Illumina) paired-end (2×100 bp) sequencing performed by Macrogen Incorporated.

**Differential expression (DE) analyses of WTS data**

The raw data (FASTQ) were imported into AltAnalyze software (v2.1.4), which uses the embedded software Kalisto and Ensemble72 annotations. Raw count values were obtained and used for DE analysis via R DESeq2 package (v1.34.0). Genes with a total read count less than 10 were excluded from further analysis. Differentially expressed genes (DEGs) were identified by pairwise comparison through DESeq2. Volcano plots were drawn using R EnhancedVolcano package (v1.12.0). Gene Ontology (GO) analysis was performed using R compareCluster function in clusterProfiler package (v4.2.2). The top 10 terms from either up-DEGs or down-DEGs are displayed in a dot plot.

**DE analysis of validation datasets (VDs)**

Bulk RNA-seq datasets of HCC PBMCs were downloaded from NCBI BioProject under accession codes PRJNA739257, PRJNA909469, and PRJNA717231. The raw data (FASTQ) were imported into AltAnalyze. Raw count values were obtained and used for DE analysis via R DESeq2 package. Genes with a total read count less than 1 were excluded from further analysis. The significance of differences in expression of buffy coat DEGs was validated at p.adj<0.05. Count values were obtained by plotCounts function in DESeq2 and visualized as haploid plots by ggplot and geom_boxjitter functions in R ggplot2 (v3.4.2) and ggpol (v0.0.7) packages.

**Rank-rank hypergeometric overlap (RRHO)**

Log2-fold change values were obtained from DESeq2 results. All genes annotated in the buffy coat WTS and validation datasets were ranked by log fold change. These ranked lists were further processed to include only genes common to both the buffy coat and each VD. These input files were loaded into a simple web-based version of RRHO (https://systems.crump.ucla.edu/rankrank/rankrank-simple.php). The step size was set to 100 to generate a Benjamini-Yekutieli-corrected hypergeometric matrix and RRHO heatmaps.

**Targeted proteome screening of plasma samples**

Plasma protein levels were measured using the proximity extension immunoassay technology (Olink Proteomics, Uppsala, Sweden) and an Olink Target 96 Immuno-Oncology (v.3113) panel. To conduct DE analysis, Normalized Protein Expression files, log2 scale values, were imported into R OlinkAnalyze package (v3.4.1). Differentially expressed proteins (DEPs) were identified by pairwise comparison through olink_ttest function in OlinkAnalyze package. Volcano plots were drawn using R EnhancedVolcano package.

**Single-cell RNA-seq analysis of the validation dataset**

Targeted single-cell RNA-seq datasets of HCC from BD Rhapsody Immune Response Targeted Panel (human) were downloaded from NCBI Gene Expression Omnibus under accession codes GSE179795 and GSE195648. Gene expression matrices containing distribution-based error correction-adjusted molecule counts were imported into R Seurat package (v4.3.0.1). Single-cell QC was performed filtering for a total of 1,500 reads. Batch-balanced KNN correction was conducted using R bbknnR package (v1.0.2). Cells expressing CD19 or CD14 were excluded for elimination of contaminating B cells and monocytes. Clustering was repeated after filtering contaminating cells. Cell types were annotated using canonical markers reported in several recent publications. Violin plots were drawn using VlnPlot function in Seurat.

**Quantitative reverse transcription PCR (qRT-PCR)**

mRNA expression levels in buffy coat samples were measured using qRT–PCR. Buffy coat RNA was reverse
transcribed to cDNA using SuperScript™ IV VILO™ Master Mix (Invitrogen). qRT–PCR was performed using amfiSure qGreen Q-PCR Master Mix (GenDEPOT, Barker, TX, USA) with individual primer sets (Supplementary Table 1) and monitored in real time using CFX Connect Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Relative gene expression levels were calculated using the 2^−ΔΔCt method, and GAPDH was used as the internal control. All measurements were performed in triplicate.

Area under the ROC curve (AUC) analysis

Receiver operating characteristic (ROC) analysis was performed using GraphPad Prism software (v10.0; GraphPad Software, San Diego, CA, USA), and the area under the ROC curve (AUC) was calculated, providing a quantitative measure of the overall diagnostic effectiveness. This analysis was complemented by 95% confidence intervals (CIs) for AUCs, offering a statistical range within which the true area under the curve is expected to lie and thereby indicating the precision of our assessment.

RESULTS

Study design

In our study, blood samples, along with etiology information, were obtained from healthy controls, nonalcoholic steatohepatitis patients, liver cirrhosis patients and HCC patients (Fig. 1, Supplementary Table 2). After centrifugation, buffy coat and plasma samples were obtained for WTS and targeted proteome screening, respectively. In the transcriptome analysis, buffy coat samples were divided into HCC and non-HCC groups; in the proteome analysis, plasma samples were classified into HCC and healthy groups. For identification of etiology-specific biomarkers, the samples were subdivided into viral (HBV) or nonviral groups according to their HBV infection status. Using three comparison sets, DE analyses were performed to identify etiology-specific DEGs and DEPs in HCC at the transcriptome and proteome levels. The identified DEGs were validated by analyzing publicly available bulk RNA-seq datasets of HCC PBMCs and performing qRT–PCR of buffy coat samples with AUC analysis. Further validation was conducted at the single-cell level using publicly available targeted scRNA-seq datasets.

DE analyses of buffy coat WTS data

To identify etiology-specific HCC biomarkers in the buffy coat transcriptome, we performed DE analyses comparing HCC and non-HCC samples from both HBV and nonviral groups. We further performed DE analysis between HBV-HCC and NV-HCC patients (Fig. 2A). A total of 40 and 987 DEGs were identified in the HBV-HCC and NV-HCC samples, respectively, compared to the corresponding non-HCC samples (p.adj<0.05, log|FC|>0.5). To investigate the etiology-derived functional distinctions of the identified DEGs, we performed GO analysis of the significant DEGs in each comparison (p.adj<0.05) (Fig. 2B). In HBV-HCC, the significantly enriched GO terms are involved in immune responses, including regulation of the innate immune response and activation of immune cells. In contrast, the significantly enriched GO terms in NV-HCC are related to ATP metabolism and respiration. This tendency was corroborated by GO analysis between HBV-HCC and NV-HCC, with up-DEGs in HBV-HCC enriched in the immune response pathway and down-DEGs enriched in metabolism (Fig. 2B). The significant DEGs (p.adj<0.05, log|FC|>0.5) in the buffy coat transcriptome analysis are summarized in Figure 2C; these DEGs were either etiology specific or HCC specific, regardless of etiology, with overlapping expression patterns in the two comparisons.

Validation of buffy coat DEGs

To validate the reliability of our buffy coat transcriptome dataset, we utilized three publicly available RNA-seq datasets of HCC PBMCs. To our knowledge, buffy coat transcriptome data related to HCC have not yet been published. Hence, HCC PBMC datasets were used for validation of the DE results for buffy coats. This selection was motivated by the intrinsic proximity of PBMCs to the buffy coat, as PBMCs are conventionally derived from the buffy coat. In light of the limited number of publicly available HCC PBMC transcriptome datasets, with the majority lacking comprehensive etiology information on HCC development, we identified three independent bulk RNA-seq datasets of HCC PBMCs. Notably, all datasets included sam-
Figure 1. Schematics of the study design. Buffy coat and plasma samples extracted from healthy controls, nonalcoholic steatohepatitis (NASH) patients, liver cirrhosis (LC) patients and HCC patients were used for differential expression analyses. Buffy coats were used for whole-transcriptome sequencing (WTS); plasma was used for targeted proteome screening. All samples were assigned to HBV or nonviral groups according to etiology. Validation of DEGs was performed through publicly available bulk RNA-seq and scRNA-seq datasets and qRT-PCR with AUC analysis. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; DEG, differentially expressed gene.
Figure 2. DE analysis between HCC and non-HCC samples of HBV-HCC and NV-HCC using WTS data derived from buffy coats. (A) Volcano plots visualizing significant DEGs. (B) GO results showing enriched biological processes of significant up-DEGs and down-DEGs. (C) Venn diagrams summarizing significant up-DEGs and down-DEGs. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NV, nonviral; WTS, whole-transcriptome sequencing; DEG, differentially expressed gene; GO, Gene Ontology.
Figure 3. Validation through RRHO and qPCR-AUC analyses. (A) RRHO heatmaps visualizing the overlap intensity between buffy coat WTS- and each VD-derived list of genes ranked by DE. The log fold changes are plotted in a bar graph along the x- and y-axes. (B) A table displaying significant DEGs in HBV-HCC vs. HBV-non-HCC patients validated by each VD. (C) Dot plot visualizing the overlapping number of significant DEGs in the HBV-HCC group vs. the HBV-non-HCC group compared with the VD group. (D, E) qRT-PCR and AUC results displaying the diagnostic performance of the identified DEGs. (D) C1QA results in HBV-HCC and NV-HCC. (E) SH3PXD2B results in HBV-HCC. RRHO, rank-rank hypergeometric overlap; WTS, whole-transcriptome sequencing; VD, validation dataset; DE, differential expression; DEG, differentially expressed gene; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NV, nonviral.
ples from both HBV-associated HCC patients and healthy individuals (Supplementary Table 3). Through RRHO analysis, we assessed the overlap intensity between our buffy coat transcriptome dataset and each VD. For VD1 and VD2, a significant overlap was observed, whereas VD3 was shown to have a poor correlation with the buffy coat dataset (Fig. 3A).

We analyzed the DEGs in each VD and compared each DE result with the buffy-coat DE result. Among the forty significant DEGs in the buffy coat transcriptome analysis of HBV-HCC patients compared to non-HCC patients, 27 genes were significantly differentially expressed in at least one VD (Fig. 3B). In VD1-3, 20, 16, and 9 DEGs overlapped with buffy-coat DEGs (Fig. 3B, Supplementary Fig. 1). The overlapping DEGs in each dataset are summarized in Figure 3C with the total overlap frequency. Excluding HBA2, HBA1, and HBG2, which are considered to originate from erythrocyte contamination, a total of 24 genes overlapped with the buffy coat transcriptome, suggesting that our buffy coat dataset may be reliably used for HCC blood transcriptome analysis and that these genes have the potential to serve as blood biomarkers for HBV-HCC.

To investigate the diagnostic performance of the identified DEGs in discriminating HCC patients from non-HCC patients in clinical settings, we conducted qRT‒PCR and AUC analysis of C1QA and SH3PXD2B, which were significant DEGs in the buffy coat WTS data and all VDs (Fig. 3C–E). No significant difference in qRT‒PCR results for C1QA, which was upregulated in both HBV-HCC and NV-HCC in the buffy coat transcriptome, in HBV groups was detected, though it was significantly upregulated in NV-HCC, with an AUC of 0.68 (95% CI: 0.53–0.83, P=0.05) (Fig. 3D). SH3PXD2B, upregulated only in HBV-HCC in the buffy coat transcriptome, displayed significant upregulation in HBV-HCC based on qRT‒PCR data, with an AUC of 0.73 (95% CI: 0.63–0.84, P<0.001), representing its diagnostic potential as a blood biomarker for HBV-HCC (Fig. 3E). Further examination using early-stage HCC samples compared to at-risk patients (NASH, LC) showed good diagnostic performance for detection of early-stage HBV-HCC using SH3PXD2B, with an AUC of 0.74 (Supplementary Fig. 2A, B).

Next, plasma samples derived from HCC patients and healthy controls were subjected to targeted proteome screening for 92 immuno-oncology proteins to investigate etiology-specific HCC markers at the protein level. A total of 59 and 73 DEPs were identified in the HBV-HCC and NV-HCC patients, respectively, compared to the controls (Fig. 4A, B). Compared with those in NV-HCC, a total of 6 proteins were significantly downregulated in HBV-HCC (Fig. 4A). Most DEPs were upregulated in HCC samples compared to the control samples, regardless of HCC etiology, implying that immuno-oncology proteins are typically upregulated upon HCC development (Fig. 4A). In contrast to the results of the transcriptome analysis, most of the DEPs overlapped between HBV-HCC and NV-HCC patients (Fig. 4B). To examine the correlation between the transcriptome and proteome DE results, we confirmed the expression levels of significant DEPs at the transcriptome level using buffy-coat WTS data. Among the significant DEPs, six up-DEPs exhibited correlated expression patterns at the transcriptome and proteome levels (Fig. 4C). Among the six DEPs, Gal-9 (LGALS9) and HO-1 (HMOX1) were HBV-HCC specific, while only CD70 was NV-HCC specific. Gal-1 (LGALS1) was not etiology specific but rather HCC specific for both HBV-HCC and NV-HCC patients.

These genes proven to be upregulated in HCC using multiomics data were examined by qRT‒PCR and AUC analyses to further investigate their potential as biomarkers (Fig. 4D–F). Unexpectedly, HCC-specific Gal-1 was significantly downregulated in the HBV-HCC group, with an AUC of 0.68 (95% CI: 0.55–0.81, P=0.005), but was significantly upregulated in the NV-HCC group, with an AUC of 0.73 (95% CI: 0.58–0.88, P=0.01), implying that Gal-1 might serve as an NV-HCC-specific biomarker (Fig. 4D). HBV-HCC-specific Gal-9 was significantly upregulated in patients with HBV-HCC, though it showed poor diagnostic value, with an AUC of 0.55 (95% CI: 0.44–0.67, P=0.4) (Fig. 4E). Notably, NV-HCC-specific CD70 was significantly upregulated in NV-HCC patients, with an AUC of 0.83 (95% CI: 0.71–0.96, P<0.001), showing its superior potential as an NV-HCC-specific blood biomarker (Fig. 4F). In addition, CD70 displayed great diagnostic potential for detection of early-stage NV-HCC, with an AUC of 0.82 (Supplementary Fig. 2C–E).
Figure 4. Proteome screening for targeted immuno-oncology genes using plasma samples. (A) Volcano plots visualizing significant DEPs. (B) Venn diagrams summarizing significant up-DEPs and down-DEPs. The list of significant DEPs is provided. (C) A table summarizing significant DEPs with correlating significant DE patterns in buffy-coat WTS data. Haploid plots of the transcriptome expression levels of each DEP are dis-played together. (D–F) qRT-PCR and AUC results displaying the diagnostic performance of the identified DEPs at the mRNA level. (D) LGALS1 results in HBV-HCC and NV-HCC. (E) LGALS9 results in HBV-HCC. (F) CD70 results in NV-HCC. DEP, differentially expressed protein; WTS, whole-transcriptome sequencing; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NV, nonviral.
Figure 5. Single-cell analysis of the identified DEGs from multiomics profiling data. (A) Uniform Manifold Approximation and Projection (UMAP) visualizing cell clusters after correction. (B) Violin plots showing relative expression of cell type markers. (C) Stacked bar plot visualizing the composition of cell type proportions. (D) A table summarizing significant DEGs from the buffy coat analysis that had significant DE patterns across individual cell types, as displayed with violin plots showing the relative expression level of each gene in the specified cell types. (E) A table summarizing significant DEGs identified from the buffy coat and plasma multiomics analyses that exhibited significant DE patterns across individual cell types, as displayed with violin plots showing the relative expression level of each gene in the specified cell types. DEG, differentially expressed gene.
Single-cell analysis of the identified DEGs

To assess differences in DEG expression across distinct cell populations in blood, we processed and analyzed publicly available targeted single-cell RNA-seq datasets of innate lymphoid cells (ILCs) derived from patients with HBV-HCC and NV-HCC (see Methods). After batch-balanced KNN correction, seven clusters were identified, among which two were classified as T cells and four as ILCs (Fig. 5A, B). The control and HBV-HCC patient cellular compositions exhibited considerable similarity, whereas NV-HCC patients displayed a distinct cellular profile characterized by an increased proportion of T cells and a reduced presence of ILC3 (Fig. 5C).

We first examined expression levels of DEGs in the buffy coat transcriptome in each cell type and identified a total of 8 genes with expression patterns that correlated with those of the buffy coat transcriptome (Fig. 5D, Supplementary Fig. 3A). IFITM3, one of the up-DEGs in HBV-HCC, was significantly upregulated in CD8 T cells, NK cells, and ILCs compared to healthy controls. For NV-HCC, a total of 5 genes (ANXA5, CD63, ITGB2, JUN, and LGALS1) were significantly upregulated in different cell types but TCF7 was significantly downregulated in ILCs compared to control cells. Compared with that in NV-HCC, JUN was significantly downregulated in ILCs in HBV-HCC. Additionally, energy metabolic processes such as ATP metabolism are altered in HCC and might be exploited as therapeutic targets. However, most studies do not differentiate HCC by etiology. In our study, enriched metabolic processes were identified as characteristics of NV-HCC, whereas HBV-HCC was characterized by activated immune responses. It has been reported that the microenvironment of HBV-HCC is more immunosuppressive and exhausted than that of NV-HCC. This might be linked to the different response rates to immune checkpoint inhibitors between HBV-HCC patients and NV-HCC patients. In general, patients with HBV-HCC have a better response rate. These results suggest the need for differentiated strategies for HCC treatment and biomarker identification depending on etiology, particularly in cancer immunotherapy.

Among 24 DEGs in HBV-HCC identified through buffy coat WTS analyses combined with VDs, SH3PXD2B upregulation was repeatedly confirmed by all VDs and qRT–PCR with a high AUC, indicating its great potential as an effective blood biomarker for diagnosis of HBV-HCC. In addition, NV-HCC-specific CD70, as revealed by our multiomics profiling, has not yet been studied regarding diagnostic values for HCC. CD70 upregulation in NV-HCC was confirmed by qRT–PCR, with a high AUC, suggesting that CD70 might serve as a novel NV-HCC-specific blood biomarker. Interestingly, CD70 has also been reported as an emerging target in cancer immunotherapy.

Notably, SH3PXD2B and CD70 displayed great diagnostic performance for detection of early-stage HCC compared to at-risk patients with NASH or cirrhosis, suggesting that profiling expression of these genes in the buffy coat can...
serve as an effective strategy for HCC surveillance. This approach may help to effectively distinguish HCC from chronic liver diseases that often share common signatures, such as cirrhosis, thereby enhancing early detection of HCC and consequently reducing HCC mortality.\(^{27}\) Integrating these results, we suggest that SH3PXD2B and CD70 are potent blood-based biomarkers for HCC diagnosis and potential therapeutic targets across different etiologies.

Finally, we validated cell type-specific differences in DEG expression using publicly available targeted single-cell RNA-seq datasets. Although these datasets derived from ILCs isolated from the PBMCs of HCC patients, we identified a total of 7 cell clusters, including T cells, NK cells, and ILCs. The limited number of target genes and cell types included in the datasets precluded validation of SH3PXD2B and CD70, which are proposed as effective biomarkers with diagnostic potential. Unlike SH3PXD2B, CD70 was included in the targeted gene panel, but its expression in the identified cell types was not detected (Supplementary Fig. 4A). Further investigation through the ABC portal\(^{35}\) using healthy PBMCs suggested that CD70 was expressed in B cells and dendritic cells, though SH3PXD2B was not obviously detected (Supplementary Fig. 4B). Despite these limitations, single-cell validation enabled us to investigate expression of multiple DEGs at the cell type level. Notably, IFITM3 was shown to be upregulated in all cell types except for CD4 T cells, emphasizing its reliability as a biomarker. Although the limited availability of single-cell RNA-seq datasets restricts validation of several genes at the single-cell level, their validation using bulk RNA-seq datasets and multimomics profiling emphasizes the effective utility of buffy coat mRNA signatures for HCC diagnosis.

Overall, we discerned etiology-specific blood biomarkers for HCC by employing multimomics profiling, incorporating buffy coat WTS and targeted plasma proteome data. This identification was corroborated through validation using publicly available bulk RNA-seq and single-cell RNA-seq datasets and conducting qRT–PCR with AUC analysis. Our investigation confirmed distinctive gene expression landscapes for HCC across different etiologies at the blood level, suggesting that several genes, particularly SH3PXD2B and CD70, are novel etiology-specific blood biomarkers. Our comprehensive multimomics profiling and validation across diverse datasets may serve as a resource for identifying etiology-specific blood biomarkers for HCC, with the potential to be effectively applied in clinical settings, and for facilitating exploration of etiology-specific therapeutic targets.

**Authors’ contribution**

Conceptualization: JH, JWE, HJC, SBL. Data Curation: JH. Formal Analysis: JH, JWE. Funding Acquisition: HJC, SBL. Investigation: JH, JWE, SP, HJC, SBL. Project Administration: HJC, SBL. Resources: JWE, GOB, JYC, SSK, HJC, SBL. Supervision: HJC, SBL. Validation: JWE, GOB. Visualization: JH. Writing – original draft: JH, JWE, GOB, SP, HJC, SBL. Writing – review & editing: JH, JWE, GOB, SP, HJC, SBL. All authors have read and approved the final manuscript.

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**Conflicts of Interest**

The authors have no conflicts to disclose.
SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES

25. Korean Liver Cancer Association (KLCA) and National Cancer


Phase 1 trial of the safety, pharmacokinetics, and antiviral activity of EDP-514 in untreated viremic chronic hepatitis B patients

Man-Fung Yuen¹, Wan-Long Chuang², Cheng-Yuan Peng³, Wen-Juei Jeng⁴,⁵, Wei-Wen Su⁶, Ting-Tsung Chang⁷,⁸, Chi-Yi Chen⁹, Yao-Chun Hsu¹⁰, Guy De La Rosa¹¹,¹², Alaa Ahmad¹³, Ed Luo¹⁵, and Annie L. Conery¹³

¹Department of Medicine, School of Clinical Medicine, Queen Mary Hospital, and State Key Laboratory of Liver Research, The University of Hong Kong, Hong Kong; ²Hepatobiliary Division, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan; ³Center for Digestive Medicine, China Medical University Hospital, Taichung, Taiwan; ⁴Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital, Linkou Branch, Taiwan; ⁵College of Medicine, Chang Gung University, Taoyuan, Taiwan; ⁶Department of Gastroenterology and Hepatology, Changhua Christian Hospital, Changhua, Taiwan; ⁷Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan; ⁸Infectious Disease and Signaling Research Center, National Cheng Kung University, Tainan, Taiwan; ⁹Department of Internal Medicine, Ditmanson Medical Foundation Chi-Yi Christian Hospital, Chiayi, Taiwan; ¹⁰Center for Liver Diseases and School of Medicine, E-Da Hospital/I-Shou University, Kaohsiung, Taiwan; ¹¹Formerly Enanta Pharmaceuticals, Inc., Watertown, MA, USA; ¹²Currently at Curevo Vaccine, Bothell, Washington, USA; ¹³Enanta Pharmaceuticals, Inc., Watertown, MA, USA

Graphical Abstract

Study Highlights
- CHB is a major cause of morbidity and mortality worldwide. Oral EDP-514 is a potent core protein inhibitor of HBV. This study tested the safety, tolerability, PK and antiviral activity of EDP-514. EDP-514 was well tolerated with a favorable safety and PK profile. EDP-514 reduced HBV DNA and HBV RNA in untreated viremic CHB patients.
INTRODUCTION

Infection with hepatitis B virus (HBV) is a common cause of hepatic cirrhosis and the most common cause of hepatocellular carcinoma.1 Worldwide, almost 300 million people are estimated to be chronically infected with HBV, and 1.5 million new infections/year are reported resulting in >800,000 deaths annually.2 HBV infection can be prevented with widely available HBV vaccines,3,4 however, the majority of people are unvaccinated, especially in lower- and middle-income countries. Standard treatment for HBV infection includes pegylated interferon (pegIFN) and nucleos(t)ide analogues (NUCs). These effectively suppress the infection through immunomodulation and inhibition of viral replication, respectively. NUCs are associated with an excellent safety/tolerability profile and potent antiviral activity for suppressing hepatitis B DNA levels but is needed to be taken long-term, whereas, pegIFN provides a satisfactory response in a very limited number of patients.3,4 Hence, a need exists for treatments providing a sustained clinical response and functional cure, which is defined as a sustained loss of hepatitis B surface antigen (HBsAg), with or without acquisition of hepatitis B surface

Background/Aims: Oral EDP-514 is a potent core protein inhibitor of hepatitis B virus (HBV) replication, which produced a >4-log viral load reduction in HBV-infected chimeric mice with human liver cells. This study evaluated the safety, pharmacokinetics, and antiviral activity of three doses of EDP-514 in treatment-naive viremic patients with HBeAg-positive or -negative chronic HBV infection.

Methods: Patients with HBsAg detectable at screening and at least 6 months previously were eligible. HBeAg-positive and -negative patients had a serum/plasma HBV DNA level ≥20,000 and ≥2,000 IU/mL, respectively. Twenty-five patients were randomized to EDP-514 200 (n=6), 400 (n=6) or 800 mg (n=7) or placebo (n=6) once daily for 28 days.

Results: A dose-related increase in EDP-514 exposure (AUClast and Cmax) was observed across doses. At Day 28, mean reductions in HBV DNA were −2.9, −3.3, −3.5 and −0.2 log10 IU/mL with EDP-514 200 mg, 400 mg, 800 mg, and placebo groups, respectively. The corresponding mean change from baseline for HBV RNA levels was −2.9, −2.4, −2.0, and −0.02 log10 IU/mL. No virologic failures were observed. No clinically meaningful changes from baseline were observed for HBsAg, HBeAg or HBcrAg. Nine patients reported treatment emergent adverse events of mild or moderate severity with no discontinuations, serious AEs or deaths.

Conclusions: In treatment-naïve viremic patients, oral EDP-514 was generally safe and well-tolerated, displayed PK profile supportive of once-daily dosing, and markedly reduced HBV DNA and HBV RNA. (Clin Mol Hepatol 2024;30:375-387)

Keywords: Chronic hepatitis B; Hepatitis B virus; Core protein inhibitor; Pharmacokinetics; Safety

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Abbreviations:
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CHB, chronic hepatitis B; CV, coefficient of variation; cccDNA, covalently closed circular DNA; CpAMs, core protein allosteric modulators; EC50, concentration that is 50% effective; HAV, hepatitis A virus; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBs, hepatitis B surface antibody; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDV, hepatitis D virus; HEV, hepatitis E virus; NUC, nucleos(t)ide analogue; PT, prothrombin time; PTT, partial thromboplastin time; pegIFN, pegylated interferon; TEAE, treatment-emergent adverse event; ULN, upper limit of normal
antibody (anti-HBs), and undetectable HBV DNA 6 months after completing treatment. Current therapies only achieve functional cure in a limited number of patients. Treatment with pegIFN achieves functional cure in approximately 11% of patients after 3 years, and treatment with NUC produces a functional cure in ≤10% of patients after 5 years of treatment. As a result, patients with HBV infection frequently require lifelong maintenance therapy, which imparts a substantial economic burden and may be associated with a risk of breakthrough drug resistance and drug toxicity. Further, the risk of hepatocellular carcinoma is reduced but not eliminated with these currently available treatments.

Novel drugs, which offer the potential for a functional cure, are in early clinical development for HBV infection including viral entry inhibitors, drugs for epigenetic control of cccDNA, immune modulators, RNA interference agents, ribonuclease H inhibitors, and core protein inhibitors. Two types of core protein inhibitors are recognized, both of which accelerate the kinetics of core protein interactions. Treatment with Type I inhibitors in vitro leads to the intracellular core protein aggregation and degradation, while in vitro treatment with Type II inhibitors results in production of empty capsids that lack the RNA polymerase complex required for HBV replication. Interestingly, in addition to suppressing replication by preventing pgRNA encapsidation, inhibitors of both these classes have been described to prevent de novo formation of cccDNA by preventing delivery of intact capsids to the nucleus. Treatment with core protein inhibitors in patients with HBV infection demonstrated a 2 to 3 log10 IU/mL reduction in the mean HBV DNA concentrations in viremic chronic hepatitis B (CHB) patients.

EDP-514 is a novel HBV core protein inhibitor that is in clinical development to treat chronic HBV infection. EDP-514 is a Type II core protein inhibitor that stimulates core assembly and prevents encapsulation of viral pregenomic RNA to block HBV replication resulting in the production of empty capsids (Fig. 1). EDP-514 potently inhibited encapsidation of viral RNA and production of viral DNA in stable cell lines expressing HBV and prevented HBV cccDNA establishment in cell lines or primary human hepatocytes when present at the time of viral infection. EDP-514 was equally active across HBV genotypes (A to H) and NUC-resistant variants with no cytotoxicity and exhibited a promising safety and pharmacological profile in nonclinical studies. In a first-in-human study of the safety and pharmacokinetics (PK) of oral EDP-514 in healthy volunteers and NUC-suppressed patients with CHB, EDP-514 was well-tolerated, exhibited a PK profile supportive of once daily dosing, and reduced antiviral activity in NUC-suppressed CHB patients.

This study evaluated the safety, PK, and antiviral activity of three doses of oral EDP-514 in treatment-naive, viremic patients with either hepatitis B e antigen (HBeAg)-positive or -negative chronic HBV infection. The inclusion of patients with CHB who are viremic and not currently on treatment provides an opportunity to evaluate initial safety and efficacy for EDP-514 in CHB patients. In addition, the present study would provide supportive data allowing for adequate dose selection and choice of appropriate endpoints for future studies in patients with CHB including those receiving NUC therapy.

MATERIALS AND METHODS

The present study was conducted in compliance with the International Conference on Harmonisation-Good Clinical Practices guidelines, the Declaration of Helsinki, and national regulations for clinical trials. The study protocol and informed consent were reviewed and approved by Institutional Review Boards of participating institutions. Written informed consents were obtained from all participants prior to any study procedures. This study was registered at clinicaltrials.gov: NCT04470388.

Study design

This was a randomized, double-blind, placebo-controlled, phase 1b study. The study consisted of three cohorts of viremic patients with CHB not currently on treatment. Each cohort enrolled patients who were randomized to EDP-514 200 mg, 400 mg or 800 mg or placebo once daily for 28 days. A safety follow-up was conducted 2 and 8 weeks after the last dose of study drug. Following dosing for the initial 200 mg cohort with EDP-514, subsequent cohorts were dosed following review of available blinded safety and PK data from the previous cohort.
Patient selection

Men or women aged 18 to 70 years with a body mass index (BMI) of 18 to 35 kg/m² were eligible if they had HBsAg detectable in serum/plasma at screening and in the most recent HBsAg serum/plasma testing within the past 6 months. At screening, all patients who were HBeAg-positive had a screening serum/plasma HBV DNA level ≥20,000 IU/mL or for those who were HBeAg-negative, a screening serum/plasma HBV DNA level ≥2,000 IU/mL, and no HBV DNA serum/plasma test values <1,000 IU/mL over the previous 12 months. Patients were not taking prescribed anti-HBV treatment, specifically pegIFN and/or NUC therapy for at least 12 months prior to screening.

Patients were excluded if there was a prior diagnosis of cirrhosis or history of hepatic decompensation (ascites, encephalopathy or variceal hemorrhage) or documented extensive bridging fibrosis or cirrhosis defined by any one of the following: a) Metavir ≥3 or Ishak fibrosis score ≥4 by a prior liver biopsy; b) FibroSure at screening with a score of ≥0.48 and aspartate aminotransferase (AST) to platelet ratio index ≥0.45; or c) FibroScan with a result ≥9 kPa at screening or within 6 months of screening. Patients also were excluded if they had coinfection with human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis A virus (HAV) or hepatitis E virus (HEV); prior history of hepatocellular carcinoma, evidence of hepatocellular carcinoma, by imaging in the past 3 months or screening alpha-fetoprotein ≥50 ng/mL without imaging. Patients also were excluded if there were usage of any prescription medication or St. John’s Wort or receipt of any vaccine or investigational drugs within 28 days or 5 half-lives prior to the first dose of study drug; or use of non-prescription drugs, dietary or herbal supplements, hormone replacement therapy or cytochrome P450 3A4 or p-glycoprotein inducers or inhibitors within 14 days of study drug.

Full eligibility criteria are provided in Supplementary Material A.
Study assessments

Safety was assessed by physical examination, vital signs (heart rate, blood pressure, respiratory rate, body temperature), clinical laboratory testing (chemistry, hematology, urinalysis), 12-lead electrocardiogram (ECG), and reports of adverse events (AEs). Partial thromboplastin time (PTT), prothrombin time (PT), and international normalized ratio (INR) were measured at each study visit. Patients were assessed at screening for the presence of HAV, HDV, HCV, HEV, and HIV. HBV DNA, HBsAg, hepatitis B core-related antigen (HBcrAg), HBeAg, and HBV RNA were assessed at each study visit. The lower limits of quantification for HBV DNA, HBsAg, HBcrAg, HBeAg, and HBV RNA were 1.3 log10 IU/mL, 0.05 IU/mL, 2.75 log10 U/mL, 0.59 PEI-U/mL, and 1.65 log10 U/mL, respectively. Serious AEs, grade 3 or 4 AEs considered at least possibly related to the study drug, all clinically significant grade 3 or 4 laboratory abnormalities, alanine aminotransferase (ALT) ≥2 × baseline with signs of hepatic decompensation and/or laboratory changes suggestive of worsening hepatic function, ALT elevations >3 × upper limit of normal (ULN) and ≥2 × baseline, and ALT elevations >10 × ULN were monitored and managed according to protocol-specific guidelines (Supplementary Material B). AEs and laboratory abnormalities were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events Version 5.0.

Blood samples were collected for PK analysis on Day 1 and Day 28 (or EOT) predose and at 0.5, 1, 2, 3, 4, 5, 6, and 8 hours postdose, and on Days 3, 7, 14, and 21 predose and at 1 to 3 hours postdose and at least 1 hour later but before administration of the next dose.

Statistical analysis

No formal sample size calculations were performed. A total of 24 viremic CHB patients not currently on treatment were planned to be enrolled, which was considered sufficient to characterize the efficacy, safety, tolerability, and PK for each EDP-514 dose level.

For safety data, no formal statistical analyses were performed. Plasma PK parameters for each dose level were calculated from the concentrations of EDP-514 and its major metabolites measured in predose and postdose plasma samples. For each EDP-514 dose level, descriptive statistics (sample size, arithmetic means, geometric means, standard deviation, % coefficient of variation [CV], % geometric CV, minimum, median, and maximum) were reported. Dose proportionality was assessed using a linear regression. PK parameters included Cmax, Ctrough, and AUC0-last for plasma EDP-514 and its major metabolites.

For each EDP-514 dose cohort, HBV-specific biomarkers were evaluated at baseline, on treatment through Day 28, and at 2 and 8 weeks posttreatment. The primary antiviral endpoint was HBV DNA levels through Day 28. HBV DNA levels were summarized by treatment using descriptive statistics. The percentage of patients with virologic failure/viral breakthrough defined as a confirmed increase in serum/plasma HBV DNA level ≥1.0 log10 IU/mL from nadir while receiving EDP-514 would be reported. For all patients with virologic failure, resistance testing was attempted if HBV DNA levels were adequate. Other antiviral parameters assessed over time included HBsAg, HBeAg, HBcrAg, and HBV RNA levels (log10 U/mL). Additionally, HBsAg (reflex anti-HBs) and HBeAg (reflex anti-HBe in patients who were HBeAg-positive at baseline) were assessed serologically at the end of treatment.

The safety population included all patients who received at least one dose of study drug, the PK population consisted of all patients who received active study drug and had any measurable plasma concentration of study drug, and the antiviral population was all patients who received at least one dose of study drug and had any on-treatment HBV DNA data.

RESULTS

Twenty-five patients were randomized to treatment and completed the study. Patients were mostly male (60.0%) and all were Asian. The mean age was 46.3 years (range: 24 to 60 years), and the mean BMI was 25.8 kg/m2 (range: 18.8 to 34.7 kg/m2) (Table 1). Baseline HBV DNA and HBV RNA levels were similar in all EDP-514 treatment groups and placebo. All patients had detectable HBsAg and the baseline levels were variable in all groups. Two patients in the 800 mg group, one patient each in the EDP-514 400 mg and placebo groups, and no patient in the 200 mg group were HBeAg-positive.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EDP-514 200 mg (n=6)†</th>
<th>EDP-514 400 mg (n=6)†</th>
<th>EDP-514 800 mg (n=7)†</th>
<th>Placebo (n=6)†</th>
<th>Total (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)†</td>
<td>48.7±4.8</td>
<td>42.8±6.6</td>
<td>47.9±8.0</td>
<td>45.5±12.0</td>
<td>46.3±8.0</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>4 (66.7)</td>
<td>4 (66.7)</td>
<td>6 (85.7)</td>
<td>1 (16.7)</td>
<td>15 (60.0)</td>
</tr>
<tr>
<td>Weight (kg)†</td>
<td>70.9±12.2</td>
<td>73.2±7.7</td>
<td>82.4±15.7</td>
<td>60.3±13.7</td>
<td>72.1±14.5</td>
</tr>
<tr>
<td>Body mass index (kg/m²)†</td>
<td>24.8±3.8</td>
<td>26.4±5.0</td>
<td>28.9±4.0</td>
<td>22.9±3.7</td>
<td>25.8±4.5</td>
</tr>
<tr>
<td>Asian, n (%)</td>
<td>6 (100)</td>
<td>6 (100)</td>
<td>7 (100)</td>
<td>6 (100)</td>
<td>25 (100)</td>
</tr>
<tr>
<td>HBV Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>6 (100)</td>
<td>5 (83.3)</td>
<td>5 (71.4)</td>
<td>5 (83.3)</td>
<td>21 (84)</td>
</tr>
<tr>
<td>C</td>
<td>0 (0)</td>
<td>1 (16.7)</td>
<td>2 (28.6)</td>
<td>1 (16.7)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>HBV DNA levels (log_{10} IU/mL)†</td>
<td>4.57±0.75</td>
<td>5.37±1.59</td>
<td>5.54±1.70</td>
<td>4.91±1.38</td>
<td>5.12±1.38</td>
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<tr>
<td>HBV RNA &gt;LLOQ, n (%)</td>
<td>6 (100)</td>
<td>5 (83.3)</td>
<td>6 (85.7)</td>
<td>5 (83.3)</td>
<td>22 (88)</td>
</tr>
<tr>
<td>HBV RNA levels (log_{10} U/mL)†</td>
<td>3.22±0.95</td>
<td>3.58±2.30</td>
<td>3.77±2.17</td>
<td>3.34±1.97</td>
<td>3.49±1.82</td>
</tr>
<tr>
<td>HBsAg levels (IU/mL)†</td>
<td>1,384.25±1,316.87</td>
<td>23,874.62±46,178.28</td>
<td>6,972.28±14,771.62</td>
<td>2,601.11±4,827.76</td>
<td>8,638.63±24,189.61</td>
</tr>
<tr>
<td>HBeAg positive, n (%)</td>
<td>0 (0)</td>
<td>1 (16.7)</td>
<td>2 (28.6)</td>
<td>1 (16.7)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>HBeAg levels (PEI-U/mL)†</td>
<td>0.30±0.00</td>
<td>398.39±975.14</td>
<td>45.24±118.02</td>
<td>11.21±26.73</td>
<td>111.04±478.74</td>
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<td>HBcrAg &gt;LLOQ, n (%)</td>
<td>6 (100)</td>
<td>6 (100)</td>
<td>5 (71.4)</td>
<td>4 (66.7)</td>
<td>21 (84)</td>
</tr>
<tr>
<td>HBcrAg levels (log_{10} U/mL)†</td>
<td>3.6±0.7</td>
<td>4.9±1.9</td>
<td>4.1±2.6</td>
<td>3.5±2.4</td>
<td>4.0±2.0</td>
</tr>
</tbody>
</table>

HBV, hepatitis B virus; LLOQ, lower limit of quantification; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBcrAg, hepatitis B core-related antigen.

*Mean±standard deviation; †The n applies to all values show in the table including HBV DNA, HBV RNA, HBsAg, HBeAg and HBcrAg levels, but excluding instances where a specific n is shown (i.e., to demonstrate the number of patients with values below LLOQ).
Pharmacokinetics

Following once daily oral administration, mean EDP-514 plasma concentrations increased with dose from 200 mg to 800 mg at most time points on Day 1 and Day 28, respectively (Fig. 2). EDP-514 was absorbed within approximately 2.0 to 4.0 hours post dose, with median $T_{\text{max}}$ between approximately 2.9 to 4.0 hours across all doses on Day 1 and between 2.0 to 3.4 hours across all doses on Day 28. A dose-related increase in EDP-514 exposure ($\text{AUC}_{\text{last}}$ and $C_{\text{max}}$) was observed on both Day 1 and Day 28 (Table 2). Exposures on Day 28 were higher than on Day 1 indicating some evidence for accumulation with multiple daily dosing. The PK profile was supportive of once daily dosing, with

![Figure 2. Mean plasma EDP-514 concentration-time curves on Day 1 and Day 28.](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EDP-514 200 mg (n=6)</th>
<th>EDP-514 400 mg (n=6)</th>
<th>EDP-514 800 mg (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{AUC}_{\text{last}}$, ng/mL$^\cdot$h</td>
<td>11,815 (11)</td>
<td>21,325 (23)</td>
<td>30,746 (17)</td>
</tr>
<tr>
<td>$C_{\text{max}}$, ng/mL</td>
<td>2,387 (19)</td>
<td>4,246 (21)</td>
<td>6,806 (40)</td>
</tr>
<tr>
<td>$T_{\text{max}}$, h</td>
<td>3.8 (43.2)</td>
<td>3.8 (47.3)</td>
<td>2.7 (31.8)</td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{AUC}_{\text{last}}$, ng/mL$^\cdot$h</td>
<td>16,617 (26)</td>
<td>24,600 (27)</td>
<td>41,669 (22)</td>
</tr>
<tr>
<td>$C_{\text{max}}$, ng/mL</td>
<td>3,115 (31)</td>
<td>4,660 (17)</td>
<td>7,947 (14)</td>
</tr>
<tr>
<td>$C_{\text{trough}}$, ng/mL</td>
<td>482.7 (106.9)</td>
<td>1,250.6 (54.8)</td>
<td>1,900.3 (47.1)</td>
</tr>
<tr>
<td>$T_{\text{max}}$, h</td>
<td>2.7 (62.4)</td>
<td>3.3 (32.4)</td>
<td>2.3 (52.0)</td>
</tr>
</tbody>
</table>

All values presented as geometric mean (% geometric coefficient of variation).

PK, pharmacokinetics.
median $C_{\text{trough}}$ at Day 28 ~9-fold for 200 mg, ~20-fold for 400 mg, and ~24-fold for 800 mg doses above the protein-adjusted EC$_{50}$ (71 ng/mL) (Fig. 3).

Antiviral activity

EDP-514 showed both dose- and time-dependent decreases in HBV DNA levels when administered to viremic CHB patients not currently on treatment (Fig. 4 and Supplementary Fig. 1). Mean HBV DNA levels decreased with increasing EDP-514 dose at all measurements between Day 3 and Day 28, with the greatest decrease in the EDP-514 800 mg at all time points. At Day 28, mean reductions in HBV DNA levels were $-2.9$, $-3.3$, $-3.5$, and $-0.2$ log$_{10}$ IU/mL in the 200 mg, 400 mg, 800 mg, and placebo groups, respectively. Once treatment was discontinued, HBV DNA levels returned to near baseline levels for all EDP-514 treatment groups. No virologic failures were observed.

Twenty-two patients had quantifiable HBV RNA levels at baseline (200 mg, n=6; 400 mg, n=5; 800 mg, n=6; placebo, n=5) (Table 1). EDP-514 also showed increasing inhibition of HBV RNA levels with increasing duration of treatment, but no clear dose-dependent effects were observed (Fig. 5 and Supplementary Fig. 2). By Day 28, mean change from baseline for HBV RNA levels was $-2.9$, $-2.4$, $-2.0$, and $-0.02$ log$_{10}$ U/mL with the 200 mg, 400 mg, 800 mg, and placebo groups, respectively. Following discontinuation of study treatments, HBV RNA levels increased to approach baseline levels in all treatment groups.

No clinically meaningful changes from baseline were observed in patients with quantifiable levels at baseline for HBsAg, HBeAg (n=4) or HBcrAg (n=21) (Table 1 and Supplementary Figs. 3 and 4).

Safety/Tolerability

No discontinuations, serious AEs or deaths were reported with EDP-514 treatment. Overall, nine patients reported treatment emergent adverse events (TEAEs) (Table 3). All TEAEs were mild or moderate, and none were severe. No individual TEAE occurred more than once with any dose of EDP-514. One patient in the placebo group experienced gastrointestinal disorder, prolonged activated PTT and increased INR that were considered possibly related to placebo therapy. Clinical laboratory findings were generally normal with no clinically relevant changes in the EDP-514.
Man-Fung Yuen, et al.

EDP-514 antiviral activity in chronic hepatitis B

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man-fung.yuen@hlth.gov.hk

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 treatment groups except for one patient who experienced prolonged activated PTT and increased INR and one patient who experienced prolonged PT, both in the EDP-514 200 mg group. Mean change from baseline of ALT, AST, gamma glutamyl transferase, bilirubin, triglycerides, and cholesterol are shown in Supplementary Figures 5, 6, 7, 8, 9 and 10. No ALT elevations indicative of HBV viral flares was observed in the study. No clinically relevant changes in the physical examination, vital signs or ECG were observed with EDP-514.

DISCUSSION

The HBV core protein plays an indispensable role in the viral life cycle by packaging the viral pregenomic RNA along with the viral polymerase into capsids. This packaging enables the reverse transcription of pregenomic RNA into relaxed circular DNA to produce the infectious form of the viral genome. The core protein itself also displays limited sequence polymorphisms across HBV genotypes, which coupled with the critical role of the protein in producing infectious virus and sustaining the viral cccDNA pool, makes it an important target for novel antivirals to treat HBV infection.19,20

In this phase 1b study of EDP-514, positive data were obtained from all three dose cohorts in patients with viremic CHB infection not currently treated with pegIFN or NUC. The incidence of mild or moderate AEs was similar between EDP-514 groups and placebo with no dose-related increase in AEs. No AEs occurred more than once in any patient, and no patients discontinued the study for an AE. EDP-514 displayed a PK profile demonstrating a dose-related increase in exposure and supporting once-daily dosing, with median C trough at Day 28 ~9-fold for 200 mg, ~20-fold for 400 mg, and ~24-fold for 800 mg doses above the protein-adjusted EC50 (71 ng/mL). Marked reductions in HBV DNA and HBV RNA levels occurred rapidly with all three EDP-514 doses compared with placebo, and levels returned to baseline after discontinuing study treatment. Overall, these results were consistent with a previous study of EDP-514 in healthy patients.18 At Day 28 in the study reported here, mean reductions in HBV DNA levels were −2.9, −3.3, and −3.5 with the 200 mg, 400 mg, and 800 mg doses of EDP-514, and mean reductions in HBV RNA levels were −2.9, −2.4, and −2.0. The lack of dose proportion-
al response in HBV RNA may be due to potentially attaining the maximal effect in HBV RNA reduction with 200 mg, the small number of patients evaluated, or that the majority of patients were HBeAg negative whose HBV RNA levels are typically low.\textsuperscript{21} As a whole, other studies of drug therapy for treatment-naive patients with HBV reported similar findings.\textsuperscript{13,15,22-24} While the clinical significance of circulating HBV RNA remains unknown, it has been suggested HBV RNA levels may be a marker for treatment response and cccDNA activity in patients with chronic HBV infection.\textsuperscript{25-29} This viral biomarker is one that remains largely unchanged with short term NUC therapy and highlights the differentiated mechanism of action of EDP-514. The addition of a potent core protein inhibitor such as EDP-514 to NUC treatment could potentially lower HBV DNA and RNA levels more rapidly and profoundly, which may result in a lower incidence of hepatocellular carcinoma. The addition of an early generation core protein inhibitor vebicorvir to patients taking NUCs further suppressed HBV DNA and HBV RNA more than with NUCs alone.\textsuperscript{24,30} In this manner, an EDP-514 and NUC combination treatment may have clinical benefit in treatment-naive highly viremic HBeAg-positive patients where NUC treatment takes longer than a year to achieve undetectable HBV DNA levels and NUC-treated patients with residual HBV DNA and HBV RNA are detectable in circulation by highly sensitive assays.

In the present study of 4 weeks dosing of EDP-514, a reduction in HBsAg was not observed. Recently, another core protein inhibitor ALG-000184 demonstrated an HBsAg reduction beginning at approximately 4 weeks to 36 weeks of treatment.\textsuperscript{31} According to the present study, EDP-514 appears to have a similar potency of HBV DNA and HBV RNA suppressions when compared with ALG-000184 (mean HBV DNA and HBV RNA reductions of 3.8 log\textsubscript{10} IU/mL and 1.9 log\textsubscript{10} copies/mL after 4 weeks of treatment, respectively).\textsuperscript{32} Whether a longer treatment duration of EDP-514 than 4 weeks could also show an HBsAg reduction would be interesting to explore in the future.

Limitations to this study included a small sample size, a short treatment duration, and limited diversity in the patient population. However, this study was specifically designed to evaluate safety, PK, and antiviral activity in a previously untreated population with CHB and help to confirm results from an earlier study that evaluated EDP-514 in healthy subjects and NUC-suppressed patients.\textsuperscript{18} Studies enrolling larger numbers of more diverse patients with HBV infec-

**Figure 5.** Mean (standard deviation [SD]) change from baseline in HBV RNA concentrations over time. HBV, hepatitis B virus.
Overall, these results demonstrate that EDP-514, a novel oral HBV core protein inhibitor, was well tolerated and had a favorable safety profile for viremic, treatment-naive CHB patients. In this population of patients with chronic HBV infection, EDP-514 for 28 days resulted in a mean reduction in both HBV DNA and HBV RNA of 2 to 3.5 log_{10} that is highly suggestive of antiviral activity as a potent HBV core protein inhibitor. These results encourage continued investigation of EDP-514 for viremic, treatment-naive patients with CHB.

Authors’ contribution
GDLR, AA, EL, ALC contributed to data analysis, interpretation, and manuscript preparation. All other authors conducted the clinical study and contributed to data interpretation. All authors critically reviewed and approved the final manuscript.

Acknowledgements
The authors would like to thank all volunteers, investigators and study personnel who participated in the clinical studies. The authors acknowledge Jason Yu for assistance with preparation of figures, Michael Vaine, PhD, for critical review of the manuscript, and editorial assistance of Richard S. Perry, PharmD, which were supported by Enanta Pharmaceuticals, Inc., Watertown, MA.

The study was funded by Enanta Pharmaceuticals, Inc. and was designed in conjunction with the authors. Enanta was involved in study design, data collection, data analy-

Table 3. Incidence of treatment-emergent adverse events (TEAEs) with EDP-514

<table>
<thead>
<tr>
<th>TEAE</th>
<th>Placebo (n=14)</th>
<th>EDP-514 200 mg (n=6)</th>
<th>EDP-514 400 mg (n=6)</th>
<th>EDP-514 800 mg (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any TEAE</td>
<td>2 (33.3)</td>
<td>5</td>
<td>3 (50.0)</td>
<td>4</td>
</tr>
<tr>
<td>Severe TEAE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Discontinuation for TEAE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Serious TEAE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Individual TEAEs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activated partial thromboplastin time prolonged</td>
<td>0</td>
<td>0</td>
<td>1 (16.7)</td>
<td>1</td>
</tr>
<tr>
<td>Anaemia</td>
<td>1 (16.7)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cough</td>
<td>0</td>
<td>0</td>
<td>1 (14.3)</td>
<td>1</td>
</tr>
<tr>
<td>Dermatitis</td>
<td>0</td>
<td>1 (16.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dizziness</td>
<td>1 (16.7)</td>
<td>0</td>
<td>1 (14.3)</td>
<td>1</td>
</tr>
<tr>
<td>Flatulence</td>
<td>0</td>
<td>0</td>
<td>1 (14.3)</td>
<td>1</td>
</tr>
<tr>
<td>Gastrointestinal disorder</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Headache</td>
<td>0</td>
<td>1 (16.7)</td>
<td>0</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Hepatic neoplasm</td>
<td>0</td>
<td>0</td>
<td>1 (14.3)</td>
<td>1</td>
</tr>
<tr>
<td>International normalized ratio increased</td>
<td>1 (16.7)</td>
<td>0</td>
<td>0</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Nasopharyngitis</td>
<td>0</td>
<td>1 (16.7)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Oropharyngeal pain</td>
<td>0</td>
<td>0</td>
<td>1 (14.3)</td>
<td>1</td>
</tr>
<tr>
<td>Palpitations</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Prothrombin time prolonged</td>
<td>1 (16.7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tooth development disorder</td>
<td>0</td>
<td>1 (16.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (16.7)</td>
</tr>
</tbody>
</table>

Values are presented as number of patients per measurement (%) number of total AEs.
AEs, adverse events; n, number of patients per treatment group.
sis, data interpretation, and writing of the report. All authors had full access to all the data in the study, participated in drafting and editing the manuscript and were responsible for the decision to submit for publication.

Enanta Clinical Trial Number: EDP 514-002

Conflicts of Interest

Man-Fung Yuen: Advisory/consultant for AbbVie, Aligos Therapeutics, AiCuris, Antios Therapeutics, Assembly Biosciences, Arbutus Biopharma, Bluejay Therapeutics, Clear B Therapeutics, Dicerna Pharmaceuticals, Finch Therapeutics, GlaxoSmithKline, Gilead Sciences, Janssen, Merck Sharp and Dohme, Hoffmann-La Roche, Vir Biotechnology; grant/research supports from AbbVie, Assembly Biosciences, Arrowhead Pharmaceuticals, Arbutus Biopharma, Bristol Myer Squibb, Dicerna Pharmaceuticals, Fujirebio Incorporation, GlaxoSmithKline, Gilead Sciences, Immunocore, Merck Sharp and Dohme, Hoffmann-La Roche; sponsored lectures for Menarini, Gilead Sciences, Janssen.

Wan-Long Chuang: Consultant for Gilead Sciences, AbbVie, Bristol Myers Squibb, Roche, Vaccitech, PharmaEssentia; Speaker for Gilead Sciences, AbbVie, Bristol Myers Squibb, Roche, PharmaEssentia; Sponsored lectures for Gilead Sciences, AbbVie, Bristol Myers Squibb, Roche, PharmaEssentia.

Cheng-Yuan Peng: Advisory board for AbbVie, Bristol Myers Squibb, Gilead Sciences and Hoffman-La Roche.

Wen-Juei Jeng: Speaker for Bristol Myers Squibb and Gilead Sciences; Grants from Chang Gung Medical Foundation; National Science Council, Taiwan.

Wei-Wen Su: Speaker for Gilead Sciences, Eisai, AbbVie.

Ting-Tsung Chang: Nothing to disclose.

Chi-Yi Chen: Nothing to disclose.

Yao-Chun Hsu: Consultant for Gilead Sciences; sponsored lectures for AbbVie, Bristol Myers Squibb, Gilead Sciences, Merck Sharp & Dohme, and Novartis; grants from Ministry of Science and Technology, Taiwan, Ministry of Health and Welfare, Taiwan, E-Da Hospital, Taiwan, Tomorrow Medical Foundation, and Gilead Sciences.

Annie L. Conery, Alaa Ahmad, Ed Luo, Guy De La Rosa: Employee and stockholder for Enanta Pharmaceuticals, Inc. Watertown, MA at the time the work was performed.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES


Dynamic analysis of acute deterioration in chronic liver disease patients using modified quick sequential organ failure assessment

Do Seon Song1, Hee Yeon Kim2, Young Kul Jung3, Tae Hyung Kim3, Hyung Joon Yim3, Eileen L Yoon4, Ki Tae Suk5, Jeong-ju Yoo6, Sang Gyune Kim4, Moon Young Kim7, Young Chang8, Soung Won Jeong9, Jae Young Jang9, Sung-Eun Kim5, Jung-Hee Kim5, Jung Gil Park5, Won Kim10, Jin Mo Yang1, Dong Joon Kim9, Korean Acute-on-Chronic Liver Failure (KACLIF) Study Group, Ashok Kumar Choudhury11, Vinod Arora11, Shiv Kumar Sarin11, APASL ACLF Research Consortium (AARC) for APASL ACLF Working Party

1Department of Internal Medicine, St. Vincent’s Hospital, The Catholic University of Korea, Seoul; 2Department of Internal Medicine, Uijeongbu St. Mary’s Hospital, The Catholic University of Korea, Seoul; 3Department of Internal Medicine, Korea University Ansan Hospital, Ansan; 4Department of Internal Medicine, Hanyang University College of Medicine, Seoul; 5Department of Internal Medicine, Hallym University College of Medicine, Chuncheon; 6Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, Bucheon; 7Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju; 8Department of Internal Medicine, Soonchunhyang University College of Medicine, Seoul, 9Department of Internal Medicine, Yeungnam University College of Medicine, Daegu; 10Department of Internal Medicine, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, Korea; 11Department of Hepatology, Institute of Liver and Biliary Sciences, New Delhi, India

Graphical Abstract

Chronic liver disease patients with acute deterioration

1-month transplant-free survival rate
Background/Aims: Quick sequential organ failure assessment (qSOFA) is believed to identify patients at risk of poor outcomes in those with suspected infection. We aimed to evaluate the ability of modified qSOFA (m-qSOFA) to identify high-risk patients among those with acutely deteriorated chronic liver disease (CLD), especially those with acute-on-chronic liver failure (ACLF).

Methods: We used data from both the Korean Acute-on-Chronic Liver Failure (KACLiF) and the Asian Pacific Association for the Study of the Liver ACLF Research Consortium (AARC) cohorts. qSOFA was modified by replacing the Glasgow Coma Scale with hepatic encephalopathy, and an m-qSOFA ≥2 was considered high.

Results: Patients with high m-qSOFA had a significantly lower 1-month transplant-free survival (TFS) in both cohorts and higher organ failure development in KACLiF than those with low m-qSOFA (P<0.05). Subgroup analysis by ACLF showed that patients with high m-qSOFA had lower TFS than those with low m-qSOFA. m-qSOFA was an independent prognostic factor (hazard ratios, HR=2.604, 95% confidence interval, CI 1.353–5.013, P=0.004 in KACLiF and HR=1.904, 95% CI 1.484–2.442, P<0.001 in AARC). The patients with low m-qSOFA at baseline but high m-qSOFA on day 7 had a significantly lower 1-month TFS than those with high m-qSOFA at baseline but low m-qSOFA on day 7 (52.6% vs. 89.4%, P<0.001 in KACLiF and 26.9% vs. 61.5%, P<0.001 in AARC).

Conclusions: Baseline and dynamic changes in m-qSOFA may identify patients with a high risk of developing organ failure and short-term mortality among CLD patients with acute deterioration. (Clin Mol Hepatol 2024;30:388-405)

Keywords: qSOFA; Acute-on-chronic liver failure; Organ failure; Survival

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Corresponding author: Dong Joon Kim
Department of Internal Medicine, Hallym University College of Medicine, 1 Hallimdaehak-gil, Chuncheon 24252, Korea
Tel: +82-33-240-5646, Fax: +82-33-241-8064, E-mail: djkim@hallym.ac.kr
https://orcid.org/0000-0002-5792-1500

Abbreviations:
AARC, Asian Pacific Association for the Study of the Liver Acute-on-chronic liver failure Research Consortium; ACLF, Acute-on-Chronic Liver Failure; ALT, ALanine aminOtransferase; AST, ASpartate aminOtransferase; CI, confidence interval; CLD, chronic liver disease, CLIF-C OF, Chronic Liver Failure Consortium Organ Failure; CLIF-SOFa, Chronic Liver Failure- Sequential Organ Failure Assessment; EASL-CLIF, European Association for the Study of the Liver-Chronic Liver Failure; GCS, Glasgow Coma Scale; HE, Hepatic Encephalopathy; HR, Hazard Ratio; INR, International Normalized Ratio; KACLiF, Korean Acute-on-Chronic Liver Failure; LT, Liver Transplantation; MELD, Model for End-stage Liver Disease; m-qSOFA, modified quick Sequential Organ Failure Assessment; OR, Odds Ratio; qSOFA, Quick Sequential Organ Failure Assessment; SIRS, Systemic Inflammatory Response Syndrome; WBC, White Blood Cell

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a rapid deterioration of liver function in patients with chronic liver disease (CLD).\(^1,2\) Various acute insults, such as alcoholic hepatitis, bacterial infection, viral hepatitis, and hepatotoxic drugs, not only result in hepatic derangement but can also cause extrahepatic organ failures, with high short-term mortality.\(^3,4\) However, ACLF is a disease distinguished from decompensated liver cirrhosis because it is reversible. Therefore, the prediction of poor outcomes in patients with ACLF is crucial given the potential of its reversibility without liver transplantation (LT).

Exaggerated systemic inflammation is one of the main mechanisms driving the occurrence and progression of ACLF, along with portal hypertension. Systemic inflammation causes splanchnic and systemic circulatory dysfunction, immune-mediated tissue damage, and changes in energy metabolism in ACLF patients, and these changes are associated with multiorgan failure.\(^5\) A paradoxical immunoparesis state is associated with severe infection and sepsis.\(^6\) In practice, bacterial and fungal infections develop frequently and are associated with poor clinical courses and high mortality in ACLF patients.\(^7\) The Asian Pacific Association for the Study of the Liver ACLF Research Consortium (AARC) proposes the concept of a golden window of approximately 1 week between ACLF development and sepsis. They suggest that prompt intervention in this window is necessary to reduce the development of sepsis and improve the outcome of ACLF.\(^2\)

Early identification of sepsis is important because of its high mortality. The 3rd International Consensus Definitions for Sepsis and Septic Shock recommend the quick sequential organ failure assessment (qSOFA) score. The qSOFA score includes three components (Glasgow Coma Scale [GCS], systolic blood pressure, and respiratory rate) and serves as a bedside tool to identify adult patients with suspected infection who are at risk of poor outcomes.\(^8\) Considering that infection in patients with cirrhosis is one of the leading causes of death, the usefulness of this simple score was evaluated in those with cirrhosis and infection. However, qSOFA showed conflicting results in predicting adverse outcomes in patients with cirrhosis and infections.\(^9-12\) In addition, studies on the role of the qSOFA score in patients with ACLF are limited.

In the general intensive care unit, the SOFA score has been used to assess the severity of organ failure.\(^13\) However, some components, such as GCS and platelet count, do not reflect the specificity of CLD.\(^14\) Therefore, the European Association for the Study of the Liver (EASL)-Chronic Liver Failure (CLIF) consortium developed the CLIF-SOFA score by replacing GCS with the West Haven criteria and platelet count with prothrombin time to modify the SOFA score.\(^4\) The CLIF-SOFA score has been similar or superior to the SOFA score in predicting poor outcomes in patients with ACLF or decompensated cirrhosis.\(^3,15-17\) To apply qSOFA to patients with CLD, a modification replacing the GCS with the West Haven criteria would be necessary. In this study, we aimed to evaluate the usefulness of the modified qSOFA (m-qSOFA) score in identifying high short-term mortality in CLD patients with acute deterioration, particularly those with ACLF.

MATERIALS AND METHODS

Patients

This study was conducted using patients from two different cohorts. The first cohort included patients from the prospective Korean Acute-on-Chronic Liver Failure (KACLiF) study. The KACLiF study screened and enrolled CLD patients who were hospitalized with acute deterioration in 31 university hospitals between October 2015 and May 2019. Acute deterioration of CLD in this study included ascites, hepatic encephalopathy (HE), infection, gastrointestinal bleeding, and bacterial infection, which are decompensations defined by the EASL-CLIF consortium, and liver dysfunction which is defined as serum bilirubin ≥3 mg/dL. The underlying CLD included not only cirrhosis but also noncirrhotic CLD. The etiology of CLD included hepatitis B virus infection, hepatitis C virus infection, alcohol-associated liver disease, nonalcoholic fatty liver disease, and autoimmune liver disease. Cirrhosis was diagnosed histologically or based on clinical parameters, such as radiologic or laboratory findings.\(^18\) The exclusion criteria were as follows: 1) age under 18 years, 2) absence of CLD, 3) presence of radiologically definite hepatocellular carcinoma, 4) hospitalization due to extrahepatic diseases, 5) admission for symptomatic control of CLD, and 6) human immunodefi-
ciency virus infection. A total of 1,533 patients were screened, and 1,497 patients were enrolled in this study (Supplementary Fig. 1). The study was registered on ClinicalTrials.gov (number: NCT02650011).

The second cohort included patients from the AARC database, which collected multicenter ACLF data from the Asia-Pacific region prospectively beginning in 2009. From 2009 to 2021, 5,345 patients were enrolled in more than 80 hospitals in the Asia-Pacific region. In this study, we analyzed 1,217 patients after excluding 4,128 patients with insufficient data.

This study was approved by the institutional review board at each participating hospital and was in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki.

Data collection

We collected data on patient demographics, history (including past decompensation and etiology of CLD), potential precipitating factors, laboratory measurements, and development of ACLF. Clinical data within 24 hours of admission and 7 days (5–9 days) after admission were collected. Potential precipitating factors of acute deterioration included acute infection or reactivation of underlying viral hepatitis, gastrointestinal bleeding, bacterial infection, active alcohol drinking, toxic liver injury, and others, which included the precipitants of both the AARC and EASL-CLIF definitions. Active alcoholism was defined as more than 21 drinks per week in men and more than 14 drinks per week in women within 3 months of admission. Scores predicting the prognosis of patients with liver disease, such as Child–Pugh, Model for End-stage Liver Disease (MELD), MELD-Na, CLIF-SOFA, CLIF Consortium Organ Failure (CLIF-C OF), and AARC scores, were calculated at admission.

Definitions

The m-qSOFA used the following parameters: 1) HE grades by West Haven criteria ≥2, 2) respiratory rate ≥22/min, and 3) systolic blood pressure ≤100 mmHg. When at least 2 of the 3 criteria were met, the m-qSOFA score was defined as high, and when fewer than 2 criteria were met, the m-qSOFA score was defined as low. Systemic inflammatory response syndrome (SIRS) was defined as 2 or more of the following criteria: 1) body temperature <36°C or >38°C, 2) heart rate >90 beats per minute, 3) respiratory rate >20 breaths per minute, and 4) white blood cell (WBC) count <4,000/mm³ or >12,000/mm³. ACLF was defined by the EASL-CLIF and AARC definitions. The day 7 m-qSOFA of patients who died or received LT within 7 days was considered high to prevent these patients from being excluded from analyzing the effect of dynamic changes in m-qSOFA. Organ failures included liver, renal, coagulation, cerebral, circulatory, and respiratory failures, and they were defined by the CLIF-SOFA score.

Statistics

The primary outcomes were 1-month transplant-free survival (TFS) (28 days in the KACLiF cohort and 30 days in the AARC cohort). Continuous variables were expressed as the mean ± standard deviation (SD) for normally distributed variables and median and interquartile range (IQR) for non-normally distributed variables, and categorical variables were expressed as number (%). The normal distribution was tested using the Kolmogorov–Smirnov test. Categorical variables were analyzed by the chi-square test or Fisher’s exact test, and continuous variables were analyzed by Student’s t test. TFS was calculated using the Kaplan–Meier method, and survival differences were compared using the log-rank test. A Cox proportional hazard regression model by the backward stepwise likelihood ratio method was used to identify the independent predictor of 1-month TFS. When m-qSOFA and prognostic scores were included in multivariate analysis, their components were excluded to avoid multicollinearity. For missing data, a complete case analysis was used. Variables with P<0.05 in univariate analysis were included in multivariate analysis. The optimal cut-off value of prognostic scores was determined using the Youden index defined as sensitivity + specificity - 1. A P-value of <0.05 was considered statistically significant. Statistical tests were performed using SPSS 18.0 (SPSS, Inc.; IBM Company, Armonk, NY, USA).
Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>KACLIF cohort</th>
<th>AARC cohort</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (n=1,497)</td>
<td>Low m-qSOFA (n=1,376)</td>
<td>High m-qSOFA (n=121)</td>
<td>Total (n=1,217)</td>
</tr>
<tr>
<td>Age, years</td>
<td>54.7±11.5</td>
<td>54.6±11.5</td>
<td>55.5±10.9</td>
<td>0.433</td>
</tr>
<tr>
<td>Gender (male, %)</td>
<td>1,114 (74.4)</td>
<td>1,026 (74.6)</td>
<td>88 (72.7)</td>
<td>0.657</td>
</tr>
<tr>
<td>Cirrhosis (%)</td>
<td>1,395 (93.2)</td>
<td>1,277 (92.8)</td>
<td>118 (97.5)</td>
<td>0.007</td>
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<tr>
<td>Etiology (%)</td>
<td></td>
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<td>0.041</td>
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<tr>
<td>HBV</td>
<td>180 (12.0)</td>
<td>172 (12.5)</td>
<td>8 (6.6)</td>
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<tr>
<td>HCV</td>
<td>26 (1.7)</td>
<td>23 (1.7)</td>
<td>3 (2.5)</td>
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<td>1,017 (67.9)</td>
<td>924 (67.2)</td>
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<tr>
<td>HBV+Alcohol</td>
<td>108 (7.2)</td>
<td>102 (7.4)</td>
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<td>HCV+Alcohol</td>
<td>21 (1.4)</td>
<td>17 (1.2)</td>
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<tr>
<td>Autoimmune</td>
<td>46 (3.8)</td>
<td>35 (3.6)</td>
<td>11 (4.7)</td>
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<tr>
<td>Others</td>
<td>145 (9.7)</td>
<td>138 (10.0)</td>
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<tr>
<td>Decompensation</td>
<td></td>
<td></td>
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<tr>
<td>Ascites</td>
<td>434 (29.0)</td>
<td>410 (29.8)</td>
<td>24 (19.8)</td>
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<tr>
<td>Hepatic encephalopathy</td>
<td>220 (14.7)</td>
<td>169 (12.3)</td>
<td>51 (42.1)</td>
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</tr>
<tr>
<td>Jaundice</td>
<td>524 (35.0)</td>
<td>498 (36.2)</td>
<td>26 (21.5)</td>
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</tr>
<tr>
<td>GI bleeding</td>
<td>520 (34.7)</td>
<td>468 (34.0)</td>
<td>52 (43.0)</td>
<td>0.031</td>
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<tr>
<td>Infection</td>
<td>161 (10.8)</td>
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<td>24 (19.8)</td>
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<tr>
<td>Prior decompensation</td>
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<tr>
<td>AARC definition</td>
<td>438 (29.3)</td>
<td>395 (28.7)</td>
<td>43 (35.5)</td>
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</tr>
<tr>
<td>EASL-CLIF definition</td>
<td>583 (38.9)</td>
<td>530 (38.5)</td>
<td>53 (43.8)</td>
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<tr>
<td>AARC ACLF (%)</td>
<td>188 (12.6)</td>
<td>163 (11.8)</td>
<td>25 (20.7)</td>
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<tr>
<td>AARC ACLF grade*</td>
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<td>0.026</td>
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<tr>
<td>Grade 1</td>
<td>23 (18.1)</td>
<td>23 (19.9)</td>
<td>0 (0)</td>
<td>102 (13.3)</td>
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<td>Grade 2</td>
<td>67 (52.8)</td>
<td>55 (52.4)</td>
<td>12 (54.5)</td>
<td>380 (49.4)</td>
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<td>Grade 3</td>
<td>37 (29.1)</td>
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<td>EASL-CLIF ACLF (%)</td>
<td>248 (16.6)</td>
<td>192 (14.0)</td>
<td>56 (46.3)</td>
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<td>&lt;0.001</td>
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<td>Grade 1</td>
<td>127 (51.2)</td>
<td>109 (56.8)</td>
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### Table 1. Continued

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<th>AARC cohort</th>
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<td>Total (n=1,497)</td>
<td>Low m-qSOFA (n=1,376)</td>
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<tr>
<td>Grade 2</td>
<td>81 (32.7)</td>
<td>63 (32.8)</td>
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<tr>
<td>Grade 3</td>
<td>40 (16.1)</td>
<td>20 (10.4)</td>
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<td>SIRS (%)</td>
<td>355 (23.7)</td>
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<td>Body temperature (°C)</td>
<td>36.8±0.6</td>
<td>36.8±0.6</td>
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<tr>
<td>Heart rate (/min)</td>
<td>89.9±19.7</td>
<td>88.8±18.8</td>
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<tr>
<td>Respiratory rate (/min)</td>
<td>19.8±2.5</td>
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<tr>
<td>SBP (mmHg)</td>
<td>119.6±22.8</td>
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<td>DBP (mmHg)</td>
<td>72.2±14.1</td>
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<td>WBC (×10^9/mm³)</td>
<td>7.62±5.35</td>
<td>7.38±5.03</td>
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<td>HB (mg/dL)</td>
<td>112.6±69.3</td>
<td>113.1±69.8</td>
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<tr>
<td>Hemoglobin (g/dL)</td>
<td>112.6±69.3</td>
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<td>Platelet count (×10^9/mm³)</td>
<td>2.94±0.63</td>
<td>2.96±0.63</td>
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<tr>
<td>Albumin (g/dL)</td>
<td>3.54±1.58</td>
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<td>34.0 (20.0–69.0)</td>
<td>33.0 (20.0–69.0)</td>
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<tr>
<td>AST (IU/L)</td>
<td>84.0 (44.0–188.0)</td>
<td>83.5 (43.0–188.0)</td>
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<td>INR</td>
<td>1.47 (1.27–1.78)</td>
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<td>CRP (mg/dL)</td>
<td>0.68 (0.25–2.04)</td>
<td>0.66 (0.24–1.99)</td>
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<td>Creatinine (mg/dL)</td>
<td>0.88 (0.70–1.20)</td>
<td>0.85 (0.68–1.15)</td>
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<td>Na (mEq/L)</td>
<td>135.1±6.1</td>
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<tr>
<td>Procalcitonin (ng/mL)</td>
<td>0.24 (0.12–0.56)</td>
<td>0.22 (0.11–0.48)</td>
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<tr>
<td>Lactate (mmol/L)</td>
<td>2.00 (1.24–3.70)</td>
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<td>Child-Pugh score</td>
<td>9.0±2.1</td>
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<tr>
<td>MELD score</td>
<td>18.1±7.2</td>
<td>17.8±7.0</td>
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<td>MELD-Na score</td>
<td>20.6±7.5</td>
<td>20.3±7.3</td>
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<tr>
<td>AARC score</td>
<td>7.7±1.8</td>
<td>7.5±1.7</td>
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<tr>
<td>CLIF-SOFA</td>
<td>5.6±3.0</td>
<td>5.2±2.6</td>
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RESULTS

Baseline characteristics

The baseline characteristics of the enrolled patients, including cirrhosis and non-cirrhosis, and ACLF and non-ACLF in the KACLiF and AARC cohorts are reported in Table 1. The mean age was 54.7±11.5 years and 46.4±12.2 years in the KACLiF and AARC cohorts, respectively. The sexes of both cohorts were mostly male (74.4% in the KACLiF cohort and 86.8% in the AARC cohort). Most patients (n=1,395, 93.2%) in the KACLiF cohort had cirrhosis. The most common etiology of CLD was alcohol (67.9% in the KACLiF cohort and 63.2% in the AARC cohort), followed by HBV (12.0% in the KACLiF cohort and 14.5% in the AARC cohort). High m-qSOFA was found in 121 (8.1%) and 235 (19.3%) patients in the KACLiF and AARC cohorts, respectively. The patients with high m-qSOFA had more frequent SIRS and ACLF at baseline than the patients with low m-qSOFA in both cohorts (all Ps<0.05). The patients with high m-qSOFA had a higher heart rate, respiratory rate, WBC count, absolute neutrophil count, INR, creatinine, and lactate level and lower systolic, diastolic and mean blood pressure, and albumin levels than patients with low m-qSOFA. At baseline, those with high m-qSOFA had higher Child-Pugh, MELD, MELD-Na, AARC, CLIF-SOFA, and CLIF-C OF scores (all Ps<0.001) than those with low m-qSOFA.

Survival difference according to m-qSOFA

In the KACLiF cohort, those with high m-qSOFA had a significantly lower 1-month TFS than those with low m-qSOFA (80.2% vs. 95.9%, P<0.001) (Supplementary Fig. 2A). In the AARC cohort, the patients with high m-qSOFA also had a significantly lower 1-month TFS than the patients with low m-qSOFA (46.4% vs. 69.9%, P<0.001) (Supplementary Fig. 2B).

Survival difference according to ACLF and m-qSOFA

In the KACLiF cohort, the patients with high m-qSOFA had a significantly lower TFS than those with low m-qSOFA in the patients without AARC ACLF (82.3% vs. 97.4%,
Figure 1. One-month transplant-free survival rate according to m-qSOFA and ACLF. (A) Non-AARC ACLF and (B) AARC ACLF patients in the KACLIF cohort. (C) Non-AARC ACLF and (D) AARC ACLF patients in the AARC cohort. (E) Non-EASL-CLIF ACLF and (F) EASL-CLIF ACLF patients in the KACLIF cohort. m-qSOFA, modified quick sequential organ failure assessment; ACLF, acute-on-chronic liver failure; AARC, Asian Pacific Association for the Study of the Liver ACLF Research Consortium; EASL-CLIF, European Association for the Study of the Liver-Chronic liver failure; KACLIF, Korean acute-on-chronic liver failure; AARC, Asian Pacific Association for the Study of the Liver ACLF Research Consortium.
**m-qSOFA as a predictor of mortality**

We performed Cox hazard proportional regression analysis to identify the significant predictor of 1-month TFS in both cohorts. In the KACLiF cohort, HE (grade 2), gastrointestinal bleeding, bacterial infection, SIRS, WBC count, platelet count, serum albumin, bilirubin, AST, INR, C-reactive protein, creatinine, sodium, and lactate levels, and high m-qSOFA were significant factors in the univariate analysis, and SIRS, platelet count, serum albumin, bilirubin, AST, INR, and m-qSOFA were significant factors in the multivariate analysis (Table 2). In the AARC cohort, age, ascites, HE (grade 2), SIRS, WBC count, serum bilirubin, INR, creatinine, sodium, lactate levels, and high m-qSOFA were significant factors in the univariate analysis. Age, ascites, WBC count, serum bilirubin, INR, creatinine, lactate levels, and m-qSOFA were significant factors in the multivariate analysis (Table 2). When the m-qSOFA was adjusted by CLD-specific prognostic scores and significant variables by multivariate analysis (excluding the variables included in the prognostic scores), m-qSOFA was an independent factor in both the KACLiF and AARC cohorts (all \( P_{s} < 0.05 \)) (Table 3).

**Dynamic change in m-qSOFA in subgroups according to ACLF**

In the KACLiF cohort, those with high m-qSOFA scores at both baseline and on day 7 had the worst 1-month TFS. Interestingly, patients who changed from low baseline m-qSOFA to high on the 7th day had a significantly lower 1-month TFS than those who shifted from high to low (52.6% vs. 89.4%, \( P < 0.001 \)). In the KACLiF cohort, no statistically significant difference was observed between the patients who changed from low baseline m-qSOFA to high on the 7th day and those who had a consistently high m-qSOFA in the KACLiF cohort (52.6% vs. 33.3%, \( P = 0.173 \)) (Fig. 2A). In both subgroups of the KACLiF cohort based on the presence of AARC ACLF at the baseline, those who changed from low baseline m-qSOFA to high on the 7th day had significantly lower 1-month TFS than those who shifted from high to low (25.0% vs. 75.0%, \( P < 0.001 \)) (Fig. 2B and C).

In the AARC cohort, similar results were shown. The patients with high m-qSOFA on both baseline and the 7th day had the worst 1-month TFS, and those who changed from low baseline m-qSOFA to high on the 7th day had a significantly lower 1-month TFS than those who shifted from high to low (26.9% vs. 61.5%, \( P < 0.001 \)) (Fig. 2D). In the ACLF group of the AARC cohort, those who changed from low to high m-qSOFA had a significantly lower 1-month TFS than those who changed from high to low (25.0% vs. 58.9%, \( P < 0.001 \)) (Fig. 2E). Similar results were shown in the non-ACLF group of the AARC cohort, but they were not significant (43.8% vs. 72.2%, \( P = 0.053 \)) (Fig. 2F).

The effect of dynamic changes in m-qSOFA according to the presence of EASL-CLIF ACLF was analyzed in the KACLiF cohort alone. In both subgroups, those who had changed from m-qSOFA low to high had significantly lower 1-month TFS than those who had changed from high to low (45.5% vs. 77.8%, \( P = 0.006 \) in the ACLF group and 62.5% vs. 96.6%, \( P < 0.001 \) in the non-ACLF group) and had similar TFS compared to those who had high m-qSOFA on both the baseline and 7th day (45.5% vs. 27.8%, \( P = 0.321 \) in the ACLF group and 62.5% vs. 66.7%, \( P = 0.959 \) in the non-ACLF group) (Fig. 3A and B).

Even after excluding patients who died or received LT within 7 days, the KACLiF and AARC cohorts showed similar results (Supplementary Figs. 3 and 4).

**m-qSOFA according to CLD-specific prognostic scores**

Given that the MELD score and m-qSOFA were shown to...
### Table 2. Predictors for 28-day LT-free survival in the KACLiF cohort and 30-day survival in the AARC cohort

<table>
<thead>
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<th>Variable</th>
<th>KACLiF</th>
<th>AARC</th>
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<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate*</td>
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<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
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<td>Age</td>
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<td>0.983–1.021</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>0.98</td>
<td>0.959–1.612</td>
</tr>
<tr>
<td>Ascites</td>
<td>0.97</td>
<td>0.958–1.574</td>
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<tr>
<td>Hepatic encephalopathy (≥G2)</td>
<td>3.166</td>
<td>1.951–5.138</td>
</tr>
<tr>
<td>GI bleeding</td>
<td>0.582</td>
<td>0.348–0.974</td>
</tr>
<tr>
<td>Infection</td>
<td>2.105</td>
<td>1.217–3.640</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>0.178</td>
<td>0.025–1.278</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.07</td>
<td>0.650–1.761</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.637</td>
<td>0.345–1.177</td>
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<tr>
<td>Prior decompensation (AARC definition)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within 1 year</td>
<td>1.002</td>
<td>0.574–1.749</td>
</tr>
<tr>
<td>More than 1 year</td>
<td>1.211</td>
<td>0.598–2.450</td>
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<tr>
<td></td>
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<td>SIRS</td>
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<tr>
<td>WBC (×10³ mm⁻³)</td>
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<td>1.045–1.105</td>
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<tr>
<td>Hb (g/dL)</td>
<td>0.955</td>
<td>0.883–1.033</td>
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<tr>
<td>PLT (×10⁹/mm³)</td>
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<td>0.991–0.999</td>
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<td>Bilirubin (mg/dL)</td>
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<td>1.000–1.000</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
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<td>1.000–1.000</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
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<td>1.000–1.000</td>
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</table>

†: Represents a significant difference.
be independent prognostic factors for 1-month TFS, we investigated the usefulness of m-qSOFA according to the MELD score. KACLIF and AARC cohorts were divided into three groups with the cut-off values corresponding to the highest Youden index (MELD 23 in KACLIF cohort and MELD 29 in AARC cohort). In the KACLIF cohort, those with high baseline m-qSOFA showed significantly lower 1-month TFS (all $P<0.05$) than those with low baseline m-qSOFA except in the subgroup with MELD 23-29 ($P=0.184$) (Supplementary Fig. 5A–C). The 1-month TFS of the patients who changed from low m-qSOFA at baseline to high on the 7th day was lower than those who shifted from high to low or the patients who remained at high over the 7 days, but similar to that of patients who remained at high (Supplementary Fig. 5D–F). In the AARC cohort, those with high baseline m-qSOFA showed significantly lower 1-month TFS than those with low baseline m-qSOFA in all subgroups (all $P<0.01$) (Supplementary Fig. 6A–C). The 1-month TFS of the subgroups according to the change in m-qSOFA was similar to that of KACLIF (Supplementary Fig. 6D–F). These results were similar in subgroups based on MELD-Na 24 and 34, the cut-off values with the highest Youden index in the KACLIF and AARC cohort (data not shown).

Alcohol-associated liver disease is the most common etiology of underlying CLD accounting for two-thirds in both cohorts. Patients with alcohol-induced CLD and acute insult were divided into two groups based on a Lille score of 0.45. In both the responder group (Lille<0.45) and the non-responder group (Lille$\geq$0.45), those with high m-qSOFA at day 7 showed significantly lower 1-month TFS compared to those with low m-qSOFA ($P=0.006$ and $P<0.001$, respectively, in KACLIF cohort, $P<0.001$ and $P<0.001$, respectively, in AARC cohort) (Supplementary Fig. 7).

### Development of new organ failure

In the KACLIF cohort, the new organ failure development rate was significantly higher in those with a high baseline m-qSOFA score than in those with a low score (23.1% vs. 14.4%, $P=0.009$) (Fig. 4A). The new organ failure development rate was highest in the patients with high m-qSOFA scores at both baseline and on day 7 (33.3%), followed by those with low baseline and high day 7 scores (26.3%), high baseline and low day 7 scores (20.2%), and low base-
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<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
<th>Prognostic score</th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
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<td><strong>KACLiF cohort</strong></td>
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<td><strong>AARC cohort</strong></td>
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<td>Child-Pugh score*</td>
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<td>Child-Pugh score</td>
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<td>Child-Pugh score</td>
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<td>1.460–1.764</td>
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<td>Age</td>
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<td>1.017–1.036</td>
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<td>WBC</td>
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<td>1.013–1.032</td>
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<td>Creatinine</td>
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<td>Lactate†</td>
<td>1.131</td>
<td>1.100–1.163</td>
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<td>MELD score†</td>
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<td>1.298–2.121</td>
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<td>MELD score</td>
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<td>Albumin</td>
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<td>Age</td>
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<td>WBC</td>
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<td>1.006–1.025</td>
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<td>Lactate†</td>
<td>1.141</td>
<td>1.111–1.172</td>
<td>&lt;0.001</td>
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<td>MELD-Na score*</td>
<td>3.101</td>
<td>1.897–5.067</td>
<td>&lt;0.001</td>
<td>MELD-Na score†</td>
<td>1.676</td>
<td>1.312–2.142</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MELD-Na score</td>
<td>1.141</td>
<td>1.109–1.175</td>
<td>&lt;0.001</td>
<td>MELD-Na score</td>
<td>1.105</td>
<td>1.084–1.127</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelet count</td>
<td>0.997</td>
<td>0.993–1.001</td>
<td>0.116</td>
<td>Age</td>
<td>1.028</td>
<td>1.018–1.038</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.561</td>
<td>0.366–0.861</td>
<td>0.008</td>
<td>WBC</td>
<td>1.015</td>
<td>1.006–1.025</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ascites†</td>
<td>1.868</td>
<td>0.873–3.998</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lactate†</td>
<td>1.142</td>
<td>1.112–1.173</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AARC score*</td>
<td>2.22</td>
<td>1.200–4.108</td>
<td>0.011</td>
<td>AARC score†</td>
<td>1.346</td>
<td>1.047–1.729</td>
<td>0.02</td>
</tr>
<tr>
<td>AARC score</td>
<td>1.524</td>
<td>1.311–1.772</td>
<td>&lt;0.001</td>
<td>AARC score</td>
<td>1.489</td>
<td>1.404–1.579</td>
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</tr>
<tr>
<td>AST</td>
<td>1</td>
<td>1.000–1.000</td>
<td>0.001</td>
<td>Age</td>
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<td>1.015–1.035</td>
<td>&lt;0.001</td>
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<tr>
<td>Albumin</td>
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<td>Ascites†</td>
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<td>1.020–4.613</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>WBC</td>
<td>1.015</td>
<td>1.005–1.026</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*Missing data in KACLiF cohort: Child-Pugh score 0.1%, MELD score 0.1%, MELD-Na score 0.27%, and AARC score 41%. †Missing data in AARC cohort: lactate 22.1%, creatinine 0.25%, ascites 5.4%, Child-Pugh score 4.5%, MELD score 0.2%, MELD-Na score 0.6%, and AARC score 22.2%.

HR, hazard ratio; CI, confidence interval; KACLiF, Korean acute-on-chronic liver failure; m-qSOFA, modified quick sequential organ failure assessment; MELD, Model for End-stage Liver Disease; AARC, Asian Pacific Association for the Study of the Liver ACLF Research Consortium.
Figure 2. One-month transplant-free survival rate according to the presence of AARC ACLF and dynamic changes in m-qSOFA. (A) Total patients, (B) AARC ACLF patients, and (C) non-AARC ACLF patients in the KACLiF cohort. (D) Total patients, (E) AARC ACLF patients, and (F) non-AARC ACLF patients in the AARC cohort. m-qSOFA, modified quick sequential organ failure assessment; KACLiF, Korean acute-on-chronic liver failure; ACLF, acute-on-chronic liver failure; AARC, Asian Pacific Association for the Study of the Liver ACLF Research Consortium.
DISCUSSION

The results of our study indicate that patients with high m-qSOFA had a poor 1-month TFS in the KACLIF and AARC cohorts. The m-qSOFA was an independent predictor of short-term mortality in CLD patients with acute deterioration even after considering the CLD-specific prognostic scores. In addition, a change in the m-qSOFA score can also help to identify patients with poor prognosis.

Similar to sepsis, patients with decompensated cirrhosis or ACLF have typical hemodynamic changes called hyperdynamic circulation\(^{22,23}\) and a systemic inflammatory response as the main pathophysiology.\(^{19,24}\) Bacterial infection is common in ACLF patients, as infection is present in approximately one-third of ACLF patients at presentation,\(^{25}\) and infections are important determinants of disease progression and survival. In contrast, bacterial infection could not be detected in a substantial portion of ACLF patients fulfilling the SIRS criteria.\(^{26}\) Therefore, qSOFA, a score for screening sepsis, can be considered to predict adverse outcomes in patients with acutely deteriorated CLD with similar pathophysiology to sepsis regardless of infection.

Previous studies by Piano et al.\(^{11}\) and Augustinho et al.\(^{9}\) reported that qSOFA was effective in predicting high-risk cirrhotic patients in those with infection. However, other studies that included cirrhotic patients without infection showed doubtful results on the predictive ability of qSOFA. Müller et al.\(^{26}\) reported that qSOFA does not predict in-hospital mortality, and Choi et al.\(^{27}\) showed that qSOFA was a significant predictive factor for in-hospital mortality in critically ill patients with cirrhosis but had limited value.

We investigated the usefulness of the m-qSOFA by replacing the GCS with the HE in the qSOFA, which is used in the general population. The West Haven criteria for HE are used more commonly than the GCS to assess cerebral dysfunction in CLD patients. The CLIF-SOFA score for assessing the severity of ACLF was also developed by replacing the GCS with HE grades in the generally used SOFA score, and it provided an improved prediction of prognosis compared to the original SOFA score in critically ill CLD patients\(^{3,16}\) and in cirrhotic patients with infection.\(^{9}\) Likewise, we needed to modify the qSOFA to improve the prediction of prognosis in patients with acutely deteriorated CLD, and it helped to identify those with higher mortality. However, unlike the CLIF-SOFA classifying HE grade 3–4 as organ failure, we used HE grade ≥2 as the m-qSOFA criteria. HE grade 1 is classified as covert HE, which is dif-

Figure 3. One-month transplant-free survival rate according to the presence of EASL-CLIF ACLF and dynamic changes in m-qSOFA in the KACLIF cohort. (A) EASL-CLIF ACLF patients and (B) non-EASL-CLIF ACLF patients. m-qSOFA, modified quick sequential organ failure assessment; EASL-CLIF, European Association for the Study of the Liver-Chronic liver failure; ACLF, acute-on-chronic liver failure; KACLIF, Korean acute-on-chronic liver failure.
ficult to diagnose because there is no definite clinical manifestation, such as disorientation or asterixis. Therefore, it is more reasonable to adopt HE grade 2, which is the criterion for overt HE, as a simple way to evaluate altered mentation at the bedside.

The qSOFA was originally developed as a bedside method of identifying adult patients who are likely to have poor outcomes among those with suspected infection instead of SIRS. However, the usefulness of qSOFA to make an early diagnosis of sepsis has been questioned because of its lower sensitivity compared to SIRS. On the other hand, qSOFA was better than SIRS for the prediction of hospital mortality. Since qSOFA reflects organ failure, such as respiratory and circulatory failure, it may be natural to assume that qSOFA is more effective in predicting poor outcomes. Therefore, it is appropriate to use qSOFA to predict poor outcomes rather than for diagnosing sepsis. In our study, m-qSOFA was an independent factor for 1-month TFS in both cohorts, although not SIRS in the AARC cohort (Tables 2 and 3). The m-qSOFA was able to identify patients with high mortality when combined with ACLF, and the changes in m-qSOFA for 7 days were also useful in predicting poor outcomes, including mortality and the development of new organ failure (Figs. 2–4). The prognostic predictability of m-qSOFA is likely attributed to its variables, which are associated with organ failure in ACLF and reflect disease severity. In addition, m-qSOFA at baseline and day 7 can distinguish patients with high short-term mortality regardless of the liver-specific prognostic score such as MELD, MELD-Na, and the Lille model (Supplementary Figs. 5–7). The advantage of m-qSOFA is that it does not require calculations as with MELD, MELD-Na, or the Lille model and can be obtained with fewer variables than Child-Pugh, AARC, CLIF-SOFA, and CLIF-C OF scores. According to our results, we propose a new algorithm that can distinguish the risk to patients using the presence of baseline ACLF and m-qSOFA and the change in m-qSOFA on day 7 (Fig. 5). According to this algorithm, CLD patients who are hospitalized with acute decompensation can be divided into 3 risk groups at baseline: low, moderate, and high. These patients can be reclassified using the changes in m-qSOFA at 7 days, while the moderate group at baseline cannot be reclassified into the low-risk group even if m-qSOFA is less than 2 at 7 days. If we identify patients with poor outcomes early by using m-qSOFA and perform early interventions in this golden window, we might be able to improve the prognosis of patients with acutely deteriorating CLD.

This study has some limitations. First, the baseline characteristics of the two cohorts are different. The disease severity of the AARC cohort was higher than that of the KACLIF cohort because of the AARC cohort included more ACLF.
patients. Different disease severity leads to different cut-off values of the MELD and MELD-Na scores between the two cohorts. However, since the two cohorts showed similar results on the utility of m-qSOFA, disease severity does not seem to have a significant impact on the usefulness of m-qSOFA. In the future, the validation of m-qSOFA is necessary in patients with various characteristics. Second, this study analyzed the utility of m-qSOFA in the EASL-CLIF ACLF patients in the KACLiF cohort only. There are limitations in analyzing EASL-CLIF ACLF in the AARC cohort because the data collected focuses on AARC ACLF. Therefore, it will be necessary to study the usefulness of m-qSOFA in patients with EASL-CLIF ACLF in other cohorts. Third, the etiology of CLD was predominantly alcohol-associated liver disease in both cohorts, accounting for two-thirds of all cases. Therefore, future analyses need to consider the impact according to different CLD etiologies. Fourth, it is necessary to study whether m-qSOFA, which replaces GCS with HE grades, is better than the original qSOFA in CLD patients with acute decompensation. Fifth, despite its utility, m-qSOFA has a low ability to predict 1-month mortality when used alone compared to other prognostic scores showing a significantly lower area under the receiver operating characteristic curve (Supplementary Table 1). Therefore, m-qSOFA should be used in conjunction with ACLF or another scoring system to increase its predictive ability. However, m-qSOFA has the advantage of easily screening high-risk patients at the bedside because it can be obtained simply with only three variables.

In conclusion, m-qSOFA is an independent factor for predicting short-term mortality in patients with acutely deteriorated CLD, and combining m-qSOFA with the presence of ACLF could identify high-risk patients more accurately regardless of the definition of the ACLF. In addition, using dynamic changes in m-qSOFA scores at 7 days is also useful in identifying high-risk patients at the bedside.

Authors’ contribution

Acknowledgements
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Conflicts of Interest
The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL
Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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develops in patients with acute decompensation of cirrhosis.


Original Article

Conventional and machine learning-based risk scores for patients with early-stage hepatocellular carcinoma

Chun-Ting Ho1, Elise Chia-Hui Tan2, Pei-Chang Lee1,3, Chi-Jen Chu1,3, Yi-Hsiang Huang3,4, Teh-La Huo6, Yu-Hui Su1, Ming-Chih Hou1,3, Jaw-Ching Wu4, and Chien-Wei Su1,3,4,7

1Division of Gastroenterology and Hepatology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan; 2Department of Health Service Administration, College of Public Health, China Medical University, Taichung, Taiwan; 3School of Medicine, College of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan; 4Institute of Clinical Medicine, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan; 5Division of Basic Research, Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan; 6Department of Accounting, Soochow University, Taipei, Taiwan; 7Division of General Medicine, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan

Graphical Abstract

Conventional and machine learning based risk score for patients with early-stage HCC

- Obtain serum biomarker and image features of 1411 patients with early HCC and analyze the training (n=988) cohort
- Cox proportional hazards model
- LASSO COX regression (ML)
- The CATS-IF score 8 factors
  - C: serum albumin < 3.5 g/dL
  - A: Age >= 65
  - T: AFP > 400 ng/mL
  - M: Malignant tumor ≥ 2 cm
  - S: medical treatment not received
  - L: single large HCC
  - I: inflammation (LAP)
  - F: Fibrosis (FIB-4)
- The CATS-INF score 10 factors
  - C: serum albumin < 3.5 g/dL
  - A: Age >= 65
  - T: AFP > 400 ng/mL
  - M: Malignant tumor ≥ 2 cm
  - S: medical treatment not received
  - L: single large HCC
  - I: inflammation (LAP)
  - N: fibrosis (FIB-4)
  - F: Fibrosis (FIB-4)

Validate the performance in validating (n=423) cohort

Conclusion:
Both the conventional Cox-based CATS-IF score and ML-based CATS-INF score effectively stratified patients with early-stage HCC into distinct prognostic groups and outperformed other modern risk scores, with the CATS-INF score showing slightly superior performance.

Study Highlights
- Question: Does a ML-based approach exhibit superior performance in predicting the outcomes of patients with early-stage HCC compared to the conventional Cox proportional hazards model?
- Findings: In this cohort study of 1,411 patients with early-stage HCC, both the Cox-based CATS-IF score and ML-based CATS-INF score demonstrated superior predictive performance for the overall survival compared to other prognostic scores. Notably, the CATS-INF score exhibited the lowest Akaike information criterion value.
- Meaning: These findings suggest that the ML-based CATS-INF score could stratify patients with early-stage HCC into distinct prognostic groups.
INTRODUCTION

Primary liver cancer is the seventh most frequently occurring cancer and the third leading cause of cancer mortality in the world.\(^1\) Hepatocellular carcinoma (HCC) accounts for approximately 75–90% of primary liver cancers.\(^2\) HCC typically develops over an extended period of time, often in the setting of advanced chronic liver diseases (ACLDs), such as chronic hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, alcoholism, and metabolic dysfunction-associated steatotic liver disease.\(^3\) The outcomes of HCC patients have improved through...
advances in the surveillance of high-risk patients, antiviral therapy for chronic HBV or HCV infection, surgical and local regional therapy, and the introduction of systemic therapy. One survey from the United States showed that the 5-year overall survival (OS) rates of patients with HCC increased from 3% between 1975 and 1977 to 18% between 2004 and 2010. Nevertheless, there is still room to improve.

The Barcelona Clinic Liver Cancer (BCLC) clinical algorithm is the most commonly applied system for staging and patient stratification and plays an important role in clinical decision-making, especially for the management of HCC. The BCLC algorithm mainly focuses on tumor burden, liver functional reserve, performance status, vascular invasion, and extrahepatic spread. Early stage HCC vs early-stage HCC (BCLC stage 0-A) are defined by the presence of either a single tumor irrespective of size or 2 to 3 tumors less than 3 cm in size, well-preserved liver function, good performance, and a lack of vascular invasion, extra-hepatic metastasis, and cancer-related symptoms. Patients with BCLC stage 0-A HCC usually have better outcomes compared to those with intermediate or advanced stage HCC, and the median OS time is more than 5 years. However, the clinical manifestations and outcomes of patients in stage 0-A HCC are not the same. To improve the prognoses of early stage HCC vs early-stage HCC patients, it is warranted to identify the risk factors associated with the outcomes and to adopt individual strategies for treatment and follow-up.

Machine learning (ML) has been described as a “marriage between mathematics and computer science” and has been emerging as a promising method for biomarker selection and prognostic models. Prognostic models based on ML have been developed for several different diseases, such as acute stroke, depression, and malignancy. However, ML-based models to predict the outcomes of patients with early-stage HCC have not been widely studied. Some discrepancies between conventional and ML-based methods have also been reported in several studies. Therefore, we aimed to develop risk scores using ML and conventional methods to stratify patients with early-stage HCC into distinct prognostic groups.

MATERIALS AND METHODS

Data source and study population

This retrospective cohort study analyzed patient-level data from the HCC registration system at Taipei Veterans General Hospital (TVGH), a major medical center in northern Taiwan. TVGH is a medical center located in northern Taiwan, boasting 3,160 beds. It handles a substantial volume of patient care in Taiwan, with approximately 8,000 outpatients visit per day. This registration system prospectively collects comprehensive data on demographics, etiology, baseline laboratory information, tumor factors, treatments, and outcomes for newly diagnosed HCC patients and has been used in previous studies. The diagnosis of HCC of the registration system was based on diagnostic criteria.

![Figure 1. Study flow chart. HCC, hepatocellular carcinoma; TPEVGH, Taipei Veterans General Hospital; BCLC, Barcelona Clinic Liver Cancer classification.](https://doi.org/10.3350/cmh.2024.0103)
criteria of the American Association for the Study of Liver Disease (AASLD). The study involved 3,832 consecutive treatment-naïve HCC patients from 2012 to 2021 (Fig. 1). Exclusions were made for patients with advanced HCC stages (BCLC stage B to D), missing initial serum biomarker data or diagnostic imaging, or lost to follow-up post-diagnosis. A total of 1,411 patients with BCLC stage 0–A HCC were enrolled. The index date was defined as the date on which HCC was diagnosed. The study assigned 70% of these patients randomly to the training cohort and the remaining 30% to an external validation cohort.

Curative treatments included liver transplantation, surgical resection, and local ablation therapy. Trans-arterial chemoembolization (TACE), molecular target therapy, immune checkpoint inhibitors, radiotherapy, chemotherapy, and best supportive treatment were considered non-curable treatment modalities. Patients were followed until death, loss of follow-up, or the end of the study (June 30, 2022). This study adhered to the Declaration of Helsinki and received approval from the Institutional Review Board (IRB) of Taipei Veterans General Hospital, Taiwan (IRB number: 2022-07-007BC). Informed consent was waived by the IRB since this was a retrospective observational cohort study, and patient information was de-identified before the study commenced.

Outcome measurement

The vital status of each patient was collected from the electronic health record and linked to the registry data. The primary outcome was OS, which was calculated from the index date to the date of death or the last date of follow-up.

Construction and validation of prediction model and risk score

To construct a novel prediction model, the study initially utilized univariate Cox proportional hazard models to identify significant variables for OS. These variables were then integrated into a multivariable Cox model, refined through stepwise selection based on the Akaike Information Criterion (AIC), and verified by residual analysis. The detailed methods for model construction and validation are provided in Supplementary Material.

For each prognostic factor, a Cox-based risk score was calculated and standardized on a 0–100 scale. The cumulative risk score for each patient, derived from the sum of individual factor scores, was segmented into high, medium, and low-risk categories using the 33rd and 66th percentiles for effective risk stratification.

We employed the least absolute shrinkage and selection operator (LASSO) method to overcome the common challenges of multicollinearity and overfitting in complex models. This led to the creation of a ML-based risk score utilizing the variables retained in the LASSO-based Cox model. The effectiveness of this ML-based risk score, compared to a standard Cox regression model, was then evaluated.

Validations of Cox-based and ML-based risk scores were conducted in training and validation cohorts. This encompassed a three-step process: assessing predictive performance for OS through comparisons of homogeneity, AIC, and AUROC; generating calibration plots for predicted versus observed survival; and implementing time-dependent ROC curve analysis to evaluate prognostic performance at 1, 2, 3, and 5 years post-diagnosis, acknowledging the dynamic nature of disease status and survival time.

Statistical analysis

The baseline characteristics, including demographics, treatments, tumor factors, viral hepatitis status, and HCC-related biomarkers, were collected. The ROCs and the Youden index were utilized to determine the optimal cutoff values for non-invasive serum marker scores in predicting the risk of mortality in patients with early-stage HCC.

Continuous variables were presented as the median and interquartile range (IQR) and compared using the Mann–Whitney U test. Categorical variables were expressed as frequencies and percentages and compared using the chi-squared test or Fisher’s exact test. Cumulative OS rates were estimated using the Kaplan-Meier method and compared using the Cox proportional hazards model. All statistical analyses were conducted using SPSS version 24.0 (IBM Corp., Armonk, NY, USA) and R software (version 4.2.3) (R Foundation for Statistical Computing, Vienna, Austria). SPSS was employed for the forward stepwise Cox regression, while R and the “glmnet” package were used for the LASSO Cox regression and subsequent plots, and the “timeROC” package was used to generate the time-dependent ROC curves. A two-tailed P-value of <0.05 was
considered statistically significant.

**RESULTS**

**Basic characteristics**

Among the 1,411 patients with BCLC stage 0-A HCC, 1276 underwent curative treatments: 830 had surgical resection, 5 underwent liver transplantation, 425 received radiofrequency ablation therapy, and 16 were treated with percutaneous ethanol injection therapy. Of the 135 patients who received non-curative therapy, 116 underwent TACE, 17 received radiotherapy, and 2 received best supportive care. The study divided these patients into two cohorts: 988 patients in the training cohort and 423 in the validation cohort. The clinical characteristics of patients are shown in Table 1. In both cohorts, males were the majority, and most of the etiologies were HBV or HCV-related HCC.

**Biomarker selection**

After a median follow-up of 38.0 months (IQR 18.0–57.0 months), 368 patients died, and the 5-year OS rate was 67.5%. All available clinical variables, including the clinicopathological features and serum biomarkers in Table 1, were subjected to stepwise Cox regression and LASSO Cox regression. As shown in Table 2, the multivariate analysis by conventional Cox regression showed that OS correlated with age, treatment modalities, single large (>5 cm) HCC (SLHCC), serum creatinine levels, fibrosis-4 (FIB-4), lymphocyte-to-monocyte ratio (LMR), albumin-bilirubin (ALBI) grade, and alpha-fetoprotein (AFP) levels. The risk factors associated with OS by the LASSO Cox regression

<table>
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<th>Characteristic</th>
<th>Training cohort (n=988)</th>
<th>Validating cohort (n=423)</th>
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<td>Age</td>
<td>66.0 (59.0–74.0)</td>
<td>68.0 (59.0–75.0)</td>
<td>0.676</td>
</tr>
<tr>
<td>Sex, male (%)</td>
<td>680 (68.8)</td>
<td>313 (74.0)</td>
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</tr>
<tr>
<td>Curative Tx, yes (%)</td>
<td>891 (90.2)</td>
<td>385 (91.0)</td>
<td>0.762</td>
</tr>
<tr>
<td>Tumor number, single (%)</td>
<td>889 (90)</td>
<td>370 (87.5)</td>
<td>0.164</td>
</tr>
<tr>
<td>Tumor size, &gt;3cm (%)</td>
<td>392 (39.7)</td>
<td>167 (39.5)</td>
<td>0.945</td>
</tr>
<tr>
<td>HBsAg, + (%)</td>
<td>508 (59.4)</td>
<td>215 (59.7)</td>
<td>0.921</td>
</tr>
<tr>
<td>Anti-HCV, + (%)</td>
<td>263 (32.0)</td>
<td>111 (32.0)</td>
<td>0.991</td>
</tr>
<tr>
<td>SLHCC, yes/no (%)</td>
<td>155/833 (15.7)</td>
<td>64/359 (15.1)</td>
<td>0.791</td>
</tr>
<tr>
<td>Platelet (/mm$^3$)</td>
<td>152,000 (105,000–198,000)</td>
<td>148,000 (106,000–198,000)</td>
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<tr>
<td>Albumin (g/dL)</td>
<td>4.0 (3.7–4.3)</td>
<td>4.0 (3.7–4.2)</td>
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<tr>
<td>Creatinine (mg/dL)</td>
<td>0.89 (0.76–1.09)</td>
<td>0.90 (0.76–1.14)</td>
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</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.71 (0.50–1.00)</td>
<td>0.70 (0.51–0.99)</td>
<td>0.331</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>32.0 (21.0–50.0)</td>
<td>31.0 (20.0–48.0)</td>
<td>0.653</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>33.0 (24.0–50.0)</td>
<td>34.0 (24.0–54.0)</td>
<td>0.703</td>
</tr>
<tr>
<td>LMR</td>
<td>3.19 (2.40–4.15)</td>
<td>3.08 (2.27–3.98)</td>
<td>0.033</td>
</tr>
<tr>
<td>PNI</td>
<td>47.9 (43.8–51.4)</td>
<td>47.1 (43.2–51.2)</td>
<td>0.117</td>
</tr>
<tr>
<td>FIB-4</td>
<td>2.79 (1.75–4.64)</td>
<td>2.91 (1.79–4.80)</td>
<td>0.253</td>
</tr>
<tr>
<td>ALBI, 1/2 or 3 (%)</td>
<td>564/424 (57.1/42.9)</td>
<td>247/176 (58.4/41.6)</td>
<td>0.649</td>
</tr>
<tr>
<td>AFP (ng/mL)</td>
<td>10.1 (3.5–81.8)</td>
<td>9.68 (3.44–74.6)</td>
<td>0.532</td>
</tr>
</tbody>
</table>

Continuous variables are expressed as the median with the 25th and 75th percentiles.

HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; SLHCC, single large hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LMR, lymphocyte-to-monocyte ratio; PNI, prognostic nutritional index; FIB-4, fibrosis-4 index; ALBI, albumin–bilirubin; AFP, alpha fetoprotein.
Table 2. Univariate and multivariate Cox regression and LASSO Cox regression of selected biomarkers to predict overall survival in the training cohort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
<th>LASSO Cox Reg.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P-value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;65</td>
<td>2.001 (1.538–2.604)</td>
<td>&lt;0.001</td>
<td>1.532 (1.148–2.043)</td>
</tr>
<tr>
<td>≤65</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.065 (0.814–1.392)</td>
<td>0.647</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curative Tx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>No</td>
<td>3.223 (2.365–4.390)</td>
<td>&lt;0.001</td>
<td>1.823 (1.303–2.550)</td>
</tr>
<tr>
<td>Tumor No.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>1.284 (0.890–1.852)</td>
<td>0.182</td>
<td></td>
</tr>
<tr>
<td>Max. Size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>1.304 (1.015–1.676)</td>
<td>0.038</td>
<td>0.983 (0.711–1.361)</td>
</tr>
<tr>
<td>≤3</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>SLHCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.842 (1.359–2.497)</td>
<td>&lt;0.001</td>
<td>2.291 (1.538–3.413)</td>
</tr>
<tr>
<td>No</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Platelet (/uL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100,000</td>
<td>1.841 (1.409–2.405)</td>
<td>&lt;0.001</td>
<td>1.047 (0.743–1.475)</td>
</tr>
<tr>
<td>≥100,000</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3.5</td>
<td>2.330 (1.714–3.166)</td>
<td>&lt;0.001</td>
<td>1.204 (0.832–1.744)</td>
</tr>
<tr>
<td>≥3.5</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.2</td>
<td>1.824 (1.372–2.425)</td>
<td>&lt;0.001</td>
<td>1.437 (1.062–1.945)</td>
</tr>
<tr>
<td>≤1.2</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1</td>
<td>1.309 (0.995–1.723)</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>≤1</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;40</td>
<td>1.493 (1.162–1.919)</td>
<td>0.002</td>
<td>1.030 (0.740–1.434)</td>
</tr>
<tr>
<td>≤40</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;45</td>
<td>2.346 (1.828–3.010)</td>
<td>&lt;0.001</td>
<td>1.210 (0.834–1.730)</td>
</tr>
<tr>
<td>≤45</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>FIB-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;3.25</td>
<td>2.624 (2.032–3.389)</td>
<td>&lt;0.001</td>
<td>1.685 (1.178–2.410)</td>
</tr>
<tr>
<td>≤3.25</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>
included the above eight factors and two additional ones: aspartate aminotransferase (AST) and prognostic nutritional index (PNI) (Fig. 2).

**Development of the risk scores**

The \(\beta\)-coefficients in the Cox regression for each selected factor were simplified based on their ratios, resulting in a user-friendly and clinically applicable score named the CATS-IF score (abbreviated from the contributing factors, as outlined in Table 3). Simultaneously, variables demonstrating significance in the ML-based LASSO Cox regression were identified to develop our ML-based risk score. The \(\beta\)-coefficients in the multivariate Cox regression for each selected factor in LASSO COX regression were similarly simplified based on their ratios. The coefficients be-

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
<th>LASSO Cox Reg.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>(P)-value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>LMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3.62</td>
<td>1.890 (1.432–2.494)</td>
<td>&lt;0.001</td>
<td>1.446 (1.066–1.960)</td>
</tr>
<tr>
<td>(\geq3.62)</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>PNI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45</td>
<td>2.632 (2.049–3.383)</td>
<td>&lt;0.001</td>
<td>1.102 (0.766–1.586)</td>
</tr>
<tr>
<td>(\geq45)</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>ALBI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2 or 3</td>
<td>2.459 (1.902–3.178)</td>
<td>&lt;0.001</td>
<td>1.548 (1.103–2.174)</td>
</tr>
<tr>
<td>AFP (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>1.780 (1.387–2.284)</td>
<td>&lt;0.001</td>
<td>1.578 (1.206–2.064)</td>
</tr>
<tr>
<td>(\leq20)</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

HBSAg, hepatitis B surface antigen; HCV, hepatitis C virus; SLHCC, single large hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LMR, lymphocyte-to-monocyte ratio; PNI, prognostic nutritional index; FIB-4, fibrosis-4 index; ALBI, albumin-bilirubin; AFP, alpha fetoprotein; HR, hazard ratio; CI, confidence interval.
Chun-Ting Ho, et al.  
ML-based risk score for HCC  
https://doi.org/10.3350/cmh.2024.0103

Table 3. Parameter, coefficient, and formula of the conventional Cox-based CATS-IF score and ML-based CATS-INF score from the training cohort

### Conventional Cox-Based CATS-IF score

<table>
<thead>
<tr>
<th>If fulfill=1, else=0</th>
<th>β-coefficient</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt;65 years</td>
<td>0.419</td>
<td>53</td>
</tr>
<tr>
<td>No curative treatment received</td>
<td>0.592</td>
<td>75</td>
</tr>
<tr>
<td>SLHCC</td>
<td>0.788</td>
<td>100</td>
</tr>
<tr>
<td>Serum creatinine &gt;1.2 mg/dL</td>
<td>0.359</td>
<td>46</td>
</tr>
<tr>
<td>FIB-4 &gt;3.25</td>
<td>0.559</td>
<td>71</td>
</tr>
<tr>
<td>LMR &lt;3.62</td>
<td>0.350</td>
<td>44</td>
</tr>
<tr>
<td>ALBI grade 2 or 3</td>
<td>0.468</td>
<td>59</td>
</tr>
<tr>
<td>AFP &gt;20 ng/mL</td>
<td>0.442</td>
<td>56</td>
</tr>
</tbody>
</table>

SLHCC, single large hepatocellular carcinoma; FIB-4, fibrosis-4 index; LMR, lymphocyte-to-monocyte ratio; ALBI, albumin–bilirubin; AFP, alpha fetoprotein.

**Formula:**

\[
\text{CATS-IF score} = 53 \times (\text{Age >65 years}) + 75 \times (\text{No curative treatment received}) + 100 \times \text{SLHCC} + 46 \times (\text{Serum creatinine >1.2 mg/dL}) + 71 \times (\text{FIB-4 >3.25}) + 44 \times (\text{LMR <3.62}) + 59 \times (\text{ALBI grade 2 or 3}) + 56 \times (\text{AFP >20 ng/mL})
\]

between these two models were compared and the results showed high consistency (Supplementary Fig. 1). Both the Cox-based CATS-IF score and ML-based CATS-INF score exhibited good predictive capability for OS in the training cohort, with respective AUC values of 0.723 and 0.729. To stratify patients into risk groups, we sorted them based on their CATS-IF score and CATS-INF score, establishing cut-offs for high, intermediate, and low-risk groups at the 33rd and 67th percentiles of the patients’ scores. The Kaplan–Meier plots depicting the survival outcomes in the training cohort are presented in Figure 3A and Figure 3B for the CATS-IF score and CATS-INF score, respectively.

Table 3. Continued

### ML-based CATS-INF score

<table>
<thead>
<tr>
<th>If fulfill=1, else=0</th>
<th>β-coefficient</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt;65 years</td>
<td>0.419</td>
<td>53</td>
</tr>
<tr>
<td>No curative treatment received</td>
<td>0.592</td>
<td>75</td>
</tr>
<tr>
<td>SLHCC</td>
<td>0.788</td>
<td>100</td>
</tr>
<tr>
<td>Serum creatinine &gt;1.2 mg/dL</td>
<td>0.359</td>
<td>46</td>
</tr>
<tr>
<td>AST &gt;45 IU/L</td>
<td>0.216</td>
<td>27</td>
</tr>
<tr>
<td>FIB-4 &gt;3.25</td>
<td>0.559</td>
<td>71</td>
</tr>
<tr>
<td>LMR &lt;3.62</td>
<td>0.350</td>
<td>44</td>
</tr>
<tr>
<td>PNI &lt;45</td>
<td>0.147</td>
<td>19</td>
</tr>
<tr>
<td>ALBI grade 2 or 3</td>
<td>0.468</td>
<td>59</td>
</tr>
<tr>
<td>AFP &gt;20 ng/mL</td>
<td>0.442</td>
<td>56</td>
</tr>
</tbody>
</table>

SLHCC, single large hepatocellular carcinoma; FIB-4, fibrosis-4 index; LMR, lymphocyte-to-monocyte ratio; PNI, prognostic nutritional index; ALBI, albumin–bilirubin; AFP, alpha fetoprotein.

**Formula:**

\[
\text{CATS-INF score} = 53 \times (\text{Age >65 years}) + 75 \times (\text{No curative treatment received}) + 100 \times \text{SLHCC} + 46 \times (\text{Serum creatinine >1.2 mg/dL}) + 27 \times (\text{Serum AST >45 IU/L}) + 71 \times (\text{FIB-4 >3.25}) + 44 \times (\text{LMR <3.62}) + 19 \times (\text{PNI <45}) + 59 \times (\text{ALBI grade 2 or 3}) + 56 \times (\text{AFP >20 ng/mL})
\]

Calibration, validation and performance of the Cox-based CATS-IF and ML-based CATS-INF score

To evaluate the predictive efficacy of both the Cox-based CATS-IF score and ML-based CATS-INF score for OS, we calculated these scores in the validation cohort and stratified patients into high, intermediate, and low risk groups. In the validation cohort, both the CATS-IF score and CATS-INF score exhibited well-predictive capabilities for OS (AUC=0.695 and 0.707, respectively) and accurately strati-
fied patients into low, intermediate, and high-risk groups. The 5-year OS rates in these groups stratified by CATS-IF were 81.8%, 62.8%, and 43.3%, while groups stratified by CATS-INF were 83.5%, 62.0%, and 42.9%, respectively (Fig. 3C and 3D, \( P<0.001 \)).

We subsequently generated calibration plots by plotting observed and predicted probabilities, stratified by 10 percentiles of the predicted probability from both CATS-IF score and CATS-INF score (Supplementary Fig. 2A and 2B). The calibration plot matched well with the ideal 45-degree line and showed good correlation between predicted and observed outcomes. We then compared the ability of the modern prognostic scores for HCC. As shown in Table 4, the CATS-INF score and CATS-IF scores had better pre-

**Figure 3.** (A) Kaplan–Meier survival analysis of training cohort according to the conventional Cox-based CATS-IF score (low risk: 0–102, intermediate risk: 103–211, high risk: 212+). (B) Kaplan–Meier survival analysis of training cohort according to the ML-based CATS-INF score (low risk: 0–114, intermediate risk: 104–223, high risk: 224+). (C) Kaplan–Meier survival analysis of the validation cohort according to the conventional Cox-based CATS-IF score (low risk: 0–102, intermediate risk: 103–211, high risk: 212+). (D) Kaplan–Meier survival analysis of the validation cohort according to the ML-based CATS-INF score (low risk: 0–102, intermediate risk: 104–211, high risk: 212+). ML, machine learning.
dictive capability when compared to the ALBI score, AST-to-platelet ratio index (APRI), LMR, PNI, FIB-4, and the model for end-stage liver disease (MELD) score. Notably, the ML-based CATS-INF score exhibited the lowest AIC value.

The time-dependent ROC curves were generated for the Cox-based CATS-IF and ML-based CATS-INF scores (Fig. 4). While the two scores had similar performance in predicting OS within the first year after diagnosis (AUROC 0.672 vs. 0.672), the ML-based CATS-INF score exhibited enhanced predictive accuracy during the second, third, and fifth years post-diagnosis compared to the conventional Cox-based CAT-IF score (AUROC 0.722 vs. 0.712, 0.712 vs. 0.704, and 0.704 vs. 0.690, respectively).

<table>
<thead>
<tr>
<th>Score</th>
<th>Homogeneity</th>
<th>AUC</th>
<th>AIC</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATS-INF</td>
<td>47.656</td>
<td>0.707</td>
<td>1259.825</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CATS-IF</td>
<td>43.398</td>
<td>0.695</td>
<td>1264.421</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PNI</td>
<td>33.202</td>
<td>0.672</td>
<td>1275.337</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALBI score</td>
<td>30.364</td>
<td>0.670</td>
<td>1278.611</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LMR</td>
<td>11.850</td>
<td>0.586</td>
<td>1292.475</td>
<td>0.001</td>
</tr>
<tr>
<td>MELD score</td>
<td>15.430</td>
<td>0.654</td>
<td>1296.598</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>APRI</td>
<td>0.069</td>
<td>0.583</td>
<td>1307.147</td>
<td>0.793</td>
</tr>
<tr>
<td>FIB-4 score</td>
<td>0.089</td>
<td>0.604</td>
<td>1307.182</td>
<td>0.680</td>
</tr>
</tbody>
</table>

PNI, prognostic nutritional index; ALBI, albumin–bilirubin; LMR, lymphocyte-to-monocyte ratio; MELD, model for end-stage liver disease; AST, aspartate aminotransferase; APRI, AST to platelet ratio index; FIB-4, fibrosis-4 index.

**Figure 4.** (A) Time-dependent ROC curve of Cox-based CATS-IF score. (B) Time-dependent ROC curve of ML-based CATS-INF score. (C) Consecutive comparison of time-dependent area under the ROC curve (AUROC) of the Cox-based CATS-IF score and ML-based CATS-INF score. Both scores showed good predictability and were equally competitive, while ML-based CATS-INF score had slightly greater AUC in the long term (≥2 years). ML, machine learning.
DISCUSSION

In this study, we showed that age, SLHCC, serum creatinine and AFP levels, FIB-4, LMR, ALBI grade, and treatment modalities are crucial risk factors for determining OS in patients with HCC in BCLC stage 0 or A. Both the CATS-INF and CATS-IF scores had excellent ability in prognosis prediction and risk stratification of the patients. It was validated by the validation cohort, and the calibration plot was well matched. Moreover, it outperformed currently available prognostic scores. Hence, the novel CATS-INF and CATS-IF scores could provide individual risk identification for patients with early-stage HCC and could benefit patients with HCC by allowing for more individualized medical services and treatment plans.

The factors that determine the prognoses of patients with HCC include tumor factors, field factors in the background liver (including the grade of inflammation and steatosis and the stage of fibrosis), and treatment factors. For patients with early-stage HCC, the impact of tumor factors might be less critical, while the field factors play a more important role in determining the outcomes of patients.

Studies have shown that systemic inflammation can promote cancer growth, invasion, and metastasis in patients with malignant tumors. Leukocytes play an important role in the immune response. Lymphocytes participate in cytotoxic cell death and inhibition of tumor-cell proliferation and migration. Conversely, monocytes can promote tumor progression and metastasis. As a result, the LMR has been used as a serum biomarker to predict the prognosis of several cancers and has shown good predictive capability of prognosis for patients with HCC in previous studies. Our research revealed that low LMR is related to poorer OS in patients with early-stage HCC, indicating that inflammation was an important factor in the outcomes of patients.

Besides inflammation status, fibrosis also plays a crucial role in hepatic carcinogenesis as well as deteriorating liver function. The FIB-4 score combines standard biochemical values (platelets, alanine aminotransferase, and AST) and age and has been recognized as an accurate, convenient, and non-invasive serum marker for evaluating the status of liver fibrosis for patients with ACLD. We demonstrated that patients with high FIB-4 scores had poor OS compared to their counterparts, suggesting that fibrosis was also an important prognostic factor.

Moreover, the significance of nutritional status in predicting the outcomes of patients has been explored in diverse malignancies, including HCC. It was postulated that a better nutritional function indicated a better body reserve against disease burden and could promote the immune response to combat malignancies. By incorporating the PNI, a well-validated marker for assessing a patient’s nutritional status, into the risk scores, we could gain additional insights into their nutritional well-being. This inclusion enabled a more comprehensive understanding of a patient’s prognosis, considering the role of nutritional status as a contributing factor to OS.

The ALBI score has been widely validated as a reliable tool for evaluating liver functional reserve, as well as predicting the prognosis of patients with HCC. In the current study, we confirmed that the ALBI grade was an independent factor in the outcomes of patients with early-stage HCC. Taken together, our results revealed the importance of inflammatory status, fibrosis status, and liver functional reserve in the survival of patients with early-stage HCC. The prognostic model that we developed based on the results could serve as a convenient, accessible, and economical way to predict patients’ outcomes and facilitate management of patients individually.

The categorization of SLHCC as BCLC stage A or B has been controversial in some studies, especially in Asia. In the 2022 updated BCLC strategy for HCC, SLHCC was classified as BCLC stage A. However, our previous study showed that patients who had SLHCC had a 5-year OS rate of 42.6%, which showed significant differences compared to those in BCLC stage A (57.0%) and stage B (27.3%) HCC. Therefore, we proposed that SLHCC might be a distinctive stage between A and B. In the current study, SLHCC was an independent risk factor associated with poorer OS among patients with HCC and BCLC stage 0-A and was a component of the CATS-IF score and CATS-INF score. Consequently, it is suggested that patients with SLHCC should be closely followed up or given adjuvant systemic therapy because they have a higher risk of mortality. More prospective studies are warranted to elucidate this issue.

For patients with HCC in BCLC stage 0-A, curative treatment modalities are recommended as front-line therapy. In our study, around 90% of patients received curative treat-
ments. Moreover, non-curative treatment was an independent risk factor associated with poorer OS. This indicates that curative treatment modalities should be performed for patients with early-stage HCC if there are no contraindications.

There were some disparities in the significance of our variables when comparing the conventional Cox regression model with the ML-based LASSO Cox regression model. Notably, serum AST levels and PNI were independent risk factors for OS in the ML model but not in the conventional model. Cox logistic regression has traditionally been a stalwart in survival analysis due to its accuracy in identifying factors influencing survival. However, its precision can be compromised when variables interact with each other. In our study, both the conventional Cox regression model and ML-based methods demonstrated superior performance in predicting the prognoses of early-stage HCC patients compared to other current prognostic scores. It is noteworthy that the ML-based CATS-INF score, with its lowest AIC value, particularly excelled in long-term prognostication (Fig. 4). The results highlighted the impact of nutritional status on the outcomes of patients with early-stage HCC. Furthermore, they validated the potential of ML-based methods, such as LASSO Cox regression, as valuable complements to traditional analytic approaches and promising tools for future model development.

Despite the good performance of the CATS-IF score and CATS-INF score, there are still several limitations that need to be addressed. First, this study was a single-center retrospective cohort study, and further validation with prospective cohorts will be needed to confirm the predictive capability for prognosis. Second, the model was developed based on a limited database, so further research on possible prognostic factors and biomarkers will be required to predict the outcome of patients with HCC. Third, most of the patients in our study cohort had viral HCC, and further study is needed to determine whether etiologies of HCC interfere with the result. Fourth, microvascular invasion (MVI) is a critical factor to determine OS and recurrence for patients with early-stage HCC who underwent surgical resection. However, it is important to note that the diagnosis of MVI relies on pathological examination. In our study, approximately 40% of the patients received non-surgical treatments, which limited the availability of detailed pathological specimens necessary for determining MVI status. Consequently, we were unable to adequately incorporate MVI into our analysis. Fifth, in our study, the treatment modality emerged as a significant parameter in determining the OS of patients with early-stage HCC. The results were consistent with previous studies. However, the proportion of patients receiving non-curative treatment modalities was relatively small, potentially impacting the significance of our analysis. Lastly, some of the biomarkers involved in this study still lack universal acknowledgement of cutoff values and clinical applicability. Hence, further studies with larger scale and more detailed information are needed.

The CATS-IF score developed by conventional Cox regression and the CATS-INF score developed by ML-based methods both showed excellent prognostic ability in early HCC patients, while the ML-based CATS-INF score showed slightly superior performance, especially in the long-term follow-up.

**Author’s contribution**

Tan ECH and Su CW had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: Tan ECH, Lee PC, Su CW. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: Ho CT, Tan ECH, Su CW. Critical review of the manuscript for important intellectual content: Chu CJ, Huang YH, Huo TI, Hou MC, Wu JC. Statistical analysis: Ho CT, Tan ECH, Su YH. Administrative, technical, or material support: Ni. Supervision: Tan ECH, Su CW.

All authors approved the final version of the article, including the authorship list.

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A part of this study was presented as a poster exhibition at the annual meeting of the annual meeting of the American Association for the Study of the Liver, Boston, USA.
November 10-14, 2023. It has been identified as a “Poster of Distinction.”

Writing assistance: American Manuscript Editors.

Conflicts of Interest


SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES


Ischemia-free liver transplantation improves the prognosis of recipients using functionally marginal liver grafts

Shuai Wang1,2,3,*, Xiaohong Lin4,*, Yunhua Tang1,2,3, Yichen Liang1,2,3, Min Zhang1,2,3, Zhonghao Xie1,2,3, Yiweng Guo1,2,3, Yuqi Dong1,2,3, Qiang Zhao1,2,3, Zhiyong Guo1,2,3, Dongping Wang1,2,3, Xiaoshun He1,2,3, Yiwen Guo1,2,3, Yuqi Dong1,2,3, Qiang Zhao1,2,3, Zhiyong Guo1,2,3, Dongping Wang1,2,3, Xiaoshun He1,2,3, and Maogen Chen1,2,3

1Organ Transplant Center, First Affiliated Hospital of Sun Yat-sen University; 2Guangdong Provincial Key Laboratory of Organ Donation and Transplant Immunology; 3Guangdong Provincial International Cooperation Base of Science and Technology (Organ Transplantation); 4Department of Thyroid and Breast Surgery, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, People’s Republic of China

Graphical Abstract

Study Highlights
- It was challenging to balance the benefits and drawbacks of marginal livers in liver transplantation. Functionally marginal liver grafts were associated with worse prognosis than other marginal livers. Ischemia-free liver transplantation can significantly alleviate liver injury via inhibiting the infiltration of NK cells and pyroptosis level, which contributed to a better clinical benefit. This provided us a novel direction when addressing the marginal liver issue.
**Background/Aims:** The shortage of donor liver hinders the development of liver transplantation. This study aimed to clarify the poor outcomes of functionally marginal liver grafts (FMLs) and provide evidence for the improvement of ischemia-free liver transplantation (IFLT) after FML transplantation.

**Methods:** Propensity score matching was used to control for confounding factors. The outcomes of the control group and FML group were compared to demonstrate the negative impact of FMLs on liver transplantation patients. We compared the clinical improvements of the different surgical types. To elucidate the underlying mechanism, we conducted bioinformatic analysis based on transcriptome and single-cell profiles.

**Results:** FMLs had a significantly greater hazard ratio (HR: 1.969, \(P=0.018\)) than did other marginal livers. A worse 90-day survival (Mortality: 12.3% vs. 5.0%, \(P=0.007\)) was observed in patients who underwent FML transplantation. Patients who received FMLs had a significant improvement in overall survival after IFLT (Mortality: 10.4% vs 31.3%, \(P=0.006\)). Pyroptosis and inflammation were inhibited in patients who underwent IFLT. The infiltration of natural killer cells was lower in liver grafts from these patients. Bulk transcriptome profiles revealed a positive relationship between IL-32 and Caspase 1 \((R=0.73, P=0.01)\) and between IL-32 and Gasdermin D \((R=0.84, P=0.0012)\).

**Conclusions:** FML is a more important negative prognostic parameter than other marginal liver parameters. IFLT might ameliorate liver injury in FMLs by inhibiting the infiltration of NK cells, consequently leading to the abortion of IL-32, which drives pyroptosis in monocytes and macrophages.  

**Keywords:** Marginal liver grafts; Liver transplantation; Ischemia-free liver transplantation; Static cold storage; Normothermic machine perfusion; Transplantation immunology

**Corresponding author:** Maogen Chen
Organ Transplant Center, The First Affiliated Hospital, Sun Yat-sen University, No. 58 Zhongshan Er Road, Guangzhou 510080, China
Tel: +86-020-87306082, Fax: +86-020-87306082, E-mail: chenmg3@mail.sysu.edu.cn
https://orcid.org/0000-0001-9319-5334

Weiqiang Ju
Organ Transplant Center, The First Affiliated Hospital, Sun Yat-sen University, No. 58 Zhongshan Er Road, Guangzhou 510080, China
Tel: +86-020-87306082, Fax: +86-020-87306082, E-mail: weiqiangju@163.com
https://orcid.org/0000-0002-7544-8745

Xiaoshun He
Organ Transplant Center, The First Affiliated Hospital, Sun Yat-sen University, No. 58 Zhongshan Er Road, Guangzhou 510080, China
Tel: +86-020-87306082, Fax: +86-020-87306082, E-mail: gdtrc@163.com
https://orcid.org/0000-0001-5317-9612

*These authors share first authorship.

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**Abbreviations:**
FMLs, functionally marginal liver grafts; IFLT, ischemia-free liver transplantation; PSM, propensity score matching; SCS, static cold storage; NMP, normothermic machine perfusion; HMP, hypothermic machine perfusion; EAD, early allograft dysfunction; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; NK Cells, natural killer cells; IL-32, interleukin 32; Caspase 1, Caspase 3; IL1B, interleukin 1 beta; IL18, interleukin 18; GSDMD, gasdermin D; ECDs, extended criteria donors; ICU, intensive care unit; BMI, body mass index; PNF, primary nonfunction; EAD, early allograft dysfunction; ITBLs, ischemic-type biliary lesions; GGT, gamma-glutamyl transpeptidase; HCC, hepatocellular carcinoma; DCD, donation after circulatory death; HBV, hepatitis B virus; MELD Score, model for end-stage liver disease score; AKI, acute kidney injury; GSVA, gene set variation analysis; CLT, conventional liver transplantation; EP, end of preservation; PR, post-graft revascularization; SNPs, single nucleotide polymorphisms; GWAS, genome-wide association studies; scRNAseq, single-cell RNA-sequencing; PP, pre-procurement; ssGSEA, single-sample gene set enrichment analysis; IL-27, interleukin 27; I/R, ischemia-reperfusion; IL-2RB, interleukin 2 receptor subunit beta; TNF-α, tumor necrosis factor-alpha

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http://www.e-cmh.org
INTRODUCTION

Liver transplantation is a life-saving treatment for patients with end-stage liver disease, such as cirrhosis, liver failure, and hepatocellular carcinoma (HCC). An elevated burden of liver disease leads to an increased demand for liver transplantation. In 2021, 34,944 liver transplantations were performed globally. However, organ shortage poses an obstacle to the development of liver transplantation. The mortality rate of patients on the waiting list has continued to increase since 2009. Therefore, surgeons and clinical researchers have attempted to extend this donor pool.

In the liver transplantation field, extended-criteria donors (ECDs) are defined as steatosis, age up to 80 years, serum sodium >165 mmol/L, serum alanine aminotransferase (ALT) >105 U/L, intensive care unit (ICU) stay with ventilation >7 days, body mass index (BMI) >30, serum aspartate transaminase (AST) >90 U/L, and total bilirubin (Tbil) >3 mg/dL. With donations after cardiac death, advanced age, and hepatic B virus (HBV)-infected patients assumed to be eligible donors, the number of liver transplantations increases annually. However, recipients with ECD livers have a greater incidence of primary non-function (PNF), early allograft dysfunction (EAD), and ischemic-type biliary lesions. Donors with hypernatremia, advanced age, or steatosis have been shown to have increased mortality due to liver transplantation. However, the prognosis of donors with high serum ALT, AST, and Tbil levels has not yet been clarified. Accordingly, functionally marginal liver grafts (FMLs) are defined as those with ALT >105 U/L, AST >90 U/L, or Tbil >3 mg/dL. Therefore, two questions need to be answered: What are the consequences of FML usage, and how can we alleviate liver injury during the FML transplantation process?

In 2018, a novel and promising technique called ischemia-free liver transplantation (IFLT) was introduced at the First Affiliated Hospital of Sun Yat-sen University. Recently, a randomized controlled trial of IFLT demonstrated that patients could gain greater clinical benefits from IFLT than from conventional liver transplantation. More specifically, there were lower incidences of EAD, postreperfusion syndrome, and non-anastomotic biliary strictures. Moreover, several studies have widened the application of IFLT. Compared with conventional liver transplantation, IFLT reduced postoperative peak AST, gamma-glutamyl transpeptidase, and creatine levels, and steatotic patients achieved better survival with a lower occurrence of EAD (IFLT: 0%, conventional liver transplantation: 60%). Based on transcriptome and metabolome profiles, IFLT significantly abrogated graft ischemia-reperfusion injury and suppressed inflammation. In addition, the incidence of recurrence was low in patients with HCC who underwent IFLT. All these results suggested that IFLT could function as a novel and promising surgery for the transplantation of FMLs, but this needs to be further examined. Here, we investigated the impact of FMLs on liver transplantation outcomes and clarified the effect of IFLT on the prognosis of FMLs from both clinical and molecular perspectives.

MATERIALS AND METHODS

Population

From January 1, 2015, to October 1, 2023, 1,309 patients underwent liver transplantation at the Organ Transplantation Center at the First Affiliated Hospital of Sun Yat-sen University. The grafts during machine perfusion were discarded if the parameters of viability did not reach the VIT-TAL criteria (lactate ≤2.5 mmol/L and two or more of bile production, pH ≥7.30, glucose metabolism, hepatic arterial flow ≥150 mL/min and portal vein flow ≥500 mL/min, or homogeneous perfusion). All adult liver transplants with both donor and recipient ages ≥18 years (n=1,093) were included in this study. The data of 871 eligible participants were acquired by excluding donations after cardiac death; donation after brain death followed by circulatory death; and individuals with missing information about donor type, serum ALT, AST, and Tbil. We defined FMLs as ALT >105 U/L, AST >90 U/L, or Tbil >3 mg/dL, according to a previous review. Ultimately, 353 FMLs were identified. More specifically, 279, 24, and 50 FML donors underwent surgery using different preservation techniques, such as static cold storage (SCS), normothermic machine perfusion (NMP), and IFLT, respectively.

This study involving humans was approved by the Institutional Ethics Committee for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University.
sity. The studies were conducted in accordance with local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement for written informed consent for participation from the participants or their legal guardians/next of kin due to the retrospective, minimal-risk nature of the study.

**Data collection**

For the donors, the clinical parameters of sex, age, BMI, diabetes status, hypertension status, HBV infection status, and serum concentrations of sodium, potassium, hemoglobin, Tbil, ALT, AST, creatinine, and steatosis status were collected before the operation. For recipients, clinical characteristics, including sex, age, BMI, model for end-stage liver disease score, diagnosis (decompensated cirrhosis, liver failure, or liver tumor), operation time, blood loss, respiratory support time, ICU stay time, reintubation ratio, duration of resumed diet, PNF, EAD, acute kidney injury (AKI), thrombosis ratio (in the hepatic artery, portal vein, or postcaval vein), biliary fistula, biliary stricture, wound infection, and pulmonary infection, were acquired from our hospital management system. In addition, overall and 90-day survival information was recorded through postoperative follow-up. The ALT, AST, and Tbil levels of the recipients were measured daily, and the data were collected seven days after liver transplantation.

**Bioinformatic analysis**

Using gene set variation analysis (GSVA), we evaluated the activity of different cell death pathways (PANoptosis, ferroptosis, pyroptosis, autophagy, necroptosis, and apoptosis) to determine the mechanism related to liver injury using previously published transcriptome data from six conventional liver transplantation (CLT) and six IFLT samples.11 The gene lists for PANoptosis and Cuproptosis were acquired from two published reviews,14,15 whereas other gene lists were downloaded from the Molecular Signatures Database (MSigDB).16,17 Liver biopsy tissues were collected at the end of preservation (EP), and resting samples were acquired at post-graft revascularization (PR). To determine the gene pattern associated with liver injury, we extracted single nucleotide polymorphisms (SNPs) related to serum ALT, AST, and Tbil levels in both European and Asian populations. With the “twoSampleMR” package,18 we downloaded the corresponding genome-wide association studies (GWAS) data from the IEU Open GWAS project (https://gwas.mrcieu.ac.uk/).19 Detailed information regarding these data were provided in Supplementary Table 6. SNPs with a P-value <1e-5 were identified as significant liver injury-related SNPs. We then used the g:Profiler tool to map the SNPs to gene names (https://biit.cs.ut.ee/gprofiler/snpense).19 The corresponding genes were defined as liver injury genes. We further conducted a differential gene expression analysis using previously described transcriptome data. Liver injury-related genes with logFC >1 and false discovery rate <0.05 were identified, and the corresponding expression patterns were shown in the heatmap. In addition, we performed enrichment analysis for both upregulated and downregulated genes using the online enrichment tool Metascape (https://metascape.org/gp/index.html#main/step1).20

To further investigate the relationship between liver injury and the immune system, we reanalyzed single-cell RNA-sequencing (scRNA-seq) data that included liver tissue samples at pre-procurement (PP), EP, and PR. We extracted EP and PR samples for further analysis to ensure consistency with the transcriptome data. The “Single R” package21 and the online tool CellMarker (http://xteam.xbio.top/CellMarker/)22 were utilized to define cell types. The expression of the previously described significant liver injury-related genes was detected in different cell types. Using single-sample gene set enrichment analysis (ssGSEA), we used the top 10 DEGs to estimate the proportions of different immune cells in the transcriptome data. The Wilcoxon test was used to compare cell proportions between the SCS and IFLT groups. In addition, we carried out correlation analysis among different immune cells with the package “corrplot.”

**Statistical analysis**

All analyses were performed using R version 4.2.0. Graphs were drawn using both R 4.2.0 and GraphPad Prism software. The package ‘gstsummary’ was used to compare different groups. We evaluated the outcomes of these groups using a K-M plot. Propensity score matching (PSM) analyses were performed to control for bias. Categorical variables were presented as the frequencies (per-
percentages) and were compared using Pearson's chi-square test and Fisher's exact test. Continuous variables with a normal distribution were presented as the means (standard deviation, SD) and were analysed using a t test and repeated measures analysis of variance. Continuous variables with a non-normal distribution were presented as the medians (interquartile ranges, IQRs) and were analysed using the Wilcoxon test. Statistical significance was set at \( P<0.05 \).

RESULTS
FMLs contribute to poor prognosis for LT patients

In total, 871 participants were enrolled in this study. The parameters used to define the extended criteria for donor livers were presented in Supplementary Table 1. Multivariate Cox regression analysis was used to compare the impact of different ECD parameters (FMLs, donor liver steatosis, donor BMI, donor serum sodium levels, and donor age) on postoperative 90-day survival. Compared with the other factors, FMLs had a significantly greater hazard ratio (HR: 1.969, \( P=0.018 \)) (Fig. 1A). The baseline characteristics of the normal and FML groups before and after PSM were shown in Supplementary Table 2. The age and BMI of the FML group were lower than those of the control group. There were 32.8% steatotic livers in the FML donors. FML donors had higher serum levels of sodium, ALT, AST, and Tbil. Considering the differences at baseline, we performed PSM analysis to control for confounders: age, BMI, serum sodium level, serum creatine level, liver steatosis status of donors, and primary diagnosis of cirrhosis (Supplementary Table 2). Recipients who received FMLs exhibited a longer ICU stay (40.8 hours vs. 35.8 hours, \( P=0.294 \)); resumed diet time (114 hours vs. 108 hours, \( P=0.277 \)); and had a greater probability of reintubation (11.4% vs. 5.1%, \( P=0.076 \), PNF (6.6% vs. 3.7%, \( P=0.174 \)), and pulmonary infection (14.6% vs. 7.8%, \( P=0.023 \)) (Table 1). The percentage of deceased patients within 90 days increased from 5.0% to 12.3% (\( P=0.007 \)), and the overall death ratio increased from 23.3% to 30.1% (\( P=0.105 \)). Similarly, a poor survival plot was generated for both overall and 90-day
Table 1. Different characteristics of prognosis between FMLs and normal

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Before PSM</th>
<th>P-value</th>
<th>After PSM (1:1)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (n=436)</td>
<td>FMLs (n=279)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal (n=219)</td>
<td>FMLs (n= 219)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outcomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory support time (h)†</td>
<td>18.0 (12.7, 43.3)</td>
<td>17.0 (11.9, 44.5)</td>
<td>0.330</td>
<td>17.0 (12.0, 35.0)</td>
</tr>
<tr>
<td>ICU stay time (h)†</td>
<td>39.0 (23.0, 91.9)</td>
<td>42.5 (23.0, 89.0)</td>
<td>0.659</td>
<td>35.8 (22.0, 82.0)</td>
</tr>
<tr>
<td>Reintubation (%)</td>
<td>13.0 (5.9)</td>
<td>16.0 (10.9)</td>
<td>0.086</td>
<td>6.0 (5.1)</td>
</tr>
<tr>
<td>Resume diet (h)‡</td>
<td>109.5 (82.6, 149.4)</td>
<td>114.0 (85.0, 164.3)</td>
<td>0.412</td>
<td>108.0 (86.0, 137.0)</td>
</tr>
<tr>
<td>HA thrombosis (%)</td>
<td>16.0 (4.1)</td>
<td>13.0 (5.3)</td>
<td>0.486</td>
<td>5.0 (2.6)</td>
</tr>
<tr>
<td>PV thrombosis (%)</td>
<td>9.0 (2.3)</td>
<td>4.0 (1.6)</td>
<td>0.559</td>
<td>4.0 (2.1)</td>
</tr>
<tr>
<td>PC thrombosis (%)</td>
<td>4.0 (1.0)</td>
<td>4.0 (1.6)</td>
<td>0.494</td>
<td>1.0 (0.5)</td>
</tr>
<tr>
<td>Biliary fistula (%)</td>
<td>2.0 (0.5)</td>
<td>5.0 (2.0)</td>
<td>0.115</td>
<td>2.0 (1.0)</td>
</tr>
<tr>
<td>Biliary stricture (%)</td>
<td>13.0 (3.3)</td>
<td>9.0 (3.6)</td>
<td>0.830</td>
<td>7.0 (3.6)</td>
</tr>
<tr>
<td>PNF (%)</td>
<td>23.0 (5.4)</td>
<td>15.0 (5.5)</td>
<td>0.930</td>
<td>8.0 (3.7)</td>
</tr>
<tr>
<td>EAD (%)</td>
<td>197.0 (45.2)</td>
<td>114.0 (41.2)</td>
<td>0.290</td>
<td>89.0 (40.6)</td>
</tr>
<tr>
<td>AKI (%)</td>
<td>19.0 (4.8)</td>
<td>14.0 (5.7)</td>
<td>0.637</td>
<td>11.0 (5.6)</td>
</tr>
<tr>
<td>Pulmonary infection (%)</td>
<td>41.0 (9.4)</td>
<td>41.0 (14.7)</td>
<td>0.030</td>
<td>17.0 (7.8)</td>
</tr>
<tr>
<td>Wound infection (%)</td>
<td>6.0 (1.4)</td>
<td>5.0 (1.8)</td>
<td>0.758</td>
<td>2.0 (0.9)</td>
</tr>
<tr>
<td>Overall mortality (%)</td>
<td>117.0 (26.9)</td>
<td>80.0 (28.7)</td>
<td>0.604</td>
<td>51.0 (23.3)</td>
</tr>
<tr>
<td>90-day mortality (%)</td>
<td>38.0 (8.7)</td>
<td>34.0 (12.2)</td>
<td>0.132</td>
<td>11.0 (5.0)</td>
</tr>
</tbody>
</table>

Adjusted variables: donor age, body mass index, serum sodium level, serum creatine level, liver steatosis, and recipient liver cirrhosis. †Categorical variables were exhibited as frequencies (percentages). ‡Continuous variables with non-normal distribution were exhibited as median (interquartile range).
FMLs, functionally marginal liver grafts; PSM, propensity score matching; ICU, intensive care unit; HA, hepatic artery; PV, portal vein; PC, postcaval vein; PNF, primary non function; EAD, early allograft disfunction; AKI, acute kidney injury.
survival (Fig. 1B and C). The serum levels of ALT, AST, and Tbil of the recipients after the operation were significantly greater in the FML group (Fig. 1D–F).

**NMP is insufficient for improving the outcome of FML patients**

The baseline parameters of FMLs who underwent NMP and SCS were compared in Supplementary Table 3. There were clear increases in donor age, BMI, ratio of liver decompensated cirrhosis, and liver failure in the NMP group. Therefore, we conducted PSM analysis based on these factors, and the corresponding results were presented in Supplementary Table 4. Patients in the NMP group had a lower AKI ratio (0.0% vs. 4.7%, \( P=0.533 \)) and better overall and 90-day survival rates. The percentage of deceased patients within 90 days decreased from 14.6% to 12.5% (\( P=0.099 \)), whereas the overall percentage of deceased patients decreased from 22.9% to 12.5% (\( P=0.359 \)). However, the respiratory support time (19.5 hours vs. 16.0 hours, \( P=0.388 \)), ICU stay time (57.5 hours vs. 53.5 hours, \( P=0.400 \)), resumed diet time (232 hours vs. 114 hours, \( P=0.076 \)), reintubation ratio (33.3% vs. 23.8%, \( P=0.633 \)), pulmonary infection rate (12.5% vs. 10.4%, \( P>0.999 \)), and EAD rate (69.6% vs. 39.1%, \( P=0.017 \)) were greater in the NMP group. In addition, there was no significant difference in the K‒M plot between the NMP and SCS groups (Supplementary Fig. 1A and B). The improvements in ALT and AST levels after surgery were not significant (Supplementary Fig. 1C and D). The serum Tbil levels were greater in the NMP group (Supplementary Fig. 1E).

**IFLT significantly improves the prognosis of FML patients**

After conducting PSM analysis to adjust for confounders, creatine, and steatosis status (Supplementary Table 5), we compared the effects of SCS and IFLT on the survival of LT patients (Table 2). There were no differences in the baseline characteristics between the two groups. There was a shorter ICU stay (35.3 hours vs. 38.8 hours, \( P=0.151 \)); shorter resumed diet time (86.0 hours vs. 102.1 hours, \( P=0.143 \)); and lower prevalence of PNF (0.0% vs. 6.7%, \( P=0.092 \)), EAD (29.2% vs. 40.6%, \( P=0.179 \)), AKI (5.4% vs. 5.8%, \( P>0.999 \)), reintubation (6.9% vs. 15.8%, \( P=0.320 \)), and pulmonary infection (6.3% vs. 15.6%, \( P=0.109 \)) in IFLT patients. IFLT significantly increased the 90-day survival rate from 88.5% to 95.8% (\( P=0.022 \)) and reduced the overall mortality rate from 31.3% to 10.4% (\( P=0.006 \)). Similarly, the K‒M plot revealed a better prognosis for patients who received IFLT (Fig. 2A and B). Postoperative serum ALT, AST, and Tbil levels were distinctly lower in the IFLT group than in the SCS group (Fig. 2C–E).

**IFLT alleviates liver injury through anti-cell death and anti-inflammatory effects**

Using GSVA, we investigated the effect of IFLT on liver injury from a microscopic perspective. Pyroptosis, autophagy, and necroptosis were inhibited in IFLT patients (Fig. 3A). Pyroptosis was the most significantly altered pathway in the IFLT and SCS groups. The expression patterns of liver injury-related genes were presented in Figure 3B and C. Most of these genes, including the inflammatory factors interleukin 27 (IL-27) and IL-32, were immune-associated and were significantly downregulated in patients who underwent IFLT. Through enrichment analysis, we demonstrated that most inflammation-related pathways, including cytokine signalling in the immune system, signalling by interleukins, and the Fc gamma R-mediated phagocytosis pathway, were downregulated in patients who received IFLT (Fig. 3E).

**IFLT constructs a microenvironment characterized by low NK cell infiltration**

The strong correlation between IFLT and immune reactions led us to investigate the microenvironmental characteristics of patients undergoing IFLT and SCS. The immune cell types of patients with SCS in the ER and PR stages were annotated into nine clusters: hepatocytes, smooth muscle cells, endothelial cells, monocytes, macrophages, NK cells, T cells, B cells, and erythroblasts (Fig. 4A). Using the ssGSEA method, we estimated cell types in patients with IFLT and SCS. NK cells, T cells, and monocytes tended to infiltrate together (Fig. 4B). There was a significantly lower infiltration of NK cells in IFLT patients than in SCS patients, whereas the change in other cell types did not reach significance (Fig. 4C). In addition, the expression of four classical pyroptosis-related genes (CASP1, GSDMD,
### Table 2. Different characteristics between IFLT and SCS after PSM

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Before PSM</th>
<th>After PSM (2:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCS (n=279)</td>
<td>IFLT (n=50)</td>
</tr>
<tr>
<td>Outcomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory support time (h)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>17.0 (11.9, 44.5)</td>
<td>16.0 (12.3, 34.8)</td>
</tr>
<tr>
<td>ICU stay time (h)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>42.5 (23.0, 89.0)</td>
<td>35.3 (18.3, 58.5)</td>
</tr>
<tr>
<td>Reintubation (%)&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>16.0 (10.9)</td>
<td>2.0 (6.7)</td>
</tr>
<tr>
<td>Resume diet (h)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>114.0 (85.0, 164.3)</td>
<td>86.0 (64.0, 121.5)</td>
</tr>
<tr>
<td>HA thrombosis (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>13.0 (5.3)</td>
<td>1.0 (2.6)</td>
</tr>
<tr>
<td>PV thrombosis (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>4.0 (1.6)</td>
<td>1.0 (2.6)</td>
</tr>
<tr>
<td>PC thrombosis (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>4.0 (1.6)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>Biliary fistula (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>5.0 (2.0)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>Biliary stricture (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>9.0 (3.6)</td>
<td>2.0 (5.1)</td>
</tr>
<tr>
<td>PNF (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>15.0 (5.5)</td>
<td>1.0 (2.0)</td>
</tr>
<tr>
<td>EAD (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>114.0 (41.2)</td>
<td>15.0 (30.0)</td>
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<tr>
<td>AKI (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>14.0 (5.7)</td>
<td>2.0 (5.1)</td>
</tr>
<tr>
<td>Pulmonary infection (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>41.0 (14.7)</td>
<td>3.0 (6.0)</td>
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<tr>
<td>Wound infection (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>5.0 (1.8)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>Overall mortality (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>80.0 (28.7)</td>
<td>6.0 (12.0)</td>
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<tr>
<td>90-day mortality (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>34.0 (12.2)</td>
<td>3.0 (6.0)</td>
</tr>
</tbody>
</table>

Adjusted variables: donor liver steatosis and serum creatine level. <sup>☆</sup>Categorical variables were exhibited as frequencies (percentages). <sup>‡</sup>Continuous variables with non-normal distribution were exhibited as median (interquartile range).

IFLT, ischemia-free liver transplantation; SCS, static cold storage; PSM, propensity score matching; ICU, intensive care unit; HA, hepatic artery; PV, portal vein; PC, postcaval vein; PNF, primary nonfunction; EAD, early allograft disfunction; AKI, acute kidney injury.
IL1B, and IL-18) in different cells was determined from the scRNA-seq data. IL-32 was significantly upregulated in NK and T cells, whereas CASP1, IL1B, and IL-18 levels were elevated in monocytes and macrophages (Fig. 4D). Furthermore, the correlations between the expression of IL-32 and genes in the classical pyroptosis pathway (CASP1, GSDMD, IL1B, and IL-18) were evaluated using bulk RNA-seq data. A significant positive relationship was observed between the expression of CASP1, GSDMD, and IL-32 (Fig. 4E).

**DISCUSSION**

The large imbalance between the waiting list and organ pool presents an urgent need to expand the donor pool. ECDs are considered promising donor resources. Based on the definition of ECDs in 35 organ transplant centers, we innovatively summarized a novel type of ECD called FMLs (a detailed definition is provided in the Methods section). In this cross-sectional study, we clarified the negative effect of FMLs on the prognosis of LT patients, which was not influenced by the primary disease of the recipients. The impact of FMLs on postoperative 90-day survival was greater than that of other ECD parameters, including steatosis, BMI, serum sodium, and age. In contrast to normal donors, patients who received FMLs were more likely to develop PNF and pulmonary infections. This led to more frequent medical interventions during the postoperative period, including longer ICU stays, resumption of diet, and a greater risk of reintubation. These patients spent more time recovering and had poorer long-term outcomes. Therefore, more care should be given to the utilization of FMLs to expand the liver donor pool. It is also important to find a method to alleviate liver injury caused by FMLs.

The results showed that both SCS and NMP preservation were insufficient to improve liver function in FMLs, and comparable outcomes were observed in recipients. Although conventional SCS can significantly reduce metabolism, ROS accumulation leads to severe reperfusion injury. In recent years, clinical research has focused on a new organ preservation method, NMP, which is characteristic of livers perfused with oxygenated blood. Although NMP avoids the cooling process and reduces graft injury by 50%, there is still an ischemia-reperfusion (I/R) process during the operation. Another kind of machine perfu-
Figure 3. IFLT alleviates liver injury by suppressing cell death and inflammation. (A) GSVA scores of different cell death pathways between the CLT (underwent SCS) and IFLT groups. For both the European (B) and Asian (C) populations, the expression patterns of genes significantly related to liver injury among tissues from CLT and IFLT patients are depicted. The enrichment analysis for upregulated (D) and downregulated (E) liver injury-related genes in IFLT subjects. IFLT, ischemia-free liver transplantation; GSVA, gene set variation analysis; CLT, conventional liver transplantation; SCS, static cold storage.
Figure 4. IFLTs induce an immune microenvironment with low infiltration of NK cells. (A) The annotation of cell types in the scRNA-seq profiles. (B) The co-infiltration of immune cells among patients who underwent liver transplantation. (C) The proportions of different immune cells measured by ssGSEA between the CLT (underwent SCS) and IFLT groups. (D) The expression patterns of four classical pyroptosis genes (CASP1, GSDMD, IL1B, and IL18) and IL-32 are shown among different immune cells. (E) The correlation between the expression of IL-32 and that of four classical pyroptosis genes according to bulk RNA-seq profiles from the CLT and IFLT groups. IFLT, ischemia-free liver transplantation; scRNA-seq, single-cell RNA-sequencing; ssGSEA, single-sample gene set enrichment analysis; CLT, conventional liver transplantation; SCS, static cold storage; CASP1, caspase 1; IL1B, interleukin 1 beta; GSDMD, gasdermin D; IL1B, interleukin 1 beta; IL18, interleukin 18. *P<0.05, **P<0.01.
sion, HMP (hypothermic machine perfusion), combines static cold storage and machine perfusion. Preclinical research revealed that HMP could restore mitochondrial function, inhibit the immune response, and alleviate liver injury.\textsuperscript{26-27} Several single- or multicenter clinical trials have demonstrated that HMP reduces the incidence of hepatobiliary preservation injury, nonanastomotic biliary strictures and EAD.\textsuperscript{28-31} However, these preservation methods cannot address the interruption of blood flow. Recently, a novel type of liver transplantation surgery, IFLT, has shown significant clinical benefits. In contrast to traditional surgery (SCS and NMP), IFLT can reduce I/R injury without disrupting the blood cycle during organ procurement, preservation, and implantation.\textsuperscript{9} In this cohort study, different organ preservation methods were compared among recipients with FMLs. The protective effect of NMP on the short- and long-term outcomes of FMLs could not be verified in our study. The incidence of EAD was significantly greater in the NMP group than in the SCS group. Surprisingly, patients with FMLs who underwent IFLT had a shorter ICU stay, resumed diet, and were less likely to be reintubated, which indicated a lower cost of IFLT than SCS. In addition, the liver function factors ALT, AST, and Tbil decreased rapidly, which was consistent with the lower incidence of complications during the early postoperative period, including PNF, EAD, AKI, and pulmonary infection. Therefore, IFLT can significantly reduce the mortality rate in patients undergoing liver transplantation. The results indicate that IFLT can assist with the utilization of FMLs and reduce the risk to an acceptable level.

Mechanistically, pyroptosis was significantly inhibited in IFLT. Pyroptosis is originally called caspase-1-dependent programmed cell death and induces the secretion of IL-1b and IL-8.\textsuperscript{32-34} Many studies have revealed that pyroptosis-induced inflammation accounts for I/R injury in liver transplantation.\textsuperscript{33,35-37} Therefore, considering the proinflammatory role of pyroptosis, we investigated the effect of IFLT on FML inflammation. Most of the downregulated liver injury-related genes were proinflammatory genes, such as IL-32, IL-27, and interleukin 2 receptor subunit beta (IL-2RB). Specifically, IFLT significantly reduced NK cell infiltration. The function of NK cells in liver transplantation remains controversial owing to conflicting clinical and experimental results.\textsuperscript{38} However, accumulating evidence suggests that NK cells are involved in the development of I/R injury during conventional liver transplantation.\textsuperscript{39} The depletion of NK cells can protect the liver from I/R injury, as evidenced by decreased ALT and AST levels.\textsuperscript{40} The presence of NK cells in the liver perfusate is strongly associated with acute cellular rejection.\textsuperscript{41} Therefore, we concluded that IFLT alleviates liver injury by reversing pyroptosis-induced inflammation and inhibiting NK cell infiltration.

Additionally, IL-32 was significantly upregulated in the NK cells of SCS patients who underwent I/R injury. IL-32 is originally cloned in human NK cells and is a proinflammatory factor that contains three isoforms: IL-32a, IL-32b, and IL-32g.\textsuperscript{42} IL-32g is a proinflammatory mediator that promotes the expression of IL-1b, IL-18, and tumor necrosis factor-alpha (TNF-a).\textsuperscript{13,44} The immune cell patterns of CASP1, IL-1b, and IL-18 were remarkable in monocytes and macrophages. There was a positive relationship between IL-32, CASP1, and GSDMD in both SCS and IFLT patients. Thus, we can infer that IFLT alleviates liver injury in FMLs by inhibiting the infiltration of NK cells, thereby leading to the abortion of IL-32-driven pyroptosis and decreased secretion of downstream inflammatory factors (IL-1b and IL-18) in monocytes and macrophages.

In summary, clinical data analysis reveals that FMLs lead to poorer outcomes in LT patients than other ECDs (steatosis, advanced age, advanced BMI, and hypernatremia) in terms of postoperative complications and median survival. Transplant surgeons should be prudent regarding the utilization of this type of marginal liver. Surprisingly, we demonstrate that IFLT significantly improves the survival of patients who underwent FMLs. Furthermore, IFLT ameliorates liver injury in FMLs by inhibiting the infiltration of NK cells, leading to the abortion of IL-32-driven pyroptosis and the release of downstream inflammatory factors (IL-1b and IL-18) in monocytes and macrophages (Fig. 1B).

**Limitations**

Our study has three main limitations. First, the small number of patients in the NMP and IFLT groups weakened the statistical results. Larger population studies are required to address this issue (Supplementary Table 7). Second, the mechanism underlying the improvement of IFLT was revealed using bioinformatic analysis. Although a pyroptosis-immune network was constructed, more detailed experiments should be conducted to elucidate the mecha-
nism of liver injury in the SCS and NMP groups as compared with IFLT. Third, some low-quality grafts tended to be evaluated under machine perfusion, which could have led to an underestimation of the protective effect of NMP in our study.

Authors’ contribution
Conceptualization: S.W., M.C., and W.J; Writing the original draft: S.W., X.L., and Y.T; Date collection: Y.L., M.Z., Z.X., Y.G., and Y.D; Statistical analyses: S.W. and X.L; Bioinformatics: S.W; Review & editing: Q.Z., Z.G., D.W., and X.H. Founding: X.H., W.J., and M.C.

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Conflicts of Interest
The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL
Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES


Global prevalence of metabolic dysfunction-associated fatty liver disease-related hepatocellular carcinoma: A systematic review and meta-analysis

Harry Crane1, Guy D. Eslick2, Cameron Gofton1,3, Anjya Shaikh4, George Cholankeril5, Mark Cheah6, Jian-Hong Zhong7, Gianluca Svegliati-Baroni8, Alessandro Vitale9, Beom Kyung Kim10, Sang Hoon Ahn10, Mi Na Kim10, Simone I Strasser11, and Jacob George1

1Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, Sydney, NSW, Australia; 2NHMRC Centre for Research Excellence in Digestive Diseases, Hunter Medical Research Institute (HMRI), The University of Newcastle, Newcastle, NSW, Australia; 3Department of Gastroenterology, Royal North Shore Hospital, St Leonards, NSW, Australia; 4Department of Medicine, University of Connecticut School of Medicine, Farmington, CT, USA; 5Department of Internal Medicine, Baylor College of Medicine, Houston, TX, USA; 6Department of Gastroenterology and Hepatology, Singapore General Hospital, Singapore; 7Hepatobiliary Surgery Department, Guangxi Liver Cancer Diagnosis and Treatment Engineering and Technology Research Center, Guangxi Medical University Cancer Hospital, Nanning, China; 8Liver Injury and Transplant Unit, Polytechnic University of Marche, Ancona, Italy; 9Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy; 10Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea; 11AW Morrow Gastroenterology and Liver Centre, Royal Prince Alfred Hospital, Camperdown, VIC, Australia

Graphical Abstract

Global prevalence of MAFLD-related Hepatocellular carcinoma: A systematic review and meta-analysis

Aim: Estimate the global proportion of HCC attributable to MAFLD as:
- A sole liver disease
- In combination with other liver diseases

22 studies, 56,565 individuals with HCC included

GLOBAL BURDEN
- 12% of HCC globally is due to MAFLD as sole liver disease
- 49% in total have MAFLD (with or without other liver diseases)

Study Highlights
- The proportion of HCC for which MAFLD is the aetiological cause or contributing factor is unknown.
- In this global systematic review and meta-analysis of over 56,000 individuals with HCC, we define the prevalence of MAFLD in the presence and absence of other liver diseases.
- 49% of all individuals with HCC have MAFLD; however, MAFLD as a sole liver disease accounts for 12% of HCC.
- Individuals with MAFLD plus another liver disease appeared phenotypically distinct from both “pure” MAFLD and non-MAFLD HCC.
- This supports the use of positive diagnostic criteria and systematically ascertaining for MAFLD in all individuals with HCC.
INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality, with annual deaths predicted to rise substantially in coming decades from 800,000 in 2020 to 1,300,000 by 2040.¹ A major reason for this is the global rise in fatty liver disease driven by epidemics of obesity, diabetes, and metabolic dysfunction.² Metabolic (dysfunction)-associated fatty liver disease (MAFLD) is an increasingly important contributor to global HCC incidence, yet the true scale of its contribution remains unknown. In part, this is due to the previous non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH) diagnostic criteria which relied on exclusion of other causes of hepatic steatosis. Thus, in HCC cohorts, NAFLD/NASH-related HCC is often diagnosed and reported only when it is the sole identifiable liver disease. The MAFLD definition proposed in 2019³,⁴ allows a positive diagnosis to be made in the presence of steatosis plus defined markers of metabolic dysfunction, irrespective of concurrent liver disease. In HCC due to chronic hepatitis B, C, and alcohol-related liver disease, mixed-MAFLD prevalence was 40.0% (95% CI 30.2–50.3%), 54.1% (95% CI 40.4–67.6%) and 64.3% (95% CI 52.7–75.0%), respectively. Mixed-MAFLD HCC had significantly higher likelihood of cirrhosis and lower likelihood of metastatic spread compared to single-MAFLD HCC, and a higher platelet count and lower likelihood of macrovascular invasion compared to non-MAFLD HCC.

Conclusions: MAFLD is common as a sole aetiology, but more so as a co-factor in mixed-aetiology HCC, supporting the use of positive diagnostic criteria. (Clin Mol Hepatol 2024;30:436-448)

Keywords: Fatty liver; Hepatocellular carcinoma; Epidemiology; Prevalence; Metabolic syndrome

Abbreviations:
MAFLD, metabolic dysfunction-associated fatty liver disease; HCC, hepatocellular carcinoma; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; ALD, alcohol-related liver disease; NAFLD/NASH, non-alcoholic fatty liver disease/non-alcoholic steatohepatitis; BMI, body mass index; T2DM, type 2 diabetes mellitus; FLI, fatty liver index; OR, odds ratio; MD, mean difference; AFP, alpha fetoprotein; ICD, International classification of diseases
and prospective interventional studies is to categorise HCC by a single or “dominant” aetiology. Thus, while numerous studies have reported prevalence of fatty liver disease in HCC (using either NAFLD or MAFLD definitions), these may underestimate the true impact of MAFLD by excluding individuals with multiple interacting aetiologies. Data are therefore needed to define the role of MAFLD in HCC regardless of the presence or absence of other liver diseases.

There is growing recognition that accurately classifying underlying liver disease(s) is important. Not only do patient demographics and comorbidities differ between individuals of different liver disease aetiologies, but so do the underlying aetiological mechanisms of HCC development, tumour immune microenvironments, and perhaps responses to therapy. In this regard, liver disease aetiology has garnered interest as a potential stratification tool to guide therapy. The aim of this systematic review and meta-analysis is thus to estimate the proportion of HCC attributable to MAFLD either as a sole liver disease aetiology or in combination with another liver disease. The secondary aims were to assess variation in single or combined aetiology MAFLD-HCC by geographical region, secondary liver disease aetiology, and MAFLD sub-phenotype (lean, overweight/obese, diabetic), and to compare the clinical characteristics of single-aetiology MAFLD, combined-aetiology MAFLD, and non-MAFLD HCC.

MATERIALS AND METHODS

Search strategy and inclusion criteria

The review was synthesised in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (Supplementary Table 1). A search was conducted on 16th April 2023 using Medline, Embase, PubMed, and Web of Science from database inception to April 2023 for publications that contained information on MAFLD-related HCC. The search terms were “metabol* adj2 associa* fatty liver disease” OR “mafl*” OR “mash” OR “metabol* adj2 steatohepatitis” AND “hcc” OR “liver cell carcinoma” OR “hepatocellular carcinoma” OR “hepatoma” OR “liver cancer” on the search of title and abstracts. The references were compiled on Endnote and duplicates were removed.

Eligibility and selection criteria

Two reviewers (H.C. and C.G.) independently reviewed titles and abstracts to screen for eligible studies. Full texts of any potentially relevant studies were obtained for further evaluation. Inclusion criteria were as follows: an original article or abstract published in a peer-reviewed journal; the study reported on the prevalence of MAFLD in a cohort of individuals with HCC. HCC cohorts were defined as any group of consecutive HCC cases of any aetiology (apart from MAFLD-only cohorts). MAFLD was defined as the presence of hepatic steatosis with metabolic dysregulation as defined by the international expert consensus panel criteria. Steatosis could be defined by any method including radiology, histology or blood-based investigations such as fatty liver index. Overweight/obese was defined as BMI ≥23 kg/m² in Asian cohorts and ≥25 kg/m² in Western cohorts, and individuals under these respective cut-offs were defined as “lean”. We excluded studies which did not reference the criteria used to diagnose MAFLD or used a definition inconsistent with the internationally accepted criteria (including region-specific BMI and waist circumference cut-offs) unless authors were able to provide the appropriate clarifications on their MAFLD diagnostic criteria. Studies were also excluded if there was ambiguity as to whether their MAFLD prevalence data referred to single-MAFLD or total-MAFLD prevalence within their cohort unless clarifications were provided by authors. In the case of multiple studies reporting on overlapping cohorts, the most recently published study was included in the analysis. However, if a non-overlapping subgroup could be extracted from another study, then that subgroup was eligible for inclusion in the overall analysis.

Three prevalence estimates were made:

- Single-MAFLD HCC, defined as the proportion of HCC whereby MAFLD is the sole identifiable liver disease.
- Mixed-MAFLD HCC, defined as the prevalence of MAFLD amongst individuals with HCC with another (non-MAFLD) liver disease aetiology.
- Total-MAFLD HCC, defined as the total prevalence of MAFLD in an HCC cohort (i.e. the total sum of single-MAFLD and mixed-MAFLD HCC).
A comparison of the clinical and tumour characteristics was performed between three groups: single-MAFLD HCC, mixed-MAFLD HCC, and non-MAFLD HCC (defined by HCC in the absence of MAFLD).

Statistical analysis

For MAFLD prevalence estimates, single proportions were transformed using the Freeman-Tukey double arcsine transformation method and weighted by inverse variance for pooling. A random-effects model was used for all analyses with between-study variance estimated using a DerSimonian and Laird model. Statistical heterogeneity was assessed by I² and Cochran’s Q test values. Pre-specified subgroup analysis by geographic region, and in the case of mixed-MAFLD prevalence by other liver disease aetiology (HBV, HCV or ALD) was performed. A post-hoc meta-regression analysis was performed to investigate study level factors influencing MAFLD prevalence heterogeneity. Covariates used for the analysis were median cohort age, gender, geographical setting (Asia vs. outside Asia), duration of study enrolment, whether the cohort was limited to early-stage (resectable) HCC, and proportion of mixed-MAFLD cohort with HBV.

To compare clinical and tumour characteristics between groups, comparative meta-analysis of odds ratios (OR) for binary variables, and mean difference (MD) for continuous variables were performed. When mean and standard deviation were not reported, they were estimated using the method described by Wan et al. Publication bias was assessed by the Egger’s test and by assessing for asymmetry on funnel plots, and if present, was adjusted using the trim-and-fill method. A P-value was considered statistically significant if ≤0.05. All analyses were conducted in RStudio (R version 4.3.1).

Quality assessment

Quality assessment for included articles was done using the critical appraisal tool proposed by Hoy et al. for assessing bias risk in prevalence studies. The tool consists of 10 items addressing four domains plus a summary risk of bias assessment, with scores of 0–3 considered to reflect a low overall risk of bias, scores of 4–6 representing moderate risk of bias, and 7–10 representing high risk of bias.

RESULTS

Summary of included studies

The initial search from Medline, Embase, PubMed, and Web of Science yielded 713 results (Fig. 1). After duplicate removal, 317 articles were screened for inclusion. Three studies were excluded due to overlapping study populations; only a non-overlapping subgroup of another study was included in the primary prevalence analysis. One additional study was excluded due to a BMI cutoff not in line with accepted ethnicity-specific cutoffs. 11 authors were contacted to provide further data or clarifications on definitions used for MAFLD diagnosis. 22 studies, comprising a total of 56,565 HCC cases, were included in the final analysis. Eleven studies reported prevalence in

Figure 1. PRISMA flow diagram. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-analyses; MAFLD, metabolic dysfunction-associated fatty liver disease; HCC, hepatocellular carcinoma; BMI, body mass index.
Asian cohorts, four from Europe, three from Australia, one from North America, one from Central America, one reporting prevalence in a mixed cohort from Europe and Asia, and one in a mixed cohort from North America and Europe. All included studies used ethnicity-appropriate BMI cut-offs and T2DM status in their MAFLD diagnostic criteria. The method of hepatic steatosis assessment was variable between studies and is described in Supplementary Table 2.

The risk of bias for each of the included studies in the meta-analysis is shown in Supplementary Table 3. Each of the included studies had an overall low risk of study bias.

**Single-MAFLD HCC**

Eleven studies (comprising 39,381 HCC cases) reported the prevalence of single-aetiology MAFLD HCC with a pooled overall prevalence of 12.4% (95% confidence interval [CI] 8.3–17.3%) (Fig. 2A). There was high heterogeneity between study estimates (I²=99%, Cochran’s Q <0.01). Individual single-MAFLD prevalence estimates varied between cohorts from 4–34%. Subgroup analysis based on geographical region showed highest prevalence in Central America (30%), followed by Australia (19%, 95% CI 12–28%), North America (18%), Europe (12%, 95% CI 6–18%), and Asia (6%, 95% CI 3–9%) (Table 1). There was no evidence of publication bias.

**Mixed-MAFLD HCC**

Seventeen studies (comprising 51,556 HCC cases) reported the prevalence of mixed-MAFLD with a pooled prevalence estimate of 41.3% (95% CI 34.3–48.5%) (Fig. 2B). There was significant heterogeneity between studies (I²=99%, Cochran’s Q <0.01) with individual prevalence estimates ranging from 14% to 80%. A subgroup analysis by geographic region showed highest prevalence in North America (64%), followed by Europe (54%, 95% CI 37–71%), Asia (37%, 95% CI 28–47%), and Australia (18%). Notably, there was evidence of funnel plot asymmetry (P=0.032), and after adjustment for publication bias, the adjusted prevalence estimate was 55.1% (95% CI 47.9–62.2%).

Mixed-MAFLD HCC was stratified by other liver disease aetiology. Twelve studies (comprising 20,166 HCC cases) reported the prevalence of MAFLD in individuals with HBV-related HCC cohorts with a pooled total prevalence of 40.0% (95% CI, 30.1–50.3%). Five studies (comprising 13,090 HCC cases) reported the prevalence of MAFLD in individuals with HCV-related HCC with a pooled total prevalence of 54.1% (95% CI 40.4–67.6%). Five studies (comprising 6,841 HCC cases) reported the prevalence of MAFLD in ALD-related HCC cohorts with a pooled total prevalence of 64.3% (95% CI 52.7–75.0%) (Table 1).

**Total-MAFLD HCC**

Eight studies (comprising 33,590 HCC cases) reported the total-MAFLD prevalence in HCC cohorts with a pooled prevalence estimate of 48.7% (95% CI 34.5–63.0%) (Fig. 2C). Heterogeneity was high (I²=99.7%, Cochran’s Q <0.01) with individual studies reporting total-MAFLD prevalence from 20% to 81%. There was significant funnel plot asymmetry (P=0.037), and after adjustment for publication bias, the adjusted prevalence estimate was 74.1% (95% CI 62.1–84.5%).

**MAFLD HCC phenotypes**

**MAFLD HCC with T2DM**

11 studies (comprising 32,169 MAFLD-related HCC cases) reported the prevalence of T2DM in individuals with MAFLD-related HCC (as a single or mixed aetiology), with a total pooled prevalence of 35% (95% CI 26–44%). Four studies (comprising 5,624 HCC cases) reported the prevalence of T2DM in single-aetiology MAFLD HCC cohorts, with a pooled prevalence of 58% (95% CI 35–80%). Nine studies (26,548 HCC cases) reported T2DM prevalence in mixed-aetiology MAFLD HCC cohorts with a pooled prevalence of 36% (95% CI 26–45%) (Supplementary Fig. 2).

**Lean MAFLD**

Nine studies (comprising 31,373 HCC cases) reported the proportion of individuals with a “lean” phenotype in MAFLD HCC cohorts (single or mixed aetiology), with a total pooled prevalence of 35% (95% CI 13–32%). Four studies (5,623 HCC cases) reported lean phenotype in individuals with single-aetiology MAFLD HCC with pooled prevalence of 30% (95% CI 23–38%). Seven studies (25,934 HCC cases) reported lean phenotype in mixed-aetiology MAFLD HCC with pooled prevalence of 44% (95% CI 34–54%) (Supplementary Table 4).
Figure 2. Forrest plots and pooled prevalence estimates for single (A), mixed (B), and total (C) MAFLD-HCC. MAFLD, metabolic dysfunction-associated fatty liver disease; HCC, hepatocellular carcinoma; CI, confidence interval.
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Table 1. MAFLD HCC prevalence

<table>
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<tr>
<th>Patient group</th>
<th>Sample size (number of studies)</th>
<th>MAFLD prevalence (%) (95% CI)</th>
<th>I² (%)</th>
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<td>Single-MAFLD HCC</td>
<td></td>
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<tr>
<td>Overall prevalence</td>
<td>39,381 (11)</td>
<td>12.4 (8.3–17.3)</td>
<td>99.1</td>
<td>&lt;0.01</td>
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<td>Geographical Region</td>
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<tr>
<td>Central America</td>
<td>547 (1)</td>
<td>30.0 (26.2–33.9)</td>
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<tr>
<td>Australia</td>
<td>225 (3)</td>
<td>19.4 (8.5–33.0)</td>
<td>78</td>
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<tr>
<td>North America</td>
<td>23,245 (1)</td>
<td>18.2 (17.7–18.7)</td>
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<td>Europe</td>
<td>8,736 (2)</td>
<td>11.5 (5.7–19.0)</td>
<td>97.3</td>
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<tr>
<td>Asia</td>
<td>6,628 (4)</td>
<td>5.3 (3.6–7.4)</td>
<td>89.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clinical phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAFLD with T2DM</td>
<td>5,691 (5)</td>
<td>52.1 (30.1–73.7)</td>
<td>97.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lean MAFLD</td>
<td>5,690 (5)</td>
<td>31.4 (24.9–38.4)</td>
<td>84.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mixed-MAFLD HCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall prevalence</td>
<td>51556 (17)</td>
<td>41.3 (34.3–48.5)</td>
<td>99.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Geographic region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North America</td>
<td>23,245 (1)</td>
<td>63.6 (63.0–64.2)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Europe</td>
<td>8,914 (4)</td>
<td>53.8 (43.2–64.3)</td>
<td>96.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Asia</td>
<td>19,260 (11)</td>
<td>37.2 (27.6–47.4)</td>
<td>99.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Australia</td>
<td>137 (1)</td>
<td>17.5 (11.6–24.4)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Primary liver disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>20,166 (12)</td>
<td>40.0 (30.2–50.3)</td>
<td>99.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HCV</td>
<td>13,090 (5)</td>
<td>54.2 (40.4–67.6)</td>
<td>99.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALD</td>
<td>6,841 (5)</td>
<td>64.3 (52.7–75.0)</td>
<td>97.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clinical phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAFLD with T2DM</td>
<td>26,548 (9)</td>
<td>35.6 (26.3–45.4)</td>
<td>97.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lean-MAFLD</td>
<td>25,934 (7)</td>
<td>21.2 (9.8–35.5)</td>
<td>99.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total-MAFLD HCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall prevalence</td>
<td>33,590 (8)</td>
<td>48.7 (34.6–63.0)</td>
<td>99.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total-MAFLD phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAFLD with T2DM</td>
<td>32,169 (11)</td>
<td>34.7 (25.9–44.0)</td>
<td>98.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lean-MAFLD</td>
<td>31,373 (9)</td>
<td>21.7 (12.6–32.6)</td>
<td>99.8</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

MAFLD, metabolic dysfunction-associated fatty liver disease; HCC, hepatocellular carcinoma; CI, confidence interval; T2DM, type 2 diabetes mellitus; HBV, hepatitis B virus; HCV, hepatitis C virus; ALD, alcohol-related liver disease.

MAFLD HCC with a pooled prevalence of 21% (95% CI 10–35%) (Supplementary Fig. 3).

Clinical characteristics of single-MAFLD, mixed-MAFLD and non-MAFLD HCC

Compared to individuals with non-MAFLD HCC, single-aetiology MAFLD HCC were older (MD 6.64 years, 95% CI 1.87–11.41), more likely to be female (OR 1.21, 95% CI 1.02–1.43) and less likely to have cirrhosis (OR 0.27, 95% CI 0.15–0.51). Single-aetiology MAFLD HCC was associated with lower AFP level (MD −166.34, 95% CI −265.15 to −67.52), a lower likelihood of macrovascular invasion (OR 0.78, 95% CI 0.62–0.97) and higher likelihood of metastatic disease (OR 2.03, 95% CI 1.48–2.79).

Single-MAFLD HCCs were also distinct from mixed-
### Table 2. Comparison of clinical characteristics between single-MAFLD, mixed-MAFLD, and non-MAFLD HCC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Single-MAFLD vs. mixed-MAFLD</th>
<th>Mixed-MAFLD vs. non-MAFLD</th>
<th>Single-MAFLD vs. non-MAFLD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample size (number of studies)</td>
<td>Effect size (95% CI)</td>
<td>P-value I²</td>
</tr>
<tr>
<td>Age</td>
<td>5,401 (3) MD 5.82 (–0.64 to 12.28)</td>
<td>0.077 97.7%</td>
<td>0.057 80.3%</td>
</tr>
<tr>
<td>Female (%)</td>
<td>5,401 (3) OR 1.22 (0.47 to 3.16)</td>
<td>0.68 93.5%</td>
<td>1.12 (0.67 to 1.87)</td>
</tr>
<tr>
<td>AFP</td>
<td>5,158 (2) MD –3.95 (–73.25 to 65.35)</td>
<td>0.91 68.5%</td>
<td>7,955 (5) MD –1.20 (–240.98 to 0.10)</td>
</tr>
<tr>
<td>Platelets</td>
<td>696 (2) MD 48.06 (–6.51 to 102.63)</td>
<td>0.084 88.1%</td>
<td>3,192 (4) MD 5.74 (0.44 to 11.05)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>5,327 (3) OR 0.24 (0.10 to 0.58)</td>
<td>0.002* 84.3%</td>
<td>10,008 (7) OR 1.05 (0.90 to 1.23)</td>
</tr>
<tr>
<td>Largest HCC diameter</td>
<td>4,948 (2) MD 0.08 (–0.99 to 1.15)</td>
<td>0.88 80.1%</td>
<td>8,861 (5) MD –0.17 (–0.36 to 0.01)</td>
</tr>
<tr>
<td>Macrovascular invasion</td>
<td>4,948 (2) OR 1.06 (0.88 to 1.28)</td>
<td>0.56 0.0%</td>
<td>7,279 (3) OR 0.63 (0.45 to 0.87)</td>
</tr>
<tr>
<td>Metastatic spread</td>
<td>4,705 (1) OR 1.71 (1.30 to 2.25)</td>
<td>&lt;0.001* N/A</td>
<td>6,021 (2) OR 0.84 (0.41 to 1.73)</td>
</tr>
</tbody>
</table>

MAFLD, metabolic dysfunction-associated fatty liver disease; HCC, hepatocellular carcinoma; CI, confidence interval; AFP, alpha fetoprotein; OR, odds ratio; MD, mean difference. *Statistical significance, P<0.05.
MAFLD HCCs in that they were less likely to have cirrhosis (OR 0.24, 95% CI 0.10–0.58), with higher likelihood of metastatic disease (OR 1.71, 95% CI 1.30–2.25).

Mixed-MAFLD was compared to non-MAFLD HCC. Mixed-MAFLD had higher platelet count (MD 5.74, 95% CI 0.44–11.05), with lower likelihood of macrovascular invasion (OR 0.63, 95% CI 0.45–0.87). There was a trend towards mixed-MAFLD having lower AFP levels than non-MAFLD that did not reach significance (–120.44 CI –240.98 to 0.10, $P=0.0502$). Data is shown in Table 2.

Sensitivity analysis and meta-regression

Prevalence estimates were repeated after excluding two studies in which investigators did not confirm the presence of hepatic steatosis. Updated prevalence estimates were 11.6% (95% CI 7.0–17.0%), 39.6% (95% CI 31.3–48.1%) and 40.9% (95% CI 28.2–54.3%) for single-MAFLD, mixed-MAFLD, and total-MAFLD HCC, respectively.

Meta-regressions were performed to identify study level factors which might explain the high between-study heterogeneity (Supplementary Figs. 4–6). For single-MAFLD HCC, studies conducted within Asia, early-stage HCC cohorts, younger cohorts, and cohorts with a higher proportion of HBV were associated with lower prevalence of single-MAFLD HCC. For mixed-MAFLD HCC, early-stage HCC cohorts, shorter duration of study enrolment, and younger cohort age were associated with lower mixed-MAFLD prevalence. For total-MAFLD HCC, higher proportion of HBV was associated with lower prevalence of MAFLD. Due to the relatively small number of studies in the sample size, multivariable meta-regressions were not performed. Residual heterogeneity remained high ($I^2 >95\%$) in all single meta-regressions. Bubble plots are shown in the supplemental material.

DISCUSSION

Researchers typically report aetiology in HCC cohorts by the single disease thought to represent the most likely cause of liver dysfunction. Dichotomising aetiology into distinct groups has made sense from a pragmatic viewpoint; however, with the increasing global prevalence of MAFLD, there is now the reality that a significant proportion of HCC occurs in the setting of multiple interacting liver diseases. Our understanding regarding MAFLD-related liver disease and HCC continues to evolve,$^{14,26}$ yet little is known about the prevalence, distinct mechanisms, outcomes, and responses to treatment in these mixed-MAFLD tumours. Thus, in this study, we provide the best global estimate for the total proportion of HCCs attributable to MAFLD, as both a single and mixed aetiology. We find that approximately half of patients with HCC have MAFLD; however, HCC in individuals who have MAFLD as the sole cause of liver disease accounts for only 12% (or 1 in 8 cases). MAFLD is a common HCC cofactor in individuals with all other liver disease aetiologies. Indeed, with advancements in screening and treatment for viral hepatitis, offset by the rising prevalence of obesity and metabolic syndrome, MAFLD may in fact be dominant driver of HCC in many cases which have previously been attributed to viral aetiologies.$^{27}$ We have also described the prevalence of different MAFLD-HCC phenotypes including MAFLD with T2DM and lean MAFLD. Lean-MAFLD in particular is purported to have distinct pathophysiological mechanisms and possibly worse outcomes in a non-HCC setting,$^{28}$ although data is limited in HCC cohorts. Notably, the proportion of MAFLD-HCC classified as “lean” that we have reported is similar to the 19.2% reported in a non-HCC setting by a recent large meta-analysis.$^{29}$

To our knowledge, there exists no previous systematic review and meta-analysis estimating the global proportion of HCC attributable to MAFLD, in which systematic ascertainment for MAFLD (using MAFLD diagnostic criteria) has taken place. Numerous cohort studies have reported the proportion of HCC attributable to NAFLD, as recently summarised in the review by Huang et al.$^6$ In addition to geographic variability, limiting generalisability is the substantial heterogeneity in how NAFLD has been defined, with estimated global proportions ranging from 1% to 38%.$^{30,31}$ Several large HCC cohorts have reported NAFLD prevalence using ICD coding$^{32}$ or transplant listing diagnosis,$^{33,34}$ but such cohorts typically lack systematic evaluation of NAFLD. NAFLD is also known to be underdiagnosed more broadly$^{35}$ and thus NAFLD burden in these cohorts is likely under-reported. Smaller studies which utilise radiologic or histologic criteria to diagnose NAFLD, with or without variable components of the metabolic syndrome, still rely on exclusion of other causes of liver
A number of studies have examined differences in clinical characteristics and outcomes of HCC between different aetiologies of liver disease. A recent meta-analysis by Tan et al. compared NAFLD HCC (as defined by imaging, histology or ICD coding in the absence of significant alcohol consumption or other causes of chronic liver disease) to other causes of HCC. This study reported larger and more frequently uninodular tumours in NAFLD-HCC, but no difference in AFP, tumour stage, treatment allocation or overall survival. However, as we have shown in our meta-analysis, there is significant overlap between MAFLD and other causes of HCC which may make interpretation of this comparison difficult. Thus, in order to further explore this mixed-MAFLD phenotype, we performed a three-way comparison between single-MAFLD, mixed-MAFLD, and non-MAFLD. We report a number of phenotypic differences between the study subgroups. Consistent with Tan et al., single-MAFLD HCC tended to occur in subjects who were older with non-cirrhotic liver disease compared to non-MAFLD HCC. We also report findings to suggest differences in tumour biology and behaviour, including a lower AFP level, higher likelihood of metastatic spread, and lower likelihood of macrovascular invasion.

Importantly, we also sought to find differences between non-MAFLD and mixed-MAFLD HCC, in order to determine whether the addition of MAFLD as a co-factor to another liver disease might influence clinical phenotype or tumour biology. One might hypothesise that our finding of a higher platelet count in mixed-MAFLD compared to non-MAFLD HCC may reflect a propensity for the addition of MAFLD to cause HCC at an earlier stage of liver disease. Likewise, the lower likelihood of macrovascular invasion and trend towards lower AFP suggests these mixed-aetiology tumours may have different molecular and biological behaviour compared to “pure” non-MAFLD HCC.

The “mixed-MAFLD” category used in this analysis itself represents a heterogenous group comprised of various primary liver disease aetiologies, each with their own distinct pathological mechanisms. Nevertheless, the idea that the addition of MAFLD to another liver disease aetiology might alter tumour biology is intriguing, and at the very least, adds to the impetus to accurately diagnose and report MAFLD in future research studies so that the interaction between liver diseases can be better understood.

This study has several strengths. We analysed data from 22 studies and over 56,000 HCC cases in order to provide the best current global estimate for MAFLD prevalence in individuals with HCC. We included only studies which diagnosed MAFLD using the international expert panel criteria including use of ethnicity-specific BMI cut-offs. The objective diagnostic criteria eliminate subjectivity from diagnosis (such as estimating alcohol intake) and theoretically produce a more replicable prevalence estimate.

Nevertheless, our study has limitations. There were relatively few studies reporting MAFLD prevalence from certain geographic regions (most notably North America); thus these prevalence estimates require further validation.
and should be interpreted with caution. Secondly, few (if any) studies published data on all metabolic variables which make up the MAFLD diagnostic criteria. We included studies which defined metabolic dysfunction using a minimum of BMI and T2DM data. Of note, data from a recent large Korean MAFLD cohort suggested the non-overweight, non-diabetic phenotype accounted for only 5% of individuals with MAFLD, and thus our inclusion criteria likely capture the majority of individuals with MAFLD but may still underestimate the true prevalence. Thirdly, the method of assessing hepatic steatosis was variable across studies which may account for some heterogeneity in MAFLD prevalence estimates. In particular, two large multicentre cohorts (Italian ITA.LI.CA. HCC registry and U.S. United Network for Organ Sharing [UNOS] liver transplant registry) defined MAFLD by metabolic dysfunction alone without confirmation of present or historical hepatic steatosis. It is notable that most mixed-MAFLD HCC cases in these large Western cohorts (87% and 94%, respectively) were metabolically unhealthy individuals in the setting of either ALD or HCV infection, in whom the vast majority of individuals would be expected to have current or historical evidence of hepatic steatosis; nevertheless, these studies may have overestimated MAFLD prevalence. We have reported a sensitivity analysis excluding these studies. Data is also still lacking on HCC outcomes between these groups and is an area that requires further research.

In conclusion, MAFLD is common among individuals with HCC, both as the sole cause of liver disease and as a co-factor with other liver diseases. This study supports the benefit of systematically ascertaining for metabolic dysfunction using positive diagnostic criteria, irrespective of alcohol use or other liver disease, so that the interaction between concurrent liver diseases can be better understood.

**Authors’ contribution**

Study concept and supervision: HC, GE, JG. Study design: HC, GE, JG. Data analysis: HC, GE. Data collection: All authors. Drafting of manuscript: HC, JG. Data interpretation, review, and revision of manuscript: All authors.

**Acknowledgements**

The authors would like to acknowledge the NSW Government through the Cancer Institute NSW for grant funding that supported this work (2021/ATRG2028). JG is supported by the Robert W. Storr Bequest to the Sydney Medical Foundation, University of Sydney; a National Health and Medical Research Council of Australia (NHMRC) Program Grant (APP1053206), Project, Ideas and Investigator grants (APP2001692, APP1107178, APP1108422, APP1196492) and a Cancer Institute, NSW grant (2021/ATRG2028).

**Conflicts of Interest**

JG is on Advisory Boards and has received honoraria for talks from Novo Nordisk, Astra Zeneca, Roche, BMS, Pfizer, Cincera, Pharmaxis, Boehringer Mannheim.

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

**REFERENCES**


Ursolic acid targets secreted phosphoprotein 1 to regulate Th17 cells against metabolic dysfunction-associated steatotic liver disease

Yiyuan Zheng1,*, Lina Zhao2,3,*, Zhekun Xiong4,*, Chaoyuan Huang5,6, QiuHong Yong6,7, Dan Fang8, Yuguang Fu1, Simin Gu1, Chong Chen1, Jiacheng Li1, Yingying Zhu1, Jing Liu1, Fengbin Liu2,3, and Yong Li1

1Department of Gastroenterology, Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China; 2Department of Gastroenterology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China; 3Lingnan Medical Research Center, Guangzhou University of Chinese Medicine, Guangzhou, China; 4Department of Spleen, Stomach and Hepatobiliary, Zhongshan Hospital of Traditional Chinese Medicine, Zhongshan, China; 5Department of Gastroenterology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China; 6Department of Gastroenterology, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou, China; 7The First Clinical Medical School, Guangzhou University of Chinese Medicine, Guangzhou, China; 8Medical Affairs Department, Ton-Bridge Medical Technology Co., Ltd., Zhuhai, China

Graphical Abstract

Study Highlights
- Ursolic acid can directly bind with SPP1 to regulate its activity.
- SPP1 promotes Th17 cell differentiation via interactions with ITGB1 and CD44.
- Ursolic acid ameliorates immune inflammation in MASLD by modulating SPP1-mediated Th17 cell differentiation.
Background/Aims: Metabolic dysfunction-associated steatotic liver disease (MASLD) has become an increasingly important health challenge, with a substantial rise linked to changing lifestyles and global obesity. Ursolic acid, a natural pentacyclic triterpenoid, has been explored for its potential therapeutic effects. Given its multifunctional bioactive properties, this research further revealed the pharmacological mechanisms of ursolic acid on MASLD.

Methods: Drug target chips and bioinformatics analysis were combined in this study to explore the potential therapeutic effects of ursolic acid on MASLD. Molecular docking simulations, surface plasmon resonance analyses, pull-down experiments, and co-immunoprecipitation assays were used to verify the direct interactions. Gene knockdown mice were generated, and high-fat diets were used to validate drug efficacy. Furthermore, initial CD4+ T cells were isolated and stimulated to demonstrate our findings.

Results: In this study, the multifunctional extracellular matrix phosphorylated glycoprotein secreted phosphoprotein 1 (SPP1) was investigated, highlighting its capability to induce Th17 cell differentiation, amplifying inflammatory cascades, and subsequently promoting the evolution of MASLD. In addition, this study revealed that in addition to the canonical TGF-β/IL-6 cytokine pathway, SPP1 can directly interact with ITGB1 and CD44, orchestrating Th17 cell differentiation via their joint downstream ERK signaling pathway. Remarkably, ursolic acid intervention notably suppressed the protein activity of SPP1, suggesting a promising avenue for ameliorating the immunoinflammatory trajectory in MASLD progression.

Conclusions: Ursolic acid could improve immune inflammation in MASLD by modulating SPP1-mediated Th17 cell differentiation via the ERK signaling pathway, which is orchestrated jointly by ITGB1 and CD44, emerging as a linchpin in this molecular cascade. (Clin Mol Hepatol 2024;30:449-467)

Keywords: Metabolic dysfunction-associated steatotic liver disease; Ursolic acid; Secreted phosphoprotein 1; Th17 cells

INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) is typified as a multi-systemic metabolic stress-induced hepatic derangement, with its spectrum encompassing simple steatosis, metabolic dysfunction-associated...
steatohepatitis (MASH), and concomitant hepatic fibrosis and cirrhosis, culminating in the potential development of hepatocellular carcinoma. With global obesity trends driven by evolving lifestyles, the incidence of MASLD has been steadily increasing over the past decades. Epidemiological surveys indicate a staggering global prevalence of MASLD approaching 25%, with an estimated 20% having the propensity to evolve into MASH, posing significant health risks and substantial economic burdens on society. Most notably, a cohort study underscores that even mild steatosis can markedly augment all-cause mortality risk, which is also tightly correlated with disease severity. Contrastingly, against this dire backdrop, existing therapeutic approaches for MASLD have been underwhelming, amplifying the urgency for innovative drug exploration.

Ursolic acid, a pentacyclic triterpenoid naturally ubiquitously distributed amongst diverse plant species, is rendered particularly noteworthy owing to its intrinsic chemical structure bestowing upon it a gamut of unique bioactivities, including anti-inflammatory, anti-oxidative and anti-tumour properties. In recent years, this natural triterpenoid has manifested exceptional potential in drug development for a plethora of ailments, and researches have spotlighted its multifarious roles in lipid regulation, inflammatory and oxidative damage mitigation, as well as in gut microbiota modulation, collectively offering a bulwark against MASLD. Nonetheless, considering its multifunctional bioactive properties, unveiling the definitive pharmacological mechanisms of ursolic acid on MASLD remains paramount.

While the precise pathogenesis of MASLD is still not fully elucidated, current researches emphasize the intricate interplay of metabolic stress, immune inflammation, hypoxic injury, insulin resistance and gut microbiota dysbiosis. Of note, the dynamic equilibrium in hepatic immune homeostasis between helper T (Th) 17 cells and regulatory T (Treg) cells emerges as a cardinal nexus mediating immune stress and inflammatory responses, hence garnering widespread scrutiny. Particularly, Th17 cells, a subtype of CD4+ T-helper cells, can evolve under stimuli like transforming growth factor (TGF)-β and interleukin (IL)-6, subsequently secreting IL-17 and other critical inflammatory cytokines, thereby amplifying inflammatory cascades, and further galvanizing stellate cell activation and fibrogenesis. Given that cutting-edge discoveries accentuate the centrality of secretory factor-mediated intercellular cross-talk in metabolic inflammatory responses, this present investigation directs its focus towards secreted phosphoprotein 1 (SPP1), alternatively known as osteopontin, through the integration of bioinformatics analyses and drug target chip assays.

As a crucial component of the extracellular matrix (ECM), SPP1 is a multifunctional phosphorylated glycoprotein, secreted by a myriad of cells, including bone marrow cells, activated T cells, macrophages, endothelial cells, parenchymal cells, etc. With numerous phosphorylation and glycosylation motifs, SPP1 partakes in modulating cell adhesion and migration, immune inflammation and tissue remodelling, holding a position of significant importance within the hepatic microenvironment. The latest studies demonstrate that SPP1 is able to induce the differentiation of Th17 cells while inhibiting their apoptosis to maintain an immune-inflammatory state, subsequently facilitating the activation of hepatic stellate cells (HSCs) and promoting the progression from MASLD to MASH and liver fibrosis. Intriguingly, this current investigation discerns that apart from the canonical TGF-β/IL-6 cytokine pathway, SPP1 can also directly bind to the cell surface receptors, integrin β1 (ITGB1) and CD44, inducing the differentiation of Th17 cells by modulating their joint downstream extracellular signal-regulated kinase (ERK) signaling pathway. Moreover, the therapeutic intervention with ursolic acid suppresses the protein activity of SPP1, thereby ameliorating the immunoinflammatory state during MASLD progression.

**MATERIALS AND METHODS**

**Bioinformatic analysis**

Expression profiles pertinent to nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) were systematically retrieved from the Gene Expression Omnibus (GEO) database, utilizing the search parameters: (“fatty liver”[MeSH Terms] OR fatty liver[All Fields] OR NAFLD[All Fields] OR NASH[All Fields]) AND “Homo sapiens”[porgn]. Exclusion criteria encompassed datasets deemed as having irrelevant data, an absence of controls, or a predominance of post-operative subjects. Following a stringent review, two microarrays, GSE49541 and
GSE89632, were meticulously selected, which notably delineated the intricate comparative stages between NAFLD and NASH. To ensure coherency and mitigate potential variances arising from differing experimental conditions or platforms, batch normalization was judiciously executed on all integrated microarray datasets using the limma package in R version 4.0 (R Foundation, Vienna, Austria). The integrated datasets were subsequently processed to discern differentially expressed genes, and those with adjusted $P$-values below 0.05 were designated as statistically significant.

To acquire a holistic understanding of the biological functions and pathways associated with the identified differentially expressed genes, enrichment analysis was performed. Leveraging the Bioconductor packages, specifically org.Hs.eg.db and clusterProfiler, key pathways and functional categories were elucidated. A comprehensive protein-protein interaction network was meticulously constructed using the Search Tool for the Retrieval of Interacting Genes (STRING) database. This protein-protein interaction (PPI) network served as an invaluable framework, providing insights into the functional relationships between identified proteins, while centrality analysis, pivotal for discerning nodes of paramount importance within the network, was systematically executed based on the intricate interaction relationships between nodes.

**Drug-targets interaction analysis**

An extensive probe into the interactions between ursolic acid and potential protein targets was carried out utilizing the HuProt™20K human proteomics microarray. Ursolic acid was conjugated with biotin, generating a bio-ursolic acid probe complex. In strict adherence to the protocols delineated by Wayen Biotechnologies Co. Ltd., the chip was hybridized with both bio-ursolic acid and its biotin control. Ensuing incubation, the chip underwent analysis with a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA), and subsequent data interpretation was executed employing GenePix Pro v6.0.

The interactions between ursolic acid and specific binding proteins were verified by Surface Plasmon Resonance technology using a biacore sensor chip (28980887; Univbio, Shanghai, China). Target proteins were diluted and coupled under fixed time conditions, with ethanolamine addressing any unbound activation groups. Subsequently, varying concentrations of bio-ursolic acid were introduced, employing a running buffer as control. Parameters included a flow rate of 30 μL/min, along with binding and dissociation phases of 90 s and 120 s, respectively. Data acquisition followed a multi-cycle approach, and post double reference deduction, data extrapolation was conducted using the Biacore T200 analysis software (Cytiva, Marlborough, MA, USA).

Molecular docking simulations were executed to predict the preferential binding orientations and affinities of ligands within the active site of a target protein. The three-dimensional structure of the protein target was sourced from the Protein Data Bank and processed to remove water molecules, add missing hydrogens, and optimize the protonation states of amino acid residues. Using the AutoDock Vina software (Scripps Research, La Jolla, CA, USA) suite, grid boxes were set up around the active site with dimensions tailored to encompass key residues and potential ligand binding sub-sites. The ligands were prepared by assigning Gasteiger charges, generating 3D conformations, and optimizing their geometries. The docking procedure was executed employing the Lamarckian Genetic Algorithm, and the generated docking poses were ranked based on the Vina scoring function. To account for flexibility, the side chains of the residues within a defined radius of the active site were set as flexible. The top-ranked poses, determined by binding energy scores, were visualized and analyzed in PyMOL (DeLano Scientific, San Carlos, CA, USA) and further subjected to post-docking validation using molecular dynamics simulations to ensure the stability and feasibility of the predicted complexes.

**Animal model**

Six-week-old male C57BL/6J mice were obtained from the Medical Experimental Animal Center of Guangdong Province. Pursuant to rigorous ethical considerations, all animal experiments were conducted in strict accordance with project licenses (TCMF1-2021001 and TCMF1-2022052), which received the endorsement of the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine. Furthermore, all procedures adhered faithfully to the ARRIVE guidelines to ensure the humane treatment of the animals.
All animals were housed under standard environment and had access to food and water ad libitum. After one-week acclimatization, these mice were randomized equally into different groups, and were reared under a controlled condition. These mice were fed with high-fat diets (D12492; Research Diets, New Brunswick, NJ, USA) as well as normal control diets (NCD, D12450J, Research Diets) for 12 weeks to construct MASLD mice model, and ursolic acid (U820363, Macklin, Shanghai, China) was given to the treatment group by oral gavage at doses of 25, 50, and 100 mg/kg/d as intervention therapy, while normal saline was administered daily to the HFD and control groups.

Recombinant adeno-associated virus serotype 8 (AAV2/8) vectors were employed for in vivo gene delivery. Specifically, AAV2/8 harboring a CMV-driven shRNA targeting SPP1 (sequence: 5’-GATGACTTTAAGCAAGAAA-3’) and the control AAV2/8-CMV-Null were obtained from Obio Technology Co. Ltd., AAV vectors were propagated in HEK293T cells, purified, and then concentrated to achieve a final titer of approximately 1x10^{13} genome copies per milliliter. Adult male C57BL/6 mice, aged between 8–10 weeks, were acclimated and then randomly assigned to either the AAV2/8-shSPP1 or AAV2/8-Null groups. For delivery, 100 μL of the respective virus preparation was intraperitoneally injected into each mouse. Following the injection, mice were closely monitored, and tissue collection for assessment of AAV-mediated gene knockdown was typically conducted 4 weeks post-injection, a time frame optimized for peak AAV-mediated transgene expression in the liver.

A systematic documentation approach was implemented, wherein body weights of the subjects were recorded on a weekly basis. Upon the culmination of the experiment, liver weights were accurately determined. Extracted liver samples were either immediately snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde (PFA, BL539A; Biosharp, Beijing, China), earmarked for subsequent analyses.

**Cell culture**

Mice were euthanized in accordance with institutional and ARRIVE guidelines. Spleens were aseptically harvested from euthanized C57BL/6J mice. Following mechanical disruption, single-cell suspensions were obtained by passing the tissue through a 70 μm cell strainer (258368; NEST, Wuxi, China). Red blood cells were lysed using ACK lysis buffer (A1049201; Invitrogen, Waltham, MA, USA), and the splenocytes were washed twice with phosphate buffered saline (PBS, BL302A; Biosharp). CD4^{-} T cells were isolated using magnetic-activated cell sorting with a Mouse CD4^{-} T cell Isolation Kit (130-104-454; Miltenyi, Auburn, CA, USA), as per the manufacturer’s guidelines. Subsequently, for Th17 cell differentiation, naïve CD4^{-} T cells were cultured in RPMI 1640 medium (C11875500BT; Invitrogen) supplemented with 10% fetal bovine serum (FBS, 10091-148; Invitrogen), 1% penicillin-streptomycin (15140-122; Invitrogen), and activated with plate-bound anti-CD3 (2 μg/mL) and anti-CD28 (1 μg/mL) antibodies (11453D; eBioscience, Carlsbad, CA, USA). The culture medium was supplemented with IL-6 (20 ng/mL, AF-216-16-50; Peprotech, Rocky Hill, NJ, USA), TGF-β (3 ng/mL, RP02523; ABclonal, Wuhan, China), anti-IFN-γ (2 μg/mL, 16-7311-85; eBioscience) and anti-IL-4 (2 μg/mL, 14-7041-85; eBioscience) to promote Th17 differentiation.

Upon initiating Th17 polarization, the cells were concurrently treated with various concentrations of recombinant SPP1 protein (RP02806; ABclonal) and ursolic acid. To assess the influence of ITGB1 and CD44 modulation on Th17 differentiation, cells were additionally co-incubated with ITGB1 inhibitor GLPG0187 (1 nM, HY-100506; MedChem-Express, Monmouth, NJ, USA) or CD44 antagonist (1 μg/mL, 553131; BD Biosciences, San Jose, CA, USA) at specific concentrations recommended by preliminary dose-response studies. After a 72-hour incubation at 37°C in a 5% CO_{2} humidified atmosphere, cells were harvested for further assays. Finally, Th17 cell differentiation efficiency was evaluated by intracellular staining of IL-17A followed by flow cytometry analyses.

**IPGTT and ITT**

During the last week of the experiment, mice were fasted for 6 hours with water provided ad libitum. Prior to glucose administration, a baseline blood sample was obtained via tail vein nick for the determination of fasting blood glucose levels. Mice were then intraperitoneally injected with a solution of glucose (2 g/kg body weight, G8270; Sigma, St. Louis, MO, USA) dissolved in sterile saline. Blood glucose measurements were taken at intervals of 15, 30, 60, 90, and 120 minutes post-injection to monitor the glucose
clearance rate. After a one-day adaption, following a 4-hour fasting period, mice received intraperitoneal injections of insulin (0.75 IU/kg body weight, 91077C; Sigma), and the glucose levels were determined as described above.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Liver tissues were carefully dissected and immediately immersed in TRIzol reagent (15596026; Invitrogen) for RNA extraction, in accordance with the manufacturer’s instructions, while the concentrations were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Sunnyvale, CA, USA), with A260/A280 ratios between 1.8 and 2.0 considered acceptable for subsequent applications. Complementary DNA (cDNA) was synthesized using the PrimeScript RT Reagent Kit (RR036A; Takara, Shiga, Japan), while TB Green Premix Ex Taq II (RR420A; Takara) was used to conduct qRT-PCR based on Bio-Rad CFX96 Real-Time PCR System (Hercules, CA, USA). Thermal cycling conditions encompassed an initial denaturation phase at 95°C for 30 s, followed by 40 amplification cycles at 95°C for 5 s and 60°C for 30 s. The relative mRNA levels of target genes were calculated by $2^{-\Delta\Delta Ct}$, and beta-actin was used to normalize the samples. All primer sequences used in this study are listed in Supplementary Table 1.

**Western blotting**

Liver tissues were meticulously dissected and homogenized in RIPA lysis buffer (P0013B; Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitors (P1045; Beyotime) to ensure the retention of protein integrity. The homogenates were centrifuged at 14,000 x g for 15 minutes at 4°C. Supernatants were harvested, and the protein concentrations were determined using a BCA protein assay kit (P0010; Beyotime). Equal amounts of protein from each sample were mixed with loading buffer and boiled for 5 minutes to denature. The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels with appropriate concentrations, depending on the molecular weight of the target protein. Separated proteins in the gel were transferred onto polyvinylidene fluoride (PVDF) membranes (ISEQ00010; Millipore, Billerica, MA, USA) using a wet transfer system. The membranes were blocked in 5% milk (232100; BD Biosciences) dissolved in Tris-buffered saline containing 0.1% Tween-20 (ST825; Beyotime) for 1 hour at room temperature to prevent non-specific binding. The membranes were then incubated with primary antibodies specific to the target proteins overnight at 4°C. Following primary antibody incubation, the membranes were washed thrice with TBST and then incubated with corresponding secondary antibodies for 1 hour at room temperature. Following another series of washes, the bound antibodies were visualized using an enhanced chemiluminescence (ECL, 1705060; Bio-Rad) detection system. Images were captured by a ChemiDoc XRS System (Bio-Rad), and the intensity of the bands was quantified using ImageJ 1.8.0 (National Institutes of Health, Bethesda, MD, USA). To ensure accurate quantification, beta-actin was used as an internal control.

Primary antibodies against ITGB1 (1:2,000, 34971), ITGB3 (1:2,000, 13166), p-ERK (1:2,000, 4370), ERK (1:2,000, 4695) and beta-actin (1:2,000, 4970) were obtained from Cell Signaling Technology, against CD44 (1:1,000, ab189524) were purchased from Abcam, and against SPP1 (1:2,000, sc-21742) were purchased from Santa Cruz Biotechnology.

**Histological staining**

Liver specimens from the murine cohort underwent two distinct histological procedures for optimal morphological evaluation. Frozen liver sections were air-dried and fixed in 10% neutral buffered formalin (RL3223; Bioroyee, Beijing, China). Following fixation, sections underwent Oil Red O (C0157S; Beyotime) staining to visualize lipid droplets. Afterward, sections were rinsed and counterstained with Mayer’s hematoxylin and subsequently mounted with an aqueous mounting medium. Finally, images were observed on an Olympus BX-50 microscope (Tokyo, Japan), and for quantification of lipid accumulation, the percentage of the Oil Red O-positive area to the total area was calculated using ImageJ software.

Liver sections from paraffin blocks were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E, G1005; Servicebio, Wuhan, China) referring to the stand-
ard procedure. Following staining, the MASLD activity score was measured to assess the severity of steatosis (graded 0-3 based on the percentage of hepatocytes containing fat), lobular inflammation (graded 0-3 based on the number of inflammatory foci), and hepatocellular ballooning (graded 0-2). Thus, the cumulative score provided an overall assessment of liver pathology.

An immunohistochemistry kit (G1215; Servicebio) was applied in this research. According to the relevant protocols, liver tissues were cut into 4 μm sections from the paraffin blocks. Deparaffinized and hydrated were conducted, and sodium citrate solution was used for antigen retrieval. Liver sections were immune-stained with antibodies against SPP1 (1:50), ROR gamma (1:100, abs124841; Absin, Shanghai, China), or IL-17A (1:100, ab79056; Abcam, Cambridge, UK), and the colour reaction was developed using biotinylated immunoglobin G, horseradish peroxidase-streptavidin and diaminobenzidine. Finally, images were observed on an Olympus BX-50 microscope, and the positive staining area and intensity for each marker were quantified using ImageJ software, which was calibrated against control sections.

Flow cytometry

Liver tissues from mice were harvested and immediately frozen in liquid nitrogen to preserve cell integrity. These frozen tissues were then mechanically disrupted using a mortar and pestle under liquid nitrogen conditions, ensuring the tissues were kept thoroughly frozen throughout the process to minimize cell damage. The powdered liver tissue was then gently resuspended in a cold PBS buffer supplemented with 2% FBS to generate a single-cell suspension. Following thawing, the single-cell suspensions were filtered through a 70 μm cell strainer to eliminate large tissue debris and to obtain a cleaner cell population.

Before proceeding to stain, cells were resuspended in an RPMI 1640 medium and stimulated with phorbol myristate acetate and ionomycin (110007; Perseprch, China) in the presence of brefeldin A (110008; Perseprch) for 6 hours at 37°C in a 5 % CO₂ atmosphere. This stimulation step ensured that cytokines, especially IL-17A, were retained inside the cells, allowing for intracellular staining. Subsequently, cells were blocked with anti-mouse CD16/CD32 (Fc Block, 553141; BD Biosciences) for 15 minutes at 4°C to minimize nonspecific antibody binding. Subsequently, cells were stained with fluorescence-conjugated antibodies against CD3 (HT1610025; Otwo, Shenzhen, China) and CD4 (HT1610043; Otwo) for 30 minutes in the dark at 4°C. For intracellular staining of IL-17A, cells previously stained with surface markers were fixed and permeabilized using the Cytotix/Cytoperm Fixation and Permeabilization Solution (554714; BD Biosciences). Post-permeabilization, cells were stained with a fluorescence-conjugated antibody against IL-17A (HT1610485; Otwo) for 30 minutes in the dark at 4°C. The stained cells were then washed and re-suspended in the staining buffer, after which they were subjected to flow cytometric analysis. Finally, data acquisition was conducted on NovoCyte D2060R (Agilent, Palo Alto, CA, USA), and the analyses were performed by FlowJo v10.8 (BD Biosciences).

Pull-down assay

The Desthiobiotinylation and Pull-Down Kit (P0637S; Beyotime) was used in this study for the co-immunoprecipitation assay. Ursolic acid-conjugated agarose beads were prepared by covalently linking ursolic acid to activated agarose beads using the manufacturer’s protocol. Unreacted sites on the beads were blocked using a quenching agent, and the beads were washed thoroughly to remove any unbound ursolic acid. Fresh liver tissues were homogenized in a lysis buffer enriched with protease and phosphatase inhibitors. Gentle mechanical homogenization was implemented to ensure optimal tissue disruption. The homogenates were centrifuged at 12,000 × g for 10 minutes at 4°C, and the clarified supernatants were collected. The ursolic acid-conjugated agarose beads were added to the liver lysates and incubated overnight at 4°C on a rotating platform; this allowed for the specific binding of SPP1 or other potential interacting proteins to ursolic acid. After incubation, the beads were pelleted by brief centrifugation. The cells were then washed multiple times with lysis buffer to remove non-specifically adhered proteins. For protein elution, beads were resuspended in Laemmli buffer and boiled for 5 minutes. Eluted proteins were then separated using SDS-PAGE and transferred onto PVDF membranes for Western blotting.
Co-immunoprecipitation assay

The rProtein A/G Magnetic IP/Co-IP Kit (abs9649; Absin) was employed for the co-immunoprecipitation assay. Liver tissues and Th17 cells were initially lysed in a RIPA buffer supplemented with protease and phosphatase inhibitors to ensure the preservation of protein integrity. Tissue disruption was facilitated using gentle mechanical homogenization, ensuring uniform lysis. The resultant lysates were centrifuged at 12,000 x g for 10 minutes at 4°C, and the supernatants were collected for subsequent procedures. For pre-clearing, each supernatant was incubated with protein A/G PLUS-agarose beads for 1 hour at 4°C on a rotator. Post-incubation, the samples were centrifuged to pellet the agarose beads, and the pre-cleared supernatants were collected. SPP1-specific antibody (1 µg per 100 µg of total protein) was added to the pre-cleared lysates, followed by overnight incubation at 4°C on a rotating platform to enable the formation of immunocomplexes. Post-overnight incubation, protein A/G PLUS-agarose beads were added to the lysate-antibody mixture, followed by a further 2-hour incubation at 4°C. The agarose beads, now bound to the immunocomplexes, were subsequently pelleted through centrifugation. The pelleted beads were washed thrice with the RIPA buffer to remove non-specifically bound proteins. After the final wash, the beads were resuspended in Laemmli buffer and boiled for 5 minutes to elute the immunocomplexed proteins. Finally, SDS-PAGE and Western blotting procedures were employed to detect the presence of ITGB1, ITGB3, and CD44 in the eluted protein complexes.

Statistical analysis

All quantitative data generated during this study were collated and represented as mean ± standard error of the mean. The GraphPad Prism 7.0 software suite (GraphPad Software, San Diego, CA, USA) was enlisted for the entirety of our data visualization and subsequent statistical computations. One-way analysis of variance was conducted for data analyses among groups, and P-value <0.05 was considered statistically significant.

RESULTS

Ursolic acid exerts multifaceted therapeutic effects on MASLD

Our previous studies have demonstrated that ursolic acid has the capability to modulate the protein activity of decorin, subsequently regulating the IGF-IR and HIF-1 signaling pathways, thereby furnishing a dual protective effect against metabolic dysfunction and hepatic hypoxia during the progression of MASLD. Nevertheless, given the multifunctional biological activities associated with ursolic acid, we are inclined to further delve into its myriad pharmacological mechanisms. In this experimental research, the therapeutic efficacy of ursolic acid against MASLD-induced metabolic stress and immune inflammation was revalidated using a high-fat diet (HFD) mouse model. The results illustrated that intervention treatment with ursolic acid effectively alleviated weight gain induced by the high-fat diet in a dose-dependent manner, with statistically significant differences in weight emerging from the sixth week between the model and high-dose treatment groups and from the eighth and tenth weeks with the medium- and low-dose treatment groups, respectively (Fig. 1A). By the termination of this experiment at the twelfth week, liver weight measurements exhibited a notable reduction in both liver weight and liver index, calculated by the ratio of liver to body weight, following ursolic acid intervention (Fig. 1B); concurrently, evaluations on both serum and hepatic lipid concentrations underscored its role in improving lipid metabolism (Fig. 1C). Additionally, serum alanine aminotransferase and aspartate aminotransferase assays depicted a significant improvement in liver dysfunction, highlighting the feasibility of employing ursolic acid in the treatment of MASLD. Intrapерitoneal glucose tolerance tests (IPGTT) and insulin tolerance tests (ITT) confirmed that the high-fat diet compromised glucose tolerance and precipitated insulin resistance in mice, both of which were critical pathological foundations for MASLD, while the intervention treatment of ursolic acid rendered protection against these perturbations (Fig. 1D and Supplementary Fig. 1A). Histopathological staining of liver tissues demonstrated pronounced lipid droplet accumulation, formation of fatty vacuoles and inflammatory cell infiltration in mice subjected to the high-fat diet, and intervention with ursolic acid could offer therapeutic effects.
against all of these conditions, with the MASLD activity score, an indicator to assess the progression of MASLD, showcasing the most significant therapeutic effects in the high-dose treatment group (Fig. 1E). Lastly, serum inflammatory cytokine assays accentuated the remarkable anti-inflammatory attribute of ursolic acid, evidenced by the marked reduction in pro-inflammatory cytokines (Fig. 1F). Further assessments of gene expression levels of inflammatory cytokines in liver tissues showed that intervention with ursolic acid had the most notable improvement effects on TGF-β, IL-1β, IL-6, IL-17A, and IL-23, manifesting that even low-dose intervention treatment could significantly reduce the levels of all these genes (Supplementary Fig. 1B).

**Identification of SPP1 as a direct ursolic acid-binding protein**

To further elucidate the therapeutic mechanisms by which ursolic acid addresses NAFLD, we initiated our investigative endeavor by querying the GEO database platform for human transcriptomic microarrays associated with NAFLD. Given the pronounced anti-inflammatory effects of ursolic acid, our exploration was sharply focused on immune inflammation, a pivotal juncture in the progression

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**Figure 1.** Ursolic acid exerts multifaceted therapeutic effects on MASLD. (A) Body weights were recorded weekly. (B) Liver weights were measured by the termination of this experiment at the twelfth week, and liver indexes were calculated by the ratio of liver to body weight. (C) Serum and hepatic lipid concentrations were determined by an automatic biochemical analyzer. (D) IPGTT and ITT were performed to monitor glucose tolerance and insulin resistance. (E) Histological staining of H&E and oil red O in liver tissues was used to observe the inflammatory cytokine infiltration and lipid droplet accumulation. The MASLD activity scores were computed by steatosis, intralobular inflammation, and hepatocyte ballooning to assess disease severity. The positive area of Oil red O staining was estimated for quantification of lipid accumulation. (F) Serum inflammatory cytokines were detected to detect inflammatory reactions. MASLD, metabolic dysfunction-associated steatotic liver disease; IPGTT, intraperitoneal glucose tolerance tests; ITT, insulin tolerance tests; SD, standard deviation; HFD, high-fat diets; NCD, normal control diets. Data are represented as mean±SD. n=6-8. *P<0.05, **P<0.01, ***P<0.001.
Identification of SPP1 as a direct ursolic acid-binding protein. (A) Bioinformatic analysis was conducted in this study, 106 DEGs were identified from two microarrays, which notably delineated the intricate comparative stages between NAFLD and NASH, and a volcano plot was plotted. A PPI network was constructed for these DEGs using the STRING database, providing insights into the functional relationships between identified proteins. And centrality analysis, pivotal for discerning nodes of paramount importance within the network, was systematically executed based on the intricate interaction relationships between nodes. A pathway enrichment was performed to acquire a holistic understanding of the biological functions and pathways associated with the DEGs. (B) A schematic diagram showed that biotin-labeled ursolic acid was co-incubated with this array, followed by signal interpretation using Cy3-streptavidin. (C) 502 targets were identified that interacted directly with ursolic acid, and intersection analysis with the previously identified DEGs retained five candidates, wherein the differentially binding proteins were presented in the proteomics chip and heatmap. (D) The SPR analysis was produced using a biacore sensor chip, wherein the molecular dynamics analysis revealed that ursolic acid and SPP1 exhibited high-affinity interaction, with the equilibrium dissociation constant of 1.705E-5. (E) A pull-down assay was performed to further verify the binding affinity between ursolic acid and SPP1 in vivo. (F) The molecular docking simulation showed a mutual binding between ursolic acid and SPP1, with a binding energy of -5.8. SPP1, secreted phosphoprotein 1; DEGs, differentially expressed genes; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; STRING, Search Tool for the Retrieval of Interacting Genes; SPR, surface plasmon resonance; KD, knockdown.

Figure 2. Identification of SPP1 as a direct ursolic acid-binding protein. (A) Bioinformatic analysis was conducted in this study, 106 DEGs were identified from two microarrays, which notably delineated the intricate comparative stages between NAFLD and NASH, and a volcano plot was plotted. A PPI network was constructed for these DEGs using the STRING database, providing insights into the functional relationships between identified proteins. And centrality analysis, pivotal for discerning nodes of paramount importance within the network, was systematically executed based on the intricate interaction relationships between nodes. A pathway enrichment was performed to acquire a holistic understanding of the biological functions and pathways associated with the DEGs. (B) A schematic diagram showed that biotin-labeled ursolic acid was co-incubated with this array, followed by signal interpretation using Cy3-streptavidin. (C) 502 targets were identified that interacted directly with ursolic acid, and intersection analysis with the previously identified DEGs retained five candidates, wherein the differentially binding proteins were presented in the proteomics chip and heatmap. (D) The SPR analysis was produced using a biacore sensor chip, wherein the molecular dynamics analysis revealed that ursolic acid and SPP1 exhibited high-affinity interaction, with the equilibrium dissociation constant of 1.705E-5. (E) A pull-down assay was performed to further verify the binding affinity between ursolic acid and SPP1 in vivo. (F) The molecular docking simulation showed a mutual binding between ursolic acid and SPP1, with a binding energy of -5.8. SPP1, secreted phosphoprotein 1; DEGs, differentially expressed genes; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; STRING, Search Tool for the Retrieval of Interacting Genes; SPR, surface plasmon resonance; KD, knockdown.

from NAFLD to NASH. Consequently, two microarrays, specifically GSE49541 and GSE89632, were incorporated, which distinctly delineated the comparative stages between NAFLD and NASH, encompassing 40 and 20 samples of NAFLD, as well as 32 and 19 samples of NASH, respectively (Supplementary Fig. 2). After batch normalization, 106 differentially expressed genes (DEGs) were identified, and a PPI network was constructed for all of them, while centrality analysis disclosed that SPP1 occupied the highest weight within this framework (Fig. 2A and Supplementary Table 2). Enrichment analysis underscored the ECM-receptor interaction pathway as the most differentially enriched. It is noteworthy that numerous studies have asserted the ECM components, apart from serv-
ing as a structural scaffold, can also facilitate intercellular communication as signaling mediators and plays a crucial role in the progression of NAFLD, especially in inflammation and fibrosis processes, with SPP1 as an integral component. 

Simultaneously, the HuProt™20K human proteome microarray was employed for high-throughput screening of direct protein targets of ursolic acid. During this procedure, biotin-labelled ursolic acid was co-incubated with this array, followed by signal interpretation using Cy3-streptavidin, revealing 502 potential targets that interacted directly with ursolic acid, while intersection analysis with the previously identified DEGs retained five candidates: SPP1, PRM2, THOP1, HSD17B14 and AKR1B10 (Fig. 2B, C and Supplementary Table 3). Receiver operating characteristic curves, generated from the transcriptomic chip data, were plotted to assess the sensitivity and specificity between these candidates and NAFLD progression, and the results showed that the area under the curve values for all five genes exceeded 0.75, indicating their substantial relevance (Supplementary Fig. 3A).

Next, biomolecular interaction analyses were executed based on Surface Plasmon Resonance (SPR) technology to ascertain the binding affinity of these five proteins with ursolic acid. Surprisingly, PRM2, THOP1 and AKR1B10 manifested no detectable binding with ursolic acid, leaving only SPP1 and HSD17B14 to exhibit high-affinity interactions, with equilibrium dissociation constants of 1.705E-5 and 7.329E-6, respectively (Fig. 2D and Supplementary Fig. 3B). Relevant studies on HSD17B14 accentuated its role in the NAD-dependent conversion of estradiol to estro- 

Moving forward, it is imperative to experimentally substantiate the pivotal role of SPP1 in the progression of MASLD. To circumvent the potential perturbations of embryonic and developmental gene knockdown (KD) on the intrinsic metabolic and inflammatory phenotypes, aden- 

SPP1 gene knockdown affords protective effects against HFD-induced MASLD

In this context, considering our focal point on immune inflammatory and fibrosis processes, SPP1 is concurrently expressed by resident immune cells, parenchymal cells, and recruited blood-derived inflammatory cells, liver-specific promoters like TBG were bypassed in favor of the broad-spectrum CMV promoter. Thus, we opted for the serotype AAV2/8 to deliver CMV-driven shSPP1 into wild-type mice (Fig. 3A). While mice injected with the empty virus AAV2/8-CMV-Null were maintained as wild-type controls (WT). Ultimately, successful knockdown was affirmed by the marked reduction in SPP1 protein expression in the liver tissues derived from these KD mice.

Animal studies subsequently revealed that SPP1 KD conferred a degree of protection against HFD-induced weight gain in mice. Interestingly, this weight differential emerged from the ninth week, a time point posterior to the interventional by ursolic acid, and the magnitude of weight reduction was considerably milder. Given that HFD-induced mice were known to exhibit hepatic chronic inflammatory responses between the sixth to eighth weeks, 

While recognizing the intertwined relationship between chronic inflammation and hepatic metabolic processes, we postulated that the weight reduction ascribed to SPP1 KD stems from its anti-inflammatory effects (Fig. 3B). Multiple metrics, encompassing liver weights, liver indexes, as well as serum and hepatic triglyceride and total cholesterol levels consistently evinced that SPP1 KD could ameliorate hepatic lipid accumulation in high-fat diet-fed mice to some extent.
Data are represented as mean±SD. n=3-8. *<0.05, **<0.01, ***<0.001.

**Figure 3.** SPP1 gene knockdown affords protective effects against HFD-induced MASLD. (A) Schematic diagram revealed the protocol of SPP1 knockdown. Specifically, AAV2/8 vectors harboring a CMV-driven shRNA targeting SPP1 were employed for in vivo gene delivery. Western blot analyses demonstrated a marked reduction in SPP1 protein expression in the liver tissues from these KD mice, thus confirming the successful knockdown. Subsequently, mice were fed with HFD to construct the MASLD mice model. (B) Body weights were recorded weekly. (C) Liver weights were measured by the termination of this experiment at the twelfth week, and liver indexes were calculated by the ratio of liver to body weight. (D) Serum and hepatic lipid concentrations were determined by an automatic biochemical analyzer. (E) IPGTT and ITT were performed to monitor glucose tolerance and insulin resistance. (F) Histological staining of H&E and oil red O in liver tissues was performed to observe the inflammatory cytokine infiltration and lipid droplet accumulation. (G) Serum inflammatory cytokines were determined to detect inflammatory reactions. SPP1, secreted phosphoprotein 1; HFD, high-fat diets; MASLD, metabolic dysfunction-associated steatotic liver disease; AAV, adeno-associated virus; KD, knockdown; IPGTT, intraperitoneal glucose tolerance tests; ITT, insulin tolerance tests; SD, standard deviation. Data are represented as mean±SD. n=3-8.

extent, albeit less efficacious than ursolic acid intervention, aligning with our supposition (Fig. 3C). Furthermore, while mitigating HFD-induced liver inflammation and steatosis effectively, SPP1 KD concomitantly conferred robust protection against hepatic functional impairment and insulin resistance, which might be attributed to the alleviation of immune inflammation, a linchpin in the evolution of MASLD, thereby ameliorating associated oxidative stress, autophagy and apoptosis (Fig. 3E, F, and Supplementary Fig. 4A). Reinforcing our prior evaluations, subsequent analyses of inflammatory cytokines in both serum and hepatic tissues invariably spotlighted that SPP1 KD could substantially suppress the expression levels of IFN-γ, TGF-β, IL-6, IL-17A, IL-21 and IL-23, hinting at a potential
mechanistic nexus with Th17 cells (Fig. 3G and Supplementary Fig. 4B).

**SPP1 promotes Th17 cell differentiation via interactions with ITGB1 and CD44**

Diving into the molecular intricacies, our exploration probed the possibility of SPP1 driving Th17 cell differentiation as a therapeutic target for ursolic acid. Isolated primary CD4⁺ T cells were harvested from healthy murine spleens and subjected to a precise set of stimulatory conditions. During this process, recombinant SPP1 proteins at varying concentrations were integrated into the culture medium to foster the differentiation of Th17 cells, and flow cytometry analyses subsequently affirmed that SPP1 could dose-dependently potentiate Th17 cell differentiation, stabilizing its stimulatory prowess at a concentration of 0.2 µg/mL, which was selected for ensuing experimental endeavors (Fig. 4A). In parallel, when exposed to diverse concentrations of ursolic acid in the presence of recombinant SPP1, it was apparent that ursolic acid exerted a dose-dependent inhibition on the SPP1-induced Th17 cell differentiation, with a concentration of 5 µM emerging as optimal for subsequent investigative pursuits (Fig. 4B).

Typically, the canonical pathway attributed to SPP1-induced Th17 cell differentiation hinges on the activation of CD44 receptors on antigen-presenting cells, such as macrophages and dendritic cells. This engagement triggers

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alterations in the domain of major histocompatibility complex, facilitating the interaction with initial CD4+ T cells, thus triggering a cascade of cytokine releases including TGF-β and IL-6 that ultimately activates Th17 cells. However, recent investigations allude to the existence of an alternate IL-6/STAT3-independent pathway wherein SPP1 propels Th17 cell differentiation. Considering SPP1's intrinsic propensity as an adhesive protein to interact with an array of cell surface receptors like integrins and CD44, our study aimed to co-incubate cells, either exposed to or spared from SPP1 and ursolic acid stimuli, with recombinant SPP1, followed by Co-immunoprecipitation (Co-IP) analyses. The precipitates were probed for the protein expressions of ITGB1, ITGB3 and CD44, inspired by interaction data retrieved from online databases suggesting their putative interactions between SPP1 and integrins at the αvβ1 and αvβ3 junctures. Intriguingly, ITGB1 and CD44 protein expressions were ubiquitously detected in all cellular precipitates, with cells co-treated with ursolic acid exhibiting conspicuous reductions in these protein levels, suggesting a physiological interaction between SPP1 and both ITGB1 and CD44 within cells, which is susceptible to modulation by ursolic acid (Supplementary Fig. 5). However, ITGB3 displayed conspicuously absent across all samples counterintuitively, hinting at a potential weak affinity between SPP1 and ITGB3, precluding the formation of stable com-

**Figure 5.** Ursolic acid modulates SPP1-mediated Th17 cell differentiation to ameliorate MASLD. (A–C) Mice were derived from the experiments of SPP1 KD, wherein (A) presented that the protein levels of ITGB1 and CD44 expressed in the pull-down products were conspicuously surged by HFD administration, whereas a precipitous decline in their expression was observed in the liver tissue lysates procured from the SPP1 KD mice, while (B) demonstrated that such alterations were concomitant with trends in the phosphorylation level of ERK protein; and concurrently, (C) displayed a similar trend in hepatic Th17 cell populations. (D, E) Mice were derived from the first part of experiments, those were fed with HFD and different concentrations of ursolic acid, wherein (D) suggested that ursolic acid dose-dependently ameliorated the escalated Th17 cell populations induced by the high-fat dietary regimen, and (E) revealed consistent protein levels of SPP1, ITGB1, CD44, as well as the phosphorylation level of ERK. Data are represented as mean±SD, n=3-6, *P<0.05, **P<0.01, ***P<0.001, SPP1, secreted phosphoprotein 1; MASLD, metabolic dysfunction-associated steatotic liver disease; KD, knockdown; ITGB1, integrin β1; CD44A, CD44 antagonists; HFD, high-fat diets; ERK, extracellular signal-regulated kinase; SD, standard deviation.
plexes that could be precipitated. Since activated CD44 receptors on the surface of T cells could modulate the phosphorylation modification process of ERK via the serine/threonine protein kinase Raf-1, while the protein complex formed by SPP1 and ITGB1 could similarly regulate the ERK signaling pathway through AKT (i.e., Rac), eventually enhancing the transcription level of retinoic acid-related orphan receptor (ROR) gamma, we gauged the protein expression of their common downstream, ERK.29-31 Western blot analyses illuminated that the intervention of SPP1 significantly upregulated the phosphorylation level of ERK, whereas this effect was adeptly counteracted by ursolic acid (Fig. 4C).

To bolster our understanding of the SPP1-facilitated mechanism underlying Th17 cell differentiation, the SPP1 KD mice were revisited, isolating splenic CD4+ T cells therefrom. The empirical findings demonstrated a distinct diminution in Th17 cell differentiation in the wake of SPP1 KD, a phenomenon that the reintroduction of recombinant SPP1 could resurrect, with ERK phosphorylation patterns echoing this trend (Fig. 4D). Subsequent immunoprecipitation assays unveiled that ITGB1 and CD44 expressions were conspicuously absent in precipitates from SPP1 KD-derived cells, and their expressions were rejuvenated upon supplementation with the recombinant protein, reasserting the physiological binding of SPP1 with ITGB1 and CD44 (Fig. 4E). Finally, leveraging CD4+ T cells from healthy murine spleens, and concurrently administering recombinant SPP1 with either ITGB inhibitors (i.e., GLPG0187) or CD44 antagonists (CD44A), our results manifested that both ITGB and CD44 were instrumental in mediating the SPP1-driven Th17 cell differentiation, with an even pronounced inhibitory effect upon their combined application, which pointed towards the convergence of their downstream signaling through the ERK pathway (Fig. 4F).

**Ursolic acid modulates SPP1-mediated Th17 cell differentiation to ameliorate MASLD**

Drawing upon our preliminary experimental findings, in vivo studies were instigated to corroborate these observations. Remarkably, immunoprecipitation analyses showed that both ITGB1 and CD44 were ubiquitously expressed in the pull-down products from liver tissues of WT mice, with their expression surging conspicuously in the context of HFD administration (Fig. 5A). Conversely, a precipitous decline in their expression was observed in the liver tissue lysates procured from the SPP1 KD mice, while such alterations were concomitant with trends in the phosphorylation level of ERK protein (Fig. 5B). Flow cytometry analyses displayed an upsurge in hepatic Th17 cell populations in high-fat diet-fed mice, an anomaly rectified by SPP1 KD, and immunohistochemical staining of liver tissues for SPP1, ROR gamma, and IL-17A presented consistent results, which were also confirmed by assessments of gene expression (Fig. 5C, Supplementary Fig. 1B, and Supplementary Fig. 6A).

Lastly, our research circled back to the therapeutic potential of ursolic acid. Both flow cytometry and immunohistochemical staining unanimously demonstrated that ursolic acid could dose-dependently ameliorate the escalated Th17 cell populations in the liver tissues of mice induced by the high-fat dietary regimen (Fig. 5D, Supplementary Fig. 4B, and Supplementary Fig. 6B). Additionally, Western blot analyses illuminated that the intervention of ursolic acid conspicuously reduced the protein levels of SPP1, ITGB1 and CD44, alongside restraining the phosphorylation status of ERK in mouse liver tissues (Fig. 5E).

Taken together, all these findings validated our experimental hypothesis that ursolic acid could ameliorate the immune inflammation in MASLD by modulating SPP1-mediated Th17 cell differentiation, with the ERK signaling pathway, orchestrated jointly by ITGB1 and CD44, emerging as a linchpin in this molecular cascade.

**DISCUSSION**

In the current global health landscape, MASLD has emerged as the most prevalent chronic liver condition, with epidemiological surveys compellingly indicating that over 170 million adults worldwide are grappling with its ramifications, leading to an alarming synchronic uptick in liver transplantations, hepatocellular carcinomas, and liver-associated mortalities.5,32 Alongside, the metabolically intertwined nature of MASLD with disorders such as obesity, type 2 diabetes, and hyperlipidemia, paints a daunting picture of its economic impact on global health infrastructure, that direct medical expenses related to MASLD in the United States alone have exceeded $100 billion by estimate.2,33
A troubling trend is the downward shift in the age demographic of those affected, implying that an increasing number of individuals might suffer prolonged exposure to MASLD and its associated complications, exacerbating the strain on the global health milieu. As such, a consensus statement, crafted by a multidisciplinary consortium of global experts in 2022, has categorically designated MASLD as a chronic metabolic public health malady, underscoring its prominence in the global health quandaries whilst amplifying the dire need to address such metabolic ailments.

While the pathophysiological intricacies of MASLD, involving a labyrinthine interplay of myriad cellular entities and signaling cascades, remain not entirely deciphered, burgeoning evidence points toward metabolic disruption and immune inflammation as core players. Recent international expert consensuses have reconceptualized MASLD, emphasizing metabolic dysfunctions with the terms metabolic dysfunction-associated fatty liver disease (MAFLD) and metabolic dysfunction-associated steatotic liver disease (MASLD). Within these consensus frameworks, the term steatohepatitis is felt to be an important pathophysiological concept that should be retained, while immune inflammation is pinpointed as the crux in the progression of MASLD.

A plethora of studies denote that Th17 cell-mediated immune-inflammatory responses, acting as cardinal modulators of immune stress and inflammation, are intrinsically intertwined with every juncture of MASLD evolution. In the milieu of MASLD-afflicted hepatic tissues, a persistently chronic low-grade inflammation fosters an immunological microenvironment wherein various cytokines, inclusive of TGF-β and IL-6, experience surges. These cytokines synergistically propel Th17 cell differentiation, thus culminating in copious IL-17 production. Notably, IL-17 is a potent inflammatory mediator capable of instigating hepatocytes to unleash a plethora of chemokines, hence recruiting an even greater pool of immune cells into the liver, amplifying the inflammatory cascade. Concurrently, IL-17 wield stress influence, either directly or indirectly, over HSCs, promoting their proliferation and collagen synthesis, thereby hastening the fibrotic trajectory. Integral to this process, the ECM, a fundamental architectural component of tissue structures, not only furnishes cellular stability but is also replete with fibronectins, adhesion proteins, polysaccharides, proteases and growth factors, underpinning cellular dialogues. Given this backdrop, therapeutic strategies targeting the ECM components and Th17 cells have proffered novel therapeutic avenues for MASLD, encompassing matrix metalloproteinase inhibitors, transforming growth factor-β inhibitors and CXC chemokine receptor antagonists.

While these pharmacological agents have evinced promise in attenuating inflammation and fibrosis, they remain in the nascent stages of clinical trials, awaiting widespread validation and deployment. Ursolic acid, a natural compound endowed with an extensive array of biological activities, has garnered considerable attention in the realm of biomedical research in recent years. When employed as a prophylactic or therapeutic agent for MASLD, ursolic acid primarily augments hepatic lipid metabolic capacities, which is achieved by activating intracellular signaling pathways such as AMPK and PPARα thereby fostering the β-oxidation of fatty acids whilst concurrently curtailing their synthesis. In parallel, this compound has been empirically demonstrated to possess pronounced antiinflammatory and antioxidative attributes, as it modulates a suite of signaling molecules intricately intertwined with oxidative stress and inflammatory cascades, including but not limited to Nrf2, HO-1 and NF-κB. Additionally, ursolic acid thwarts the progression of hepatic fibrosis and decelerates the transition from MASLD to MASH by suppressing the TGF-β/Smad signaling pathway, which in turn curbs the activation of HSCs and the accumulation of ECM components.

Our previous researches have elucidated that ursolic acid exhibits dual protective capacities by modulating IGF-IR and HIF-1 signaling pathways, offering fortification against metabolic dysfunction and hepatic hypoxia. In this present investigation, we further discerned that ursolic acid could also hinder the protein activity of SPP1 within the ECM, subsequently attenuating the activation state of Th17 cells and modulating cytokine-mediated intercellular cross-talk, ameliorating immunological cascade reactions during MASLD evolution. A seminal discovery of this study is the elucidation of the mechanism whereby SPP1, beyond its traditional TGF-β/IL-6 cytokine pathway, can directly engage with ITGB1 and CD44, modulating their joint downstream ERK signaling pathway and thereby inducing Th17 cell differentiation - a molecular mechanism paramount to ursolic acid’s regulation of SPP1 and its subsequent allevi-
ation of immune inflammation. Excitingly, emerging discourse has pinpointed that exercise, a pivotal lifestyle intervention strategy for metabolic disorders, may confer systemic metabolic benefits triggered in both muscles and non-contractile tissues, and its operative mechanism could be intimately linked to ITGB1, underscoring the significance of this integrin as a potential epicentre of metabolic inflammatory responses that warrant meticulous scrutiny. Hence, a profound understanding of its underlying mechanisms might pave the way for innovative therapeutic strategies, heralding renewed hope for MASLD patients.

Limitations should be acknowledged in this exploration. As the molecular target central to our investigation, SPP1 exists two discrete isoforms: the secreted one and the intracellular one, with our emphasis predominantly casting on the former, an integral component of the ECM, due to its pivotal role as a secretory factor capable of mediating intercellular communication in metabolic inflammatory reactions; in contrast, existing research on the latter posits its pro-inflammatory role chiefly via activation of the intracellular Toll-like receptor signaling pathway, especially the critical MyD88 signal transducer, thus catalysing the secretion of various inflammatory cytokines—a domain beyond the purview of our present endeavour. On another note, a spate of innovative techniques, including single-cell sequencing and DSP spatial multi-omics analysis, have emerged as exceptionally conducive to investigating cytokine-mediated metabolic stress and immune inflammation. Yet, given they were not universally adopted at the onset of our study and hence remained unutilized herein. Nevertheless, our ongoing endeavours encompass sequencing of MASLD samples at various stages, aspiring to unearth the intricate pathophysiological mechanisms underpinning MASLD progression through pseudo-temporal analyses. This, synergized with subsequent foundational and clinical trial investigations, seeks to provide novel insights, revolutionizing prevention and therapeutic strategies, ultimately aiming to enhance the quality of life and prognosis for MASLD patients.

Authors’ contribution
YZ, FL, and YL conceived the idea for this work and designed the experiments. YZ, LZ, and ZX performed the whole experiment. CH, QY, DF, YF, SG, CC, JL, YZ and JL contributed the data. All authors read and approved the final manuscript.

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Conflicts of Interest
The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL
Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Metformin and statins reduce hepatocellular carcinoma risk in chronic hepatitis C patients with failed anti-viral therapy

Pei-Chien Tsai, Chung-Feng Huang, Ming-Lun Yeh, Meng-Hsuan Hsieh, Hsing-Tao Kuo, Chao-Hung Hung, Kuo-Chih Tseng, Hsueh-Chou Lai, Cheng-Yuan Peng, Jing-Houng Wang, Jyh-Jou Chen, Pei-Lun Lee, Rong-Nan Chien, Chi-Chieh Yang, Gin-Ho Lo, Jia-Horng Kao, Chun-Jen Li, Chia-Chi Wang, Chao-Hung Hung, Wan-Long Chung, Ming-Jong Bair, and Ming-Lung Yu

T-COACH Study Group

1Hepatobiliary Section, Department of Internal Medicine, and Hepatitis Centre, Kaohsiung Medical University Hospital; 2Hepatitis Research Centre, School of Medicine and Centre for Liquid Biopsy and Cohort Research, Kaohsiung Medical University, Kaohsiung, Taiwan; 3Health Management Centre, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan; 4Division of Hepatogastroenterology, Department of Internal Medicine, Chi Mei Medical Centre, Tainan, Taiwan; 5Division of Hepatogastroenterology, Department of Internal Medicine, Chiayi Chang Gung Memorial Hospital, Chiayi, Taiwan; 6Division of Hepatogastroenterology, Department of Internal Medicine, E-Da Hospital and School of Medicine, College of Medicine, I-Shou University, Kaohsiung, Taiwan; 7Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan; 8Division of Gastroenterology, Department of Internal Medicine, Show Chwan Memorial Hospital, Changhua, Taiwan; 9Division of Gastroenterology and Hepatology, Department of Medicine, E- Da Hospital and School of Medicine, College of Medicine, I-Shou University, Kaohsiung, Taiwan; 10Division of Gastroenterology and Hepatology, Linkou Medical Centre, Chang Gung Memorial Hospital, Keelung, Taiwan; 11Division of Gastroenterology, Department of Internal Medicine, Show Chwan Memorial Hospital, Changhua, Taiwan; 12Division of Gastroenterology and Hepatology, Department of Medicine, E-Da Hospital and School of Medicine, College of Medicine, I-Shou University, Kaohsiung, Taiwan; 13Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan; 14Division of Gastroenterology, Department of Internal Medicine, Show Chwan Memorial Hospital, Changhua, Taiwan; 15Division of Gastroenterology, Department of Internal Medicine, Changhua Christian Hospital, Changhua, Taiwan; 16Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan; 17Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Martin De Porres Hospital-Daya, Chiayi, Taiwan; 18Division of Gastroenterology and Hepatology, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan; 19Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan; 20Division of Gastroenterology and Hepatology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan; 21Institute of Clinical Medicine, Faculty of Medicine, National Yang Ming Chiayi University, Taipei, Taiwan; 22Department of Internal Medicine, Chiayi Christian Hospital, Chiayi, Taiwan; 23Division of Gastroenterology, Department of Internal Medicine, Taichung Mackay Memorial Hospital, Taichung, Mackay Medical College, Taipei, Taiwan; 24School of Medicine and Doctoral Program of Clinical and Experimental Medicine, College of Medicine and Centre of Excellence for Metabolic Associated Fatty Liver Disease, National Sun Yat-sen University, Kaohsiung, Taiwan

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Study Highlights
What is known

- Successful antiviral therapy reduces but doesn’t eliminate HCC risk.
- 25% of CHC patients failing therapy remain at high risk of HCC.
- Strategies to lower HCC risk post-therapy failure need exploration.

What is new here

- CHC patients with DM not on metformin use have a 1.51-fold higher risk of HCC than those without DM in antiviral therapy failures.
- CHC patients with HLP on statins use have a 50% reduced HCC risk than those without HLP in antiviral therapy failures.
- Metformin and statins use may prevent HCC in antiviral therapy.

Corresponding author: Ming-Lung Yu
Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan
Tel: +886-73121101 #7475, Fax: +886-73123955, E-mail: fish6069@gmail.com
https://orcid.org/0000-0001-8145-1900

Ming-Jong Bair
Division of Gastroenterology, Department of Internal Medicine, Taitung Mackay Memorial Hospital, Taitung 950, Taiwan
Tel: +886-89-310150, Fax: +886-89-361491, E-mail: a5963@mmh.org.tw
https://orcid.org/0000-0001-7069-5459

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Abbreviations:
CHC, chronic hepatitis C; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; DM, diabetes mellitus; HTN, hypertension; HLP, hyperlipidemia; LC, liver cirrhosis; LT, liver transplant; DAA, Direct-acting antiviral agent; BMI, body mass index; SVR, sustained virological response; ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis index based on the four factors; GT, genotype; SHR, sub-distribution hazard ratio; aSHR, adjusted sub-distribution hazard ratio; CI, confidence interval; ICD-9-CM, International Classification of Diseases, ninth revision; ICD-10, International Classification of Disease, tenth revision

http://www.e-cmh.org  https://doi.org/10.3350/cmh.2024.0038
**INTRODUCTION**

Hepatitis C virus (HCV) infection is a major public health concern, because it frequently leads to liver cirrhosis (LC) and hepatocellular carcinoma (HCC), which impose significant burdens on many countries. HCV treatment has changed from interferon-based therapies to direct-acting antivirals (DAAs), with significant improvements in sustained virological response (SVR) rates.\(^2\) Achieving SVR through anti-HCV therapy greatly reduces the risk of HCC, liver failure,\(^3\) and liver-related mortality.\(^4\)

The risk of HCC can be reduced but cannot be eliminated completely, even after successful antiviral therapy,\(^5\) particularly among patients with persistent advanced fibrosis, insulin resistance, and diabetes mellitus (DM), as well as among the elderly.\(^6,8\) Moreover, patients in whom antiviral therapy fails are still at a high risk of developing HCC, especially those with advanced fibrosis and DM.\(^9\)

Chronic hepatitis C (CHC) has been associated with an increased risk of DM,\(^10\) and the comorbidity of DM increases the risk of developing HCC in patients with CHC.\(^11\) Metformin use has been associated with a reduced risk of HCC compared with other oral hypoglycemic agents (OHAs) or insulin among patients with DM.\(^12\) We previously showed that metformin use in DM significantly reduces the risk of HCC in patients with CHC after successful antiviral therapy.\(^13\)

Statin use has been associated with reduced development of cirrhosis and incidence of HCC, which is mainly observed in patients receiving lipophilic statins.\(^14\) Although the chemopreventive effects of metformin in patients with DM and statins in patients with hyperlipidemia (HLP) reduce the risk of HCC in patients with CHC, which has been proven after achieving SVR,\(^15\) the impact on HCC risk reduction remains unclear in patients with CHC who have failed antiviral therapy.

The current study aimed to assess the impact of metformin for DM and/or statins for HLP on the risk of HCC among CHC patients in who failed antiviral therapy. The findings of this study will improve our understanding of the potential benefits of chemoprevention in reducing the risk of HCC in patients with difficult-to-cure CHC.

The study was approved by the institutional review boards of Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20210378) and was conducted in accordance with the Declaration of Helsinki and the ethical
guidelines. All participants provided written informed consent.

### MATERIALS AND METHODS

#### Study population

The study included patients aged ≥20 years who were diagnosed with CHC either through liver histology or by testing positive for anti-HCV or HCV RNA for > six months. These patients were drawn from a large-scale, multicenter cohort in Taiwan (Taiwanese Chronic Hepatitis C Cohort, T-COACH) and had undergone antiviral interferon-based therapy for at least four weeks since 2003.

We excluded patients who lacked virological outcome data, achieved SVR, had coinfection with human immunodeficiency virus or hepatitis B virus (HBV), died within six months of end-of-treatment (EOT), and developed HCC within 1.5 years after EOT. Finally, the analysis focused on 2,779 patients with CHC who experienced antiviral therapy failure (Fig. 1).

#### Independent variables

Data collected on the independent variables included: 1) demographic characteristics: age, sex, and body mass index (BMI); 2) medical history: DM, HLP, and hypertension (HTN); 3) laboratory data: AST, ALT, platelet count, creatinine, liver fibrosis (FIB-4 score; fibrosis index based on four factors), renal function (estimated glomerular filtration rate, eGFR); and 4) clinical features: renal function impairment (eGFR ≤60), advanced fibrosis (FIB-4 ≥3.25), LC, and sustained virological response (SVR; HCV RNA seronegativity 24 weeks after interferon-based therapy). Related assessments of the liver and renal function were performed accordingly. The FIB-4 score was calculated using age, AST, ALT, and platelet count of the patient: 

\[ \text{FIB-4} = \frac{\text{age (years)} \times \text{AST (IU/L)}}{\text{platelet (x1,000/μL)} \times \text{ALT (IU/L)}^{0.5}} \].

The eGFR was calculated using the patient’s creatinine levels, age, and sex: 

\[ \text{eGFR} = \frac{186 \times \text{creatinine (mg/dL)}^{1.154} \times \text{age (year)}^{0.203} \times 0.742}{\text{if female}} \].

LC was based on any of the following: liver histology;15 transient elastography (FibroScan®; Echosens, Paris, France) >12 kPa,16 acoustic radiation force impulse >1.98 m/s17, FIB-4 >6.5,18 or the presence of clinical, radiological, endoscopic, or laboratory evidence of cirrhosis and portal hypertension.

#### Study endpoints and linked databases

The data for the study were obtained from the National Health Insurance Research Database, which covers ap-
proximately 26 million Taiwanese people since 1995.

Participants were considered to have DM if they met any of the following criteria: a history of DM on treatment with OHAs with or without insulin, fasting glucose ≥126 mg/dL, or 2-hour plasma glucose ≥200 mg/dL. Patients with a DM diagnosis who had taken metformin for ≥84 days were considered metformin users, whereas those who had taken metformin for <84 days were considered metformin non-users after six months of EOT. Participants were considered to have HLP if they had a history of HLP and used medication. Diagnosed HLP statin users were defined as patients with an HLP diagnosis who had taken statins for ≥84 days, whereas those who had taken statins for <84 days were considered HLP statin non-users after six months of EOT.

All diseases, including HCC and liver transplant (LT), were identified using specific codes from the International Classification of Diseases, 9th or 10th revision (ICD-9-CM and ICD-10). In this study, the occurrence of HCC was determined based on data from the Cancer Registry, whereas the LT cases were identified from the registry of catastrophic illnesses. Information on patient deaths was obtained from the death registry. The relevant medication codes for the diseases were linked to the corresponding entries in the detailed health insurance inpatient/outpatient records (Supplementary Table 1).

New-onset HCC was defined as HCC occurring in patients 1.5 years after antiviral EOT. The follow-up period began 1.5 years after antiviral EOT and continued until the censored events (HCC and death/LT) or December 31, 2019.

Statistical analysis

Continuous variables are presented as mean ± standard deviation, while categorical variables are expressed as numbers (percentages). Chi-square was used to compare subgroups with categorical parameters, and Student’s t-test was used for continuous parameters, as appropriate. Person-years were calculated as the number of years that each participant contributed to the study from 1.5 years after EOT to the date of the first diagnosis of HCC, death/LT, or December 31, 2019, whichever occurred first. The annual incidence of HCC was calculated as the number of new-onset HCC cases divided by the sum of person-years and the groups were compared by Poisson method.

The study considered death or LT as competing events, meaning that patients who died or underwent LT before developing HCC were no longer at risk of developing HCC. To account for this, the study modified the Kaplan-Meier method using Gray’s cumulative incidence method. Cox proportional hazard regression was used to calculate sub-distribution hazard ratios (SHRs) for HCC development before and after adjusting for various factors such as age, sex, LC, HCV genotype (GT), HCV RNA and aspirin use. In addition, we compared the cumulative incidence of new-onset HCC between subgroups by stratifying the patients according to their LC status.

Sensitivity analyses were conducted to ensure robustness of the findings. First, advanced fibrosis was substituted with LC to validate the robustness of the results in the multivariate analysis. Second, in this study, participants were defined as metformin users when they used metformin six months after EOT. Patients who used metformin either before or during antiviral therapy were classified as metformin non-users. To strengthen our findings, we reclassified previous metformin users to confirm the effect of metformin on the risk of new-onset HCC in patients with DM. A similar approach was employed for HLP statin users. Third, to avoid potential bias related to specific clinical scenarios including treatment by DAA for IFN-failed patients or patients with diabetes and severe renal impairment (eGFR <30) who are typically not candidates for metformin therapy, we conducted further analysis to validate our findings. All statistical analyses were performed using SAS Enterprise Guide, and P-values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

The clinical characteristics of the 2,779 CHC patients who failed antiviral therapy are presented in Table 1. The mean age of the patients was 56.1±10.7 years, mean log-transformed HCV RNA levels were 6.1±0.9, 52.7% were female, 34.7% had advanced fibrosis (FIB-4 ≥3.25), 16.2% had liver cirrhosis, 6.7% had an eGFR <60 mL/min/1.73m², and 63.1% were infected with HCV GT1. Furthermore,
Table 1. The characteristics of CHC patients in whom antiviral therapy failed

<table>
<thead>
<tr>
<th>Variables</th>
<th>All (n=2,779)</th>
<th>DM patients</th>
<th>HLP patients</th>
<th>P-value</th>
<th>Non-metformin</th>
<th>Metformin</th>
<th>P-value</th>
<th>Non-statin</th>
<th>Statin</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(n=288)</td>
<td>(n=332)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(n=106)</td>
<td>(n=500)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>56.1±10.7</td>
<td>57.9±9.0</td>
<td>56.4±9.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.275</td>
</tr>
<tr>
<td>&gt;65</td>
<td>553 (19.9)</td>
<td>64 (22.2)</td>
<td>53 (16.0)</td>
<td>0.047</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.590</td>
</tr>
<tr>
<td>Female</td>
<td>1,464 (52.7)</td>
<td>128 (44.4)</td>
<td>159 (47.9)</td>
<td>0.391</td>
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<td></td>
<td></td>
<td></td>
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<td>0.860</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>25.0±3.0</td>
<td>25.9±3.8</td>
<td>25.5±2.8</td>
<td>0.088</td>
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<td></td>
<td>0.041</td>
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<tr>
<td>≥27</td>
<td>488 (17.6)</td>
<td>91 (31.6)</td>
<td>68 (20.5)</td>
<td>0.002</td>
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<tr>
<td>DM</td>
<td>620 (22.3)</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001</td>
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<td></td>
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<td></td>
<td>0.472</td>
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<tr>
<td>HTN</td>
<td>315 (11.3)</td>
<td>88 (30.6)</td>
<td>46 (13.9)</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>HLP</td>
<td>606 (21.8)</td>
<td>92 (32.0)</td>
<td>144 (43.4)</td>
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<tr>
<td>Metformin use</td>
<td>332 (12.0)</td>
<td>0 (0.0)</td>
<td>332 (100.0)</td>
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<td>Statin use</td>
<td>500 (18.0)</td>
<td>66 (22.9)</td>
<td>132 (39.8)</td>
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<tr>
<td>Aspirin use</td>
<td>289 (10.4)</td>
<td>35 (12.2)</td>
<td>60 (18.1)</td>
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<td></td>
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<td>AST (IU/L)</td>
<td>87.6±59.2</td>
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<td>90.8±52.6</td>
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<td>83.6±48.1</td>
<td>80.0±49.7</td>
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<td>≥2X (80)</td>
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<td>127 (44.1)</td>
<td>156 (47.0)</td>
<td>0.471</td>
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<td>45 (42.5)</td>
<td>180 (36.0)</td>
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<td>ALT (IU/L)</td>
<td>121.0±92.5</td>
<td>120.5±88.6</td>
<td>132.9±87.0</td>
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<td>115.9±72.4</td>
<td>117.2±82.6</td>
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<tr>
<td>≥ 2X (80)</td>
<td>1,722 (62.0)</td>
<td>173 (60.1)</td>
<td>234 (70.5)</td>
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<td>64 (60.4)</td>
<td>298 (59.6)</td>
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<td>Platelet (x1,000/μL)</td>
<td>164.6±55.7</td>
<td>158.6±72.3</td>
<td>160.7±55.1</td>
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<td></td>
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<td>171.2±55.5</td>
<td>172.0±59.1</td>
<td>0.895</td>
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<td>Creatinine (mg/dL)</td>
<td>1.00±1.23</td>
<td>1.20±1.70</td>
<td>0.92±0.75</td>
<td>0.007</td>
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<td>1.05±1.12</td>
<td>1.09±1.61</td>
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<td>FIB-4</td>
<td>3.26±2.77</td>
<td>3.90±3.32</td>
<td>3.40±3.19</td>
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<td></td>
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<td>3.11±2.05</td>
<td>2.82±1.93</td>
<td>0.159</td>
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<tr>
<td>≥3.25</td>
<td>964 (34.7)</td>
<td>121 (42.0)</td>
<td>115 (34.6)</td>
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<td>37 (34.9)</td>
<td>126 (25.2)</td>
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<td>Liver cirrhosis</td>
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<td>74 (25.7)</td>
<td>56 (16.9)</td>
<td>0.007</td>
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<td>18 (17.0)</td>
<td>64 (12.8)</td>
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<td>eGFR (mL/min/1.73m²)</td>
<td>88.6±25.5</td>
<td>87.4±30.9</td>
<td>87.8±22.6</td>
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<td>42 (14.6)</td>
<td>17 (5.1)</td>
<td>&lt;0.001</td>
<td></td>
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<td>10 (9.4)</td>
<td>44 (8.8)</td>
<td>0.835</td>
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<td>HCV RNA (log10 IU/mL)</td>
<td>6.1±0.9</td>
<td>6.0±0.9</td>
<td>6.1±0.8</td>
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<td>HCV genotype</td>
<td>GT 1</td>
<td>1,753 (63.1)</td>
<td>189 (65.6)</td>
<td>192 (57.8)</td>
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<td>69 (65.0)</td>
<td>313 (62.6)</td>
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<td>Follow-up (person-years)</td>
<td>18,668</td>
<td>1,663</td>
<td>2,848</td>
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<td>634</td>
<td>3,994</td>
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17.6% of the patients were obese (BMI ≥27), 22.3% had DM, 11.3% had HTN, and 21.8% had HLP. Among the patients with DM, 53.5% were metformin users. Among patients with HLP, 82.5% were statin users. In addition, 10.4% of the patients were aspirin users. Overall, 238 (8.6%) patients died before the development of HCC and 480 (17.3%) developed new-onset HCC during a total of 18,668 person-years of follow-up (median: 6.6 years). The annual incidence of HCC was 257.1 cases per 10,000 person-years (Table 1).

HCC risk between patients with/without DM and on/not on metformin

Of the 2,779 CHC patients in whom antiviral therapy failed, 620 (22.3%) had DM. After a median follow-up of 7.27 years, 65 died before HCC developed, and 125 developed HCC (annual incidence: 277.1 per 10,000 person-years). For the other 2,159 (77.7%) patients without DM, 173 died before HCC developed, and 355 developed HCC (annual incidence: 250.8 per 10,000 person-years) after a median follow-up of 6.56 years. The 5-year cumulative incidence rate of HCC was not significantly different between patients with and without DM (9.3% vs. 11.3%, Gray’s P =0.419) (Fig. 2A). Notably, patients with DM who were not on metformin showed a significant increase in the risk of HCC compared to metformin users (annual incidence: 408.9 per 10,000 person-years in metformin non-users vs. 200.1 per 10,000 person-years in metformin users, P<0.001, Table 1). The 5- and 10-year cumulative incidence rates of HCC were 11.3% and 21.6%, respectively, in non-DM patients; 3.1% and 19.6%, respectively, in DM metformin users; and 16.5% and 28.6%, respectively, in DM non-metformin non-users. HCC risk was significantly higher in metformin non-users than in metformin users (adjunctive metformin sub-distribution hazard ratio (aSHR=1.59, 95% CI=1.07–2.36, P=0.022) and in DM patients vs. non-metformin non-users (aSHR=1.92, 95% CI=1.22–2.98, P=0.002). However, there was no difference in HCC risk between metformin users and patients without DM (aSHR=1.06, 95% CI=0.76–1.44, P=0.763) (Fig. 2A).

<table>
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<tr>
<th>Variables</th>
<th>All (n=2,779)</th>
<th>DM patients</th>
<th>HLP patients</th>
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<td></td>
<td></td>
<td>Non-metformin (n=288)</td>
<td>Metformin (n=332)</td>
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<td>Follow-up duration (years)</td>
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<td>Mean±SD</td>
<td>6.72±3.18</td>
<td>5.78±2.90</td>
<td>8.58±3.01</td>
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<td>Median (Q1–Q3)</td>
<td>6.57 (4.41–8.64)</td>
<td>5.76 (3.41–8.05)</td>
<td>8.43 (6.25–10.35)</td>
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<tr>
<td>New-onset HCC (per 10,000 person-years)</td>
<td>480 (17.3)</td>
<td>68 (23.6)</td>
<td>57 (17.2)</td>
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<td>Annual incidence of HCC</td>
<td>257.1</td>
<td>408.9</td>
<td>200.1</td>
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<td>Competing death</td>
<td>238 (8.6)</td>
<td>40 (13.9)</td>
<td>25 (7.5)</td>
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</table>

Values are presented as mean±standard deviation (SD) or number (%). CHC, chronic hepatitis C; BMI, body mass index; DM, diabetes mellitus; HTN, hypertension; HLP, hyperlipidemia; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FIB-4, fibrosis index based on four factors; eGFR, estimated glomerular filtration rate; HCC, hepatocellular carcinoma.
HCC risk between patients with/without HLP and on/not on statins

Of 606 patients with HLP, 31 died before HCC developed, and 61 developed HCC after a median follow-up of 7.64 years (annual incidence: 131.8 per 10,000 person-years). Of the 2,173 patients without HLP, 207 died before HCC developed, and 419 developed HCC (annual incidence: 298.4 per 10,000 person-years) after a median follow-up of 6.46 years. The 5-year cumulative incidence rate of HCC was significantly lower in patients with HLP than in those without HLP (4.8% vs. 12.5%, Gray’s \( P < 0.001 \)). After further stratification by statin use in HLP patients, the annual incidences were 220.8 per 10,000 person-years in statin non-users vs. 117.7 per 10,000 person-years in statin users (\( P = 0.036 \), Table 1). The 5-year cumulative incidence rates of HCC were 12.5%, 3.8%, and 10.1% in patients without HLP, HLP statin users, and HLP statin non-users, respectively. The HCC risk was significantly lower in HLP statin users than in patients without HLP (3.8% vs. 12.5%; aSHR=0.50, 95% CI=0.36–0.68, \( P < 0.001 \)), but there was no difference between patients with HLP who were statin non-users and those who did not (Fig. 3).

Factors associated with HCC risk

After accounting for death as a competing risk, univariate Cox regression analysis showed that being elderly (≥65 years), female, having DM without metformin use, HCV GT1, high AST, high ALT, advanced fibrosis and LC were

![Figure 2](https://example.com/image.png)

**Figure 2.** The risk of HCC in CHC patients who failed antiviral therapy between with/without DM (A) and on/not on metformin (B) while considering death as a competing risk. *After considering death as a competing risk, a Kaplan–Meier plot was constructed using Gray’s cumulative incidence method. **All SHR (95% CI) and \( P \)-values were calculated using the Cox sub-distribution hazards method. *Adjusted for age, sex, LC, HCV GT1, HCV RNA, aspirin, and HLP/statin. DM, diabetes mellitus; HLP, hyperlipidemia; HCC, hepatocellular carcinoma; SHR, sub-distribution hazard ratio; LC, liver cirrhosis; GT, genotype.

http://www.e-cmh.org https://doi.org/10.3350/cmh.2024.0038
independently associated with a higher risk of HCC, while aspirin use, HLP statin use and low HCV viral load were associated with a significantly lower risk of HCC. In multivariate analysis, the significant factors associated with increased HCC risk were LC (aSHR=2.27, 95% CI=1.81–2.85), elderly (≥65 years; aSHR=1.89, 95% CI=1.52–2.36), HCV GT1 (aSHR=1.30, 95% CI=1.04–1.63) and DM without metformin use (vs. no DM; aSHR=1.51, 95% CI=1.12–2.04). Conversely, patients with HLP who were on statins had a significantly lower risk of HCC than those without HLP (aSHR=0.50, 95% CI=0.36–0.68) (Table 2). Aspirin use also exhibited a significantly lower HCC risk than non-aspirin use (aSHR=0.71, 95% CI=0.51–1.00, P=0.049) (Supplementary Fig. 1A).

**Subgroup analysis**

LC is the most significant risk factor for HCC. Therefore, we stratified the patients according to their cirrhosis status to evaluate the impact of metformin or statin use among different subgroups.

Among patients without LC, the 5-year cumulative incidence rates of HCC were 9.2%, 3.0%, and 13.5% among those without DM, metformin users, and metformin non-users, respectively. The HCC risk was significantly higher in DM metformin non-users than in patients without DM, with an aSHR of 1.73 (95% CI=1.24–2.40, P=0.001) and in patients with DM on metformin, with an aSHR of 1.71 (95% CI=1.11–2.63, P=0.014) (Fig. 4A). No significant differences were observed between metformin users and patients

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**Figure 3.** The risk of HCC in CHC patients who failed antiviral therapy between with/without HLP (A) and on/not on statin (B) while considering death as a competing risk. *After considering death as a competing risk, a Kaplan–Meier plot was constructed using Gray’s cumulative incidence method. *All SHR (95% CIs) and P-values were calculated using the Cox sub-distribution hazards method. *Adjusted for age, sex, LC, HCV GT1, HCV RNA, aspirin, and DM/metformin. DM, diabetes mellitus; HLP, hyperlipidemia; HCC, hepatocellular carcinoma; SHR, sub-distribution hazard ratio; LC, liver cirrhosis; GT, genotype.

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https://doi.org/10.3350/cmh.2024.0038  
http://www.e-cmh.org
<table>
<thead>
<tr>
<th>Variables</th>
<th>Levels</th>
<th>No.</th>
<th>New-Onset HCC, n (%)</th>
<th>Competing death, n (%)</th>
<th>Crude SHR (95% CI)</th>
<th>P-value</th>
<th>Adjusted SHR (95% CI)</th>
<th>P-value</th>
<th>Adjusted SHR (95% CI)</th>
<th>P-value</th>
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<td></td>
<td>≥65</td>
<td>553</td>
<td>145 (26.2)</td>
<td>82 (14.8)</td>
<td>1.93 (1.59–2.35)</td>
<td>&lt;0.001*</td>
<td>1.89 (1.52–2.36)</td>
<td>&lt;0.001*</td>
<td>1.89 (1.52–2.36)</td>
<td>&lt;0.001*</td>
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<td>Gender</td>
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<td>206 (15.7)</td>
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<td></td>
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<tr>
<td></td>
<td>Female</td>
<td>1,464</td>
<td>274 (18.7)</td>
<td>99 (6.8)</td>
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<td>0.025*</td>
<td>1.14 (0.92–1.40)</td>
<td>0.225</td>
<td>1.14 (0.92–1.40)</td>
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<td>BMI (kg/m²)</td>
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<td></td>
<td>≥27</td>
<td>488</td>
<td>90 (18.4)</td>
<td>52 (10.7)</td>
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<td>0.378</td>
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<td>289</td>
<td>45 (15.6)</td>
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<td>0.68 (0.50–0.91)</td>
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<td>0.71 (0.51–1.00)</td>
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<td></td>
<td>DM/metformin (+)</td>
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<td>57 (17.2)</td>
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<td>0.116</td>
<td>0.95 (0.69–1.31)</td>
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<td>DM/metformin (-)</td>
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<td>68 (23.6)</td>
<td>40 (13.9)</td>
<td>1.56 (1.20–2.03)</td>
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<td>1.51 (1.12–2.04)</td>
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<td>1.59 (1.07–2.36)</td>
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<td>Non-HLP</td>
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<td>1</td>
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<td>18 (3.6)</td>
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<td>&lt;0.001*</td>
<td>0.50 (0.36–0.68)</td>
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<td>HLP/statin (-)</td>
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<td>≥80</td>
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<td>Competing death, n (%)</td>
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<td>P-value</td>
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<td>eGFR (mL/min/1.73 m²)</td>
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<td>(0.51–1.04)</td>
<td></td>
<td>(0.51–1.04)</td>
<td></td>
</tr>
<tr>
<td>HCV genotype</td>
<td>Non-1</td>
<td>855</td>
<td>126 (14.7)</td>
<td>82 (9.6)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1,753</td>
<td>320 (18.3)</td>
<td>133 (7.6)</td>
<td>1.24</td>
<td>0.039*</td>
<td>1.30</td>
<td>0.022*</td>
<td>1.30</td>
<td>0.022*</td>
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</table>

*Due to AST, ALT and FIB-4 were associated with composition in the diagnosis of LC, we did not put these variables in the multivariate analysis.

BMI, body mass index; DM, diabetes mellitus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis index based on the 4 factors; eGFR, estimated glomerular filtration rate; HCC, hepatocellular carcinoma; SHR, sub-distribution hazard ratio.

*P<0.05.

Table 2. Continued
without DM. Among patients with LC, the 5-year cumulative incidence of HCC was 23.3%, 3.6%, and 25.1% in patients without DM, metformin users, and metformin non-users, respectively. However, the difference in HCC risk between metformin non-users and the other two groups was not significant (Fig. 4B).

Among patients without LC, the 5-year cumulative incidence rates of HCC were 10.4%, 3.1%, and 6.1% in patients without HLP, in HLP statin users, and in HLP statin non-users, respectively. The HCC risk was significantly lower in HLP statin users than in patients without HLP, with an aSHR of 0.43 (95% CI=0.30–0.61, P<0.001), while there was no difference in HCC risk between HLP statin users and HLP statin non-users (Fig. 5A). Among patients with LC, the 5-year cumulative incidence of HCC was 22.8%, 8.4%, and 30.5% in patients without HLP, HLP statin users, and HLP statin non-users, respectively. HCC incidence was significantly lower in HLP statin users than in patients without HLP (aSHR=0.47, 95% CI=0.26–0.85, P=0.012) and HLP statin non-users (aSHR=0.35, 95% CI=0.14–0.91, P=0.032), whereas there was no difference in HCC risk between patients without HLP and HLP statin non-users (Fig. 5B).

The aspirin use was not associated with a lower risk of HCC in the subgroup of non-LC (aSHR=0.76, 95% CI=0.53–1.08, P=0.122, Supplementary Fig. 1B) and LC (aSHR=0.68, 95% CI=0.39–1.18, P=0.167, Supplementary Fig. 1C).

Sensitivity analysis

Four sensitivity analyses were conducted to validate the robustness of the main findings and enhance the results.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** The risk of HCC in CHC patients who failed antiviral therapy between with/without DM and on/not on metformin stratified by baseline liver cirrhosis status: non-LC (A) and LC (B). *After considering death as a competing risk, a Kaplan–Meier plot was constructed using Gray’s cumulative incidence method. †All SHRs (95% CIs) and P-values were calculated using the Cox sub-distribution hazards method. ‡Adjusted for age, sex, HCV GT1, HCV RNA, aspirin, and HLP/statin. DM, diabetes mellitus; HLP, hyperlipidemia; HCC, hepatocellular carcinoma; SHR, sub-distribution hazard ratio; LC, liver cirrhosis; GT, genotype.
Assessment of outcomes with metformin or statin use before or after the end-of-antiviral therapy

To avoid underestimating the duration of metformin or statin use, we redefined metformin or statin use, either before or after EOT. The 5-year cumulative incidence rate of HCC was higher in patients taking metformin before or after EOT, with rates of 11.1%, 8.1%, and 29.7% in patients without DM, metformin users, and metformin non-users, respectively. HCC risk remained significantly higher in DM metformin non-users than in DM metformin users (aSHR=2.28, 95% CI=1.48–3.46, P<0.001) or patients without DM (aSHR=2.45, 95% CI=1.95–4.24, P<0.001) (Supplementary Fig. 2A). The 5-year cumulative incidence of HCC was higher in patients with HLP on statins before or after EOT, with rates of 12.7%, 5.1%, and 12.7% in patients without HLP, HLP statin users, and HLP statin non-users, respectively. HCC risk remained significantly lower in HLP statin users than in patients without HLP (aSHR=0.43, 95% CI=0.34–0.63, P<0.001) (Supplementary Fig. 2B). The results were consistent between groups using different definitions of metformin or statin use.

Outcomes using advanced fibrosis as the critical variable

We used another level of liver fibrosis, advanced fibrosis (FIB-4 score >3.25), to validate our findings. In multivariate analysis, HCC risk was significantly higher in patients with advanced fibrosis than in those without advanced fibrosis (aSHR=3.01, 95% CI=2.45–3.64, P<0.001, see Supplementary Table 2). The predicted model showed that DM metformin non-users continued to have a significantly higher risk of HCC compared to patients without DM (aSHR=1.54, 95% CI=1.16–2.03, P=0.005) and DM metformin users (aSHR=1.60, 95% CI=1.13–2.85, P=0.013). The HCC risk was significantly lower in patients with HLP on statins than in patients without HLP (aSHR=0.47, 95% CI=0.34–0.67, P<0.001). The results of LC and advanced fibrosis are summarized in Supplementary Table 2.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BL</th>
<th>1Y</th>
<th>3Y</th>
<th>5Y</th>
<th>8Y</th>
<th>10Y</th>
<th>Crude SHR (95% CI) P-value</th>
<th>Adjusted SHR (95% CI) P-value</th>
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<tr>
<td>DM metformin non-use</td>
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<td>No. at risk</td>
<td>74</td>
<td>70</td>
<td>52</td>
<td>37</td>
<td>14</td>
<td>3</td>
<td>1.17 (0.75-1.83) P=0.482</td>
<td>1.22 (0.77-1.94) P=0.394</td>
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<tr>
<td>Cumulative Incidence (%)</td>
<td>2.7</td>
<td>18.9</td>
<td>25.1</td>
<td>35.5</td>
<td>40.1</td>
<td></td>
<td>1.77 (0.49-1.99) P=0.237</td>
<td>0.84 (0.54-1.32) P=0.449</td>
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<td>DM metformin use</td>
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<tr>
<td>No. at risk</td>
<td>56</td>
<td>56</td>
<td>53</td>
<td>50</td>
<td>33</td>
<td>12</td>
<td>0.77 (0.49-1.99) P=0.237</td>
<td>0.84 (0.54-1.32) P=0.449</td>
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<tr>
<td>Cumulative Incidence (%)</td>
<td>0</td>
<td>1.8</td>
<td>3.6</td>
<td>22.1</td>
<td>38.1</td>
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<tr>
<td>Non-DM</td>
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<td>321</td>
<td>301</td>
<td>250</td>
<td>173</td>
<td>74</td>
<td>22</td>
<td>1.19 (0.76-1.86) P=0.449</td>
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<td>Cumulative Incidence (%)</td>
<td>3.4</td>
<td>14.1</td>
<td>23.3</td>
<td>33</td>
<td>39.3</td>
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Figure 4. Continued.
fibrosis models were consistent.

Minimize potential bias related to specific clinical conditions

To avoid potential bias related to specific clinical scenarios including treatment by DAAs for IFN-failed patients or patients with diabetes and severe renal impairment (eGFR <30) who are typically not candidates for metformin therapy, we conducted further analysis to validate our findings.

After excluding 78 IFN-failed patients who experienced retreatment with DAAs, we found that diabetic patients not treated with metformin had a 1.50-fold increased risk of developing HCC compared to non-diabetic individuals (aSHR=1.50, 95% CI=1.13–1.98, P=0.005) (Supplementary Fig. 3A). Conversely, hyperlipidemia patients who were prescribed statins showed a 55% decrease in HCC risk compared to those without hyperlipidemia (aSHR=0.45, 95% CI=0.33–0.60, P<0.001) (Supplementary Fig. 3B).

Similarly, after removing 49 patients with eGFR<30, the risk of HCC in diabetic patients not treated with metformin was found to be 1.56-fold higher than that in non-diabetics (aSHR=1.56, 95% CI=1.18–2.07, P=0.002, Supplementary Fig. 4A). Hyperlipidemia patients on statin therapy had a 55% decreased risk of HCC compared to those without hyperlipidemia (aSHR=0.45, 95% CI=0.33–0.60, P<0.001, Supplementary Fig. 4B).

Taken together, our findings from the current study were consistent across these sensitivity analyses.

Interaction of statin and metformin

In this study, statin non-users had a 2.42-fold higher risk of HCC compared to statin users among metformin non-users. Fifty-five percent of patients who were diagnosed with HIV and had a high viral load were found to have a higher risk of developing HCC compared to those who were diagnosed with HIV and had a low viral load (aSHR=1.55, 95% CI=1.13–1.98, P=0.005) (Supplementary Fig. 3A). Conversely, hyperlipidemia patients who were prescribed statins showed a 55% decrease in HCC risk compared to those without hyperlipidemia (aSHR=0.45, 95% CI=0.33–0.60, P<0.001) (Supplementary Fig. 3B).

Similarly, after removing 49 patients with eGFR<30, the risk of HCC in diabetic patients not treated with metformin was found to be 1.56-fold higher than that in non-diabetics (aSHR=1.56, 95% CI=1.18–2.07, P=0.002, Supplementary Fig. 4A). Hyperlipidemia patients on statin therapy had a 55% decreased risk of HCC compared to those without hyperlipidemia (aSHR=0.45, 95% CI=0.33–0.60, P<0.001, Supplementary Fig. 4B).

Taken together, our findings from the current study were consistent across these sensitivity analyses.
users, and a 2.23-fold higher risk among metformin users. Whereas, there was no significant difference in HCC risk between metformin non-users and metformin users, both among statin non-users and statin users. Consequently, our analysis did not reveal a significant interaction between statins and metformin in predicting HCC risk ($P=0.82$, Supplementary Fig. 5).

**DISCUSSION**

Of the 2,779 CHC patients, 480 (17.3%) developed new-onset HCC and 238 (8.6%) died after antiviral therapy. Patients with DM but no metformin use had a 1.51-fold higher risk of HCC than patients without DM, whereas HCC risk was comparable between patients without DM and those with DM on metformin. The 5-year cumulative HCC incidence (16.5%) was significantly higher in metformin non-users than in those without DM (11.3%, $P=0.007$) and metformin users (3.1%, $P=0.022$). Conversely, patients with HLP who used statins had a 50% lower risk of HCC than those without HLP (5-year cumulative HCC incidence: 3.8% vs. 12.5%, $P<0.001$). Notably, the unfavorable effect of metformin non-use on increased HCC risk was mainly observed among patients without LC but not among patients with LC. In contrast, a favorable effect of statins on reducing the risk of HCC was observed in patients with and without LC.

HCV infection has been linked to lower lipid profiles, and when a patient with CHC achieves SVR, their lipid profiles may worsen, potentially leading to cardiocerebral events.\(^{21-23}\) For patients with CHC, DM or HLP, treatment with the antidiabetic agent metformin and cholesterol-lowering statin is commonly used. Several studies have investigated the association between statin use and HCC risk in patients with CHC. Statins are well-known for their preventive role in many cancers, including liver cancer.\(^{24,25}\) A meta-analysis of 27 studies found strong evidence of statin-related potential in reducing the risk of HCC.\(^{24}\) Among patients with CHC, statin use has been associated with im-

![Figure 5. Continued.](https://doi.org/10.3350/cmh.2024.0038)
proved virological response rates to antiviral therapy, decreased liver fibrosis progression, and reduced HCC risk in a dose-dependent manner in both Veterans and Taiwanese insurance cohorts. These studies suggested that statin use may have a protective effect against liver cancer in patients with hepatitis C infection. In the present study, we found that statin use reduced the risk of HCC in patients with HLP and CHC after the failure of antiviral therapy. Several studies have explored the association between metformin use and HCC risk in patients with CHC and DM. A systematic review of 12 studies indicated that metformin might have a protective effect against HCC in patients with DM. A large nationwide Taiwanese study showed that metformin use was linked to a lower HCC risk in a dose-dependent manner in patients with type 2 DM and chronic liver disease. These studies suggested that metformin may exert a protective effect against HCC in patients with CHC and diabetes. Furthermore, our previous study documented that after achieving SVR, LC, and metformin nonuse in patients with DM and CHC may result in a higher risk of HCC. Similarly, we also observed a chemopreventive effect of metformin on the risk of HCC development in patients who failed antiviral therapy. Another interesting viewpoint is that among these patients, the HCC risk in the DM metformin use group initially appears lower than in the non-DM group but later aligns with it. This trend implies that the timing and effectiveness of metformin usage may vary. Maybe metformin had a real chemopreventive effect at an earlier period, but it was discontinued later due to progression or uncontrolled diabetes, and the effect faded, resulting in a comparable HCC risk as non-DM. Further validation is needed for these findings.

Our clinical findings revealed that unfavorable DM metformin non-use and favorable HLP statin use were critical in reducing the risk of HCC among patients with CHC after antiviral therapy failed. Several studies have investigated the effects of combination therapy with simvastatin (a statin) and metformin on the growth and migration of different cancer cells, including HBV-related HCC and prostate cancer. One study found that simvastatin and metformin inhibited the growth of HBV-related HCC cells by upregulating autophagy. Combining simvastatin with metformin induced G1-phase cell cycle arrest and Ripk1- and Ripk3-dependent necrosis in C4-2B osseous metastatic castration-resistant prostate cancer cells. The synergistic effects of simvastatin and metformin on osseous metastatic castration-resistant prostate cancer cells suggest that this combination may be a promising treatment option for this type of cancer.

After HCV infection, host factors, such as older age and fibrosis progression leading to LC, appear to be significant in the development of HCC. Coexisting conditions such as DM, obesity, and co-infection with HBV or HIV may accelerate the development of HCC. Conversely, successful antiviral therapy has been associated with a reduced risk of HCC. In this study, we observed that older age, advanced fibrosis, and LC were the independent risk factors for HCC. Exploring chemopreventive approaches, such as using favorable HLP statins and unfavorable DM metformin nonuse among patients with CHC in whom antiviral therapy has failed, may present another opportunity to decrease the risk of HCC. Our study had several limitations that merit attention. Firstly, the specific impact of the duration, dosage, continuity, and timing of metformin and statin use on the incidence of HCC remains to be determined. Secondly, our analysis did not differentiate between the effects of lipophilic and hydrophilic statins, nor did it consider the potential influence of other OHAs such as sulfonylureas, thiazolidinediones, and DPP4 inhibitors on HCC incidence. Thirdly, we had no information on interferon preparations, specifically pegylated interferon α-2a versus α-2b, in this study.

Despite these limitations, which make further analysis challenging, our research offers significant insights in the era of DAAs for a small subset of patients who do not achieve SVR with DAAs, are non-compliant with treatment, or have contraindications to DAAs. Additionally, this study on the chemopreventive effects of metformin and statins on HCC risk could serve as a benchmark for future research on the long-term risk of HCC among HCV patients and other patients with active liver diseases.

In summary, both metformin for DM and statins for HLP had chemopreventive effects on HCC risk in patients with CHC in whom antiviral therapy failed. These findings underscore the importance of implementing personalized preventive strategies to manage patients with these clinical profiles.

Authors’ contribution
Guarantor of the article: Ming-Lung Yu. Conception and

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Conflicts of Interest
The authors declare no conflicts of interest in this present study.

SUPPLEMENTARY MATERIAL
Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Metformin and statins on HCC risk in non-SVR CHC

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237.


Evolutionary changes in metabolic dysfunction-associated steatotic liver disease and risk of hepatocellular carcinoma: A nationwide cohort study

Seogsong Jeong1,2, Yun Hwan Oh3, Joseph C Ahn4, Seulggie Choi5, Sun Jae Park2, Hye Jun Kim2, Gyeongsil Lee6, Joung Sik Son3, Heejoon Jang6, Dong Hyeon Lee8, Meng Sha9, Lei Chen10,11, Won Kim8,12, and Sang Min Park2,6

1Department of Biomedical Informatics, Korea University College of Medicine, Seoul; 2Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul; 3Department of Family Medicine, College of Medicine, Chung-Ang University Gwangmyeong Hospital, Gwangmyeong, Korea; 4Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA; 5Department of Internal Medicine, Seoul National University Hospital, Seoul; 6Department of Family Medicine, Life Clinic, Seoul; 7Department of Internal Medicine, Hanyang University Hospital, Seoul; 8Division of Gastroenterology and Hepatology, Department of Internal Medicine, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, Korea; 9Department of Liver Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai; 10The International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Hospital, Shanghai; 11National Center for Liver Cancer, Shanghai, China; 12Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea

Graphical Abstract

Study Highlights

- Evolutionary alterations, including persistent non-metabolic dysfunction-associated steatotic liver disease (MASLD), resolved MASLD, incident MASLD, and persistent MASLD, were linked to varying hepatocellular carcinoma risks independent of metabolic risk factors and concomitant medications, offering enhanced insight into hepatocellular carcinoma risk stratification among MASLD patients.
**Background/Aims:** To determine the association between evolutionary changes in metabolic dysfunction-associated steatotic liver disease (MASLD) status and the risk of hepatocellular carcinoma (HCC) in a nationwide population-based cohort.

**Methods:** Information on study participants was derived from the Korea National Health Insurance Service database. The study population consisted of 5,080,410 participants who underwent two consecutive biennial health screenings between 2009 and 2012. All participants were followed up until HCC, death, or 31 December 2020. The association of evolutionary changes in MASLD status, as assessed by the fatty liver index and cardiometabolic risk factors, including persistent non-MASLD, resolved MASLD, incident MASLD, and persistent MASLD, with HCC risk was evaluated using multivariable-adjusted Cox proportional hazards regression.

**Results:** Among the 5,080,410 participants with 39,910,331 person-years of follow-up, 4,801 participants developed HCC. The incidence of HCC in participants with resolved, incident, and persistent MASLD was approximately 2.2-, 2.3-, and 4.7-fold higher, respectively, than that in those with persistent non-MASLD among the Korean adult population. When stratifying the participants according to the evolutionary change in MASLD status, persistent (adjusted hazard ratio [aHR], 2.94; 95% confidence interval [CI], 2.68–3.21; \( P < 0.001 \)), incident (aHR, 1.85; 95% CI, 1.63–2.10; \( P < 0.001 \)), and resolved MASLD (aHR, 1.33; 95% CI, 1.18–1.50; \( P < 0.001 \)) had an increased risk of HCC compared to persistent non-MASLD.

**Conclusions:** The evolutionary changes in MASLD were associated with the differential risk of HCC independent of metabolic risk factors and concomitant medications, providing additional information on the risk of HCC stratification in patients with MASLD. (Clin Mol Hepatol 2024;30:487-499)

**Keywords:** Liver neoplasms; Fatty liver; Epidemiology; Cohort studies

**INTRODUCTION**

Primary liver cancer is the sixth most common cancer worldwide and the third most common cause of cancer-related mortalities.\(^1\) Hepatocellular carcinoma (HCC) accounts for up to 75% of all liver cancers, with global incidence and mortality rates of 9.3 and 8.5 per 100,000 person-years (PY) in 2018, respectively.\(^2,3\) The incidence of HCC varies across different regions with regard to the proportion of etiologic factors, such as nonalcoholic fatty liver disease (NAFLD), hepatitis B virus (HBV), and hepatitis C virus (HCV).\(^4\) To date, rational and practical approaches to
surveillance, diagnosis, early detection, prevention, and treatment have been developed, showing their efficacy in reducing the incidence of HCC and cancer-related mortality. However, the incidence of HCC and cancer-related mortality continues to rise, and most patients remain undiagnosed until the advanced stages of the disease.

Chronic HBV and HCV infections, diabetes mellitus, alcohol intake, aflatoxins, and aristolochic acid are well-established risk factors for HCC. However, NAFLD and nonalcoholic steatohepatitis (NASH) have become the most common liver diseases globally and have emerged as a major factor contributing to the development of HCC. Indeed, the global proportion of NAFLD-related HCC accounts for up to 38%. Although high-quality, large-scale, population-based studies exploring the association between NAFLD and the risk of HCC are lacking, several studies have confirmed its significant association with an increased risk of HCC. In addition, NAFLD-related HCC often occurs even in patients without liver cirrhosis; a substantial number of HCC cases were found in patients with NAFLD without cirrhosis in large population-based studies. However, current evidence is not sufficient to provide a confident rationale for the appropriate management of NAFLD to prevent the development of HCC in diverse populations with varying severity of NAFLD. Furthermore, approximately one-quarter of HCC cases have been reported to develop without any known risk factors. More recently, Kim et al. found that newly suggested metabolic dysfunction-associated steatotic liver disease (MASLD), defined as steatotic liver disease (fatty liver index ≥60) and at least one of the cardiometabolic risk factors, is associated with a higher risk of HCC. However, the effects of evolutionary changes in MASLD status on the risk of HCC remain unclear.

In the present study, we sought to determine the association of evolutionary changes in MASLD status with the risk of HCC in a prospectively collected Korean nationwide cohort, which may support the establishment of better management strategies for patients with MASLD to prevent HCC.

MATERIALS AND METHODS

Study population

This South Korea nationwide retrospective cohort study derived information on the study population from the National Health Insurance Service (NHIS) of the Republic of Korea. Detailed elucidations of the NHIS cohort design, methods, and validity of the data are presented in previous studies. Briefly, the NHIS is a quasi-government entity that provides mandatory healthcare insurance with a coverage rate of up to 97% of all Korean citizens and is established under the Ministry of Health and Welfare. The NHIS database routinely and prospectively collects individual-level demographic characteristics, results of health screenings, medical treatment records, medication prescription records, and lifestyle behaviors, and undergoes quality control before being released for research purposes.

We enrolled 7,629,948 participants without alcohol consumption (<1 time/week) who underwent two consecutive biennial health screening examinations of the NHIS between 2009–2010 and 2011–2012. We excluded participants with death before January 1, 2013, diagnosis of HCC before January 1, 2013, prior history of other competing liver diseases (alcohol-related liver disease, toxic liver disease, hepatic failure, chronic hepatitis B or C virus infection, chronic hepatitis not elsewhere classified, liver cirrhosis, other inflammatory liver diseases, and other diseases of the liver; Supplementary Table 1), aged under 20 years, missing information for the covariates, and missing information for the evaluation of steatotic liver disease status (Fig. 1). Finally, 5,080,410 non-drinking adult men and women comprised the analytic cohort for the follow-up investigation against HCC. This study was conducted in accordance with both the Declarations of Helsinki and Istanbul. The institutional review board of Seoul National University Hospital approved this study (No. E-2108-136-1246). Informed consents were exempted because the NHIS database is strictly anonymized according to the Personal Data Protection Act. This study was conducted in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (https://www.equator-network.org/reporting-guidelines/strobe/).
Classification of change in MASLD status

At each health screening period, the fatty liver index (FLI) was calculated for each participant using the following formula:

$$FLI = \frac{1}{1 + \exp(-x)} \times 100,$$

where

$$x = 0.953 \times \log_e(\text{serum triglycerides}) + 0.139 \times (\text{BMI}) + 0.718 \times \log_e(\text{serum GGT}) + 0.053 \times (\text{waist circumference}) - 15.745.$$

The FLI efficiently identifies NAFLD in European and Asian populations, and previous studies have also adopted FLI in the operational definition of steatotic liver disease status.\(^{22-25}\) MASLD was defined as steatotic liver disease (FLI ≥ 60)\(^{26}\) and the presence of at least one of the following cardiometabolic risk factors: body mass index (BMI) ≥ 23 kg/m\(^2\) or waist circumference ≥ 90 cm (for men) and ≥ 85 cm (for women),\(^{27}\) fasting serum glucose ≥ 100 mg/dL or a history of antidiabetic drug prescription or type 2 diabetes, blood pressure ≥ 130/85 mm Hg or a history of antihypertensive drug prescription, triglycerides ≥ 150 mg/dL or a history of lipid-lowering drug prescription, and high-density lipoprotein cholesterol ≤ 40 mg/dL (for men) and ≤ 50 mg/dL (for women).\(^{20}\)

We created categories as follows: (1) No MASLD at both health screening periods (persistent non-MASLD; no MASLD to no MASLD), (2) MASLD at the first health screening and no MASLD at the second health screening (resolved MASLD; MASLD to no MASLD), (3) no MASLD at first health screening to MASLD at second health screening (incident MASLD; no MASLD to MASLD), and (4) MASLD at both health screening periods (persistent

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**Figure 1.** Flow diagram of study participants selected from the Korea National Health Insurance Service database.
MASLD; MASLD to MASLD). The first and second health screenings were carried out between 2009–2010 and 2011–2012, respectively.

Follow-up for HCC

The baseline period for data collection was 2011–2012, and the past MASLD status was backtracked by collecting data from 2009 to 2010. We collected medical treatment claims data from the NHIS to identify HCC events during the follow-up investigation from January 1, 2013, to December 31, 2020. We used the International Classification of Diseases, 10th Revision (ICD-10) code C22.0 and the critical condition code for cancer, as defined previously.28

Key variables for adjustment and subgroup analyses

Socioeconomic factors, including age (continuous; years), sex (categorical; men and women), and household income (categorical; 1st, 2nd, 3rd, and 4th quartiles), health examination results, including body mass index (continuous; kg/m²), systolic blood pressure (continuous; mmHg), and fasting serum glucose (continuous; mg/dL), lifestyle behaviors, including cigarette smoking (categorical; never, past, and current) and moderate-to-vigorous physical activity (MVPA; categorical; none, 1–2 times/week, 3–4 times/week, and ≥5 times/week), drug prescriptions (defined as history of specified drug prescriptions within 3 years before the date of follow-up investigation began; Supplementary Table 2), including antihypertensive medication (categorical; yes and no), antidiabetic medication (categorical; yes and no), antidyslipidemic medication (categorical; yes and no), aspirin (categorical; yes and no), acetaminophen (categorical; yes and no), and non-steroidal anti-inflammatory drugs (NSAIDs) (categorical; yes and no), and advanced fibrosis (categorical; yes and no) were included for the adjustments. Advanced fibrosis was defined as the BMI, aspartate aminotransferase/alanine aminotransferase ratio, and diabetes mellitus (BARD) score ≥2. The BARD score is calculated as follows: BMI ≥28 kg/m²=1 point, aspartate aminotransferase/alanine aminotransferase ratio ≥0.8=2 points, and diabetes mellitus=1 point.28

Statistical analysis

Beginning from January 1, 2013, all participants were followed until the date of HCC or death, or December 31, 2020, whichever came earliest. Characteristics of participants were calculated by n (%) and mean (standard deviation, SD) for categorical and continuous variables, respectively. The crude rate (i.e., incidence) was computed based on the number of total HCC events per 100,000 person-years in each group according to evolutionary changes in MASLD status. Adjusted hazard ratio (aHR) and 95% confidence interval (CI) were calculated using the Cox proportional hazards model through cause-specific analyses. The first model was adjusted for age and sex. We then developed a second adjustment model using age, sex, household income, BMI, systolic blood pressure, fasting serum glucose, cigarette smoking, exercise frequency, and the Charlson comorbidity index (CCI). In addition, the final full adjustment model was further adjusted for antihypertensive medication, antidiabetic medication, antidyslipidemic medication, aspirin, acetaminophen, NSAIDs, and advanced fibrosis.

Sensitivity analyses were performed after excluding events that occurred within 1, 3, and 5 years since the date of the follow-up investigation. We also conducted competing risk analysis using the Fine-Gray model to support primary findings by treating death (n=252,061) or liver transplantation (n=241) as competing risks. The proportional hazards assumption was not violated for the evolutionary changes in MASLD status based on the Kolmogorov-type supremum test (P=0.346). Graphical visualization was carried out using the cumulative incidence function for the incidence of HCC according to the evolutionary change in MASLD status. Subgroup analyses were carried out after stratifying participants according to the known risk factors for HCC. All data mining, collection, and statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA). P-values of less than 0.05 were considered statistically significant for all analyses.
## Table 1. Descriptive characteristics of the participants according to evolutionary changes in MASLD status

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Persistent non-MASLD (n=2,646,504)</th>
<th>Resolved MASLD (n=413,695)</th>
<th>Incident MASLD (n=391,568)</th>
<th>Persistent MASLD (n=1,628,643)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>51.7 (14.2)</td>
<td>59.0 (12.9)</td>
<td>55.3 (13.4)</td>
<td>57.4 (12.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Men</td>
<td>473,978 (17.9)</td>
<td>136,362 (33.0)</td>
<td>120,087 (30.7)</td>
<td>774,089 (47.5)</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>2,172,526 (82.1)</td>
<td>277,333 (67.0)</td>
<td>271,481 (69.3)</td>
<td>854,554 (52.5)</td>
<td></td>
</tr>
<tr>
<td>Household income*, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1st quartile</td>
<td>851,626 (32.2)</td>
<td>143,296 (34.6)</td>
<td>125,931 (32.2)</td>
<td>560,543 (34.4)</td>
<td></td>
</tr>
<tr>
<td>2nd quartile</td>
<td>651,639 (24.6)</td>
<td>100,008 (24.2)</td>
<td>96,145 (24.6)</td>
<td>412,822 (25.3)</td>
<td></td>
</tr>
<tr>
<td>3rd quartile</td>
<td>533,716 (20.2)</td>
<td>75,536 (18.3)</td>
<td>78,090 (19.9)</td>
<td>297,680 (18.3)</td>
<td></td>
</tr>
<tr>
<td>4th quartile (highest)</td>
<td>609,523 (23.0)</td>
<td>94,855 (22.9)</td>
<td>91,402 (23.3)</td>
<td>357,598 (22.0)</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>21.7 (2.2)</td>
<td>23.7 (2.1)</td>
<td>24.7 (2.3)</td>
<td>26.4 (2.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>73.9 (6.7)</td>
<td>79.6 (6.0)</td>
<td>82.6 (6.5)</td>
<td>87.1 (7.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>118.6 (14.7)</td>
<td>124.2 (15.0)</td>
<td>125.2 (14.7)</td>
<td>128.2 (14.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>73.6 (9.5)</td>
<td>76.2 (9.6)</td>
<td>77.3 (9.5)</td>
<td>79.0 (9.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>95.6 (48.9)</td>
<td>110.3 (53.5)</td>
<td>156.3 (91.3)</td>
<td>171.7 (106.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>59.5 (21.9)</td>
<td>54.2 (28.1)</td>
<td>54.3 (21.3)</td>
<td>50.9 (21.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alanine aminotransferase, IU/L</td>
<td>17.3 (9.2)</td>
<td>19.1 (8.3)</td>
<td>26.3 (22.5)</td>
<td>29.4 (20.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>γ-glutamyl transpeptidase, IU/L</td>
<td>15.8 (6.3)</td>
<td>18.3 (7.1)</td>
<td>29.4 (23.6)</td>
<td>37.7 (32.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fatty liver index at baseline</td>
<td>20.5 (16.1)</td>
<td>40.2 (14.4)</td>
<td>75.6 (10.7)</td>
<td>87.6 (11.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cigarette smoking, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Never smoker</td>
<td>2,323,812 (87.8)</td>
<td>326,206 (78.9)</td>
<td>313,249 (80.0)</td>
<td>1,115,087 (68.5)</td>
<td></td>
</tr>
<tr>
<td>Former smoker</td>
<td>134,935 (5.1)</td>
<td>42,123 (10.2)</td>
<td>30,573 (7.8)</td>
<td>222,015 (13.6)</td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>187,757 (7.1)</td>
<td>45,366 (11.0)</td>
<td>47,746 (12.2)</td>
<td>291,541 (17.9)</td>
<td></td>
</tr>
<tr>
<td>MVPA, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0 time/week</td>
<td>1,310,139 (49.5)</td>
<td>218,397 (52.8)</td>
<td>202,245 (51.7)</td>
<td>837,300 (51.4)</td>
<td></td>
</tr>
<tr>
<td>1–2 time/week</td>
<td>455,865 (17.2)</td>
<td>55,129 (13.3)</td>
<td>61,914 (15.8)</td>
<td>245,907 (15.1)</td>
<td></td>
</tr>
<tr>
<td>3–4 time/week</td>
<td>351,754 (13.3)</td>
<td>48,284 (11.7)</td>
<td>49,680 (12.7)</td>
<td>203,124 (12.5)</td>
<td></td>
</tr>
<tr>
<td>≥5 time/week</td>
<td>528,746 (20.0)</td>
<td>91,885 (22.2)</td>
<td>77,729 (19.9)</td>
<td>342,312 (21.0)</td>
<td></td>
</tr>
<tr>
<td>BARD score, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0–2</td>
<td>2,391,894 (90.4)</td>
<td>321,231 (77.6)</td>
<td>322,417 (82.3)</td>
<td>1,132,256 (69.5)</td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>254,610 (9.6)</td>
<td>92,464 (22.4)</td>
<td>69,151 (17.7)</td>
<td>496,387 (30.5)</td>
<td></td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>983,935 (37.2)</td>
<td>234,102 (56.6)</td>
<td>192,381 (49.1)</td>
<td>957,242 (58.8)</td>
<td>&lt;0.001</td>
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<tr>
<td>Antihypertensive medication</td>
<td>899,363 (34.0)</td>
<td>217,408 (52.6)</td>
<td>213,201 (54.5)</td>
<td>732,164 (45.0)</td>
<td>&lt;0.001</td>
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<tr>
<td>Diabetes mellitus</td>
<td>261,642 (9.9)</td>
<td>92,362 (22.3)</td>
<td>57,770 (14.8)</td>
<td>383,349 (23.5)</td>
<td>&lt;0.001</td>
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<tr>
<td>Antidiabetic medication</td>
<td>120,910 (4.6)</td>
<td>58,812 (14.2)</td>
<td>30,813 (7.9)</td>
<td>259,404 (15.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>467,167 (17.7)</td>
<td>143,761 (34.8)</td>
<td>112,144 (28.6)</td>
<td>618,016 (37.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antidysslipidemic medication</td>
<td>355,809 (13.4)</td>
<td>119,977 (29.0)</td>
<td>91,460 (23.4)</td>
<td>521,243 (32.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Charlson comorbidity index, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0</td>
<td>1,445,913 (54.6)</td>
<td>166,765 (40.3)</td>
<td>183,573 (46.9)</td>
<td>675,090 (41.5)</td>
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</tr>
<tr>
<td>1</td>
<td>964,178 (36.4)</td>
<td>170,090 (41.1)</td>
<td>155,266 (39.7)</td>
<td>654,587 (40.2)</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td>236,413 (8.9)</td>
<td>76,840 (18.6)</td>
<td>52,729 (13.5)</td>
<td>298,966 (18.4)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean (standard deviation) unless otherwise specified.

MASLD, metabolic dysfunction-associated steatotic liver disease; HDL, high-density lipoprotein; MVPA, moderate-to-vigorous physical activity.

*Proxy for socioeconomic status based on the insurance premium of the National Health Insurance Service.
RESULTS

Descriptive characteristics

Among 5,080,410 participants, the mean age was 51.7 years (SD, 14.2; Table 1). The mean BMI and waist circumferences were 26.4 kg/m$^2$ (SD, 2.8) and 87.1 cm (SD, 7.3), respectively, in participants with persistent MASLD. Approximately half of the participants with persistent MASLD were physically inactive (n=837,300; 51.4%; no MVPA) and 675,090 participants (41.5%) had no comorbidities (CCI=0). Of the 4 different groups stratified by the evolutionary changes in MASLD status, BMI and waist circumference showed an increasing tendency from persistent non-MASLD to resolved, incident, and persistent MASLD ($P<0.001$). The proportions of men and former or current smokers were higher in participants with persistent MASLD than in those with persistent non-MASLD ($P<0.001$). In addition, those with persistent MASLD also had higher blood pressure, triglycerides, alanine aminotransferase, and

<p>| Table 2. Association of evolutionary changes in MASLD status with the risk of hepatocellular carcinoma |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>aHR (95% CI)</th>
<th>Evolutionary changes in MASLD status</th>
<th>aHR (95% CI)</th>
<th>Evolutionary changes in MASLD status</th>
<th>aHR (95% CI)</th>
<th>Evolutionary changes in MASLD status</th>
<th>aHR (95% CI)</th>
<th>Evolutionary changes in MASLD status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistent non-MASLD</td>
<td>Resolved MASLD</td>
<td>Incident MASLD</td>
<td>Persistent MASLD</td>
<td>Persistent non-MASLD</td>
<td>Resolved MASLD</td>
<td>Incident MASLD</td>
<td>Persistent MASLD</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Number (%)</td>
<td>2,646,504 (52.1)</td>
<td>413,695 (8.1)</td>
<td>391,568 (7.7)</td>
<td>1,628,643 (32.1)</td>
<td>2,646,504 (52.1)</td>
<td>413,695 (8.1)</td>
<td>391,568 (7.7)</td>
</tr>
<tr>
<td>Event</td>
<td>1,058</td>
<td>357</td>
<td>356</td>
<td>3,030</td>
<td>1,058</td>
<td>357</td>
<td>356</td>
</tr>
<tr>
<td>Crude rate/100,000 PY</td>
<td>5.1</td>
<td>11.1</td>
<td>11.6</td>
<td>23.8</td>
<td>5.1</td>
<td>11.1</td>
<td>11.6</td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.33 (1.18–1.50)</td>
<td>1.77 (1.57–2.00)</td>
<td>2.82 (2.63–3.03)</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.30 (1.15–1.47)</td>
<td>1.75 (1.55–1.99)</td>
<td>2.77 (2.53–3.03)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.33 (1.17–1.50)</td>
<td>1.85 (1.63–2.10)</td>
<td>2.94 (2.68–3.21)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Men

<table>
<thead>
<tr>
<th>Number (%)</th>
<th>473,978 (31.5)</th>
<th>136,362 (9.1)</th>
<th>120,087 (8.0)</th>
<th>774,089 (51.5)</th>
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<tbody>
<tr>
<td>PY</td>
<td>3,654,472</td>
<td>1,045,290</td>
<td>933,707</td>
<td>6,028,331</td>
</tr>
<tr>
<td>Event</td>
<td>484</td>
<td>198</td>
<td>184</td>
<td>2,018</td>
</tr>
<tr>
<td>Crude rate/100,000 PY</td>
<td>13.2</td>
<td>18.9</td>
<td>19.7</td>
<td>33.5</td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.28 (1.08–1.51)</td>
<td>1.72 (1.45–2.03)</td>
<td>2.81 (2.55–3.11)</td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.24 (1.05–1.47)</td>
<td>1.68 (1.41–2.00)</td>
<td>2.72 (2.41–3.07)</td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.25 (1.06–1.48)</td>
<td>1.74 (1.46–2.07)</td>
<td>2.83 (2.51–3.20)</td>
</tr>
</tbody>
</table>

Women

<table>
<thead>
<tr>
<th>Number (%)</th>
<th>2,172,526 (60.8)</th>
<th>277,333 (7.8)</th>
<th>271,481 (7.6)</th>
<th>854,554 (23.9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PY</td>
<td>17,206,585</td>
<td>2,178,067</td>
<td>2,142,589</td>
<td>6,721,291</td>
</tr>
<tr>
<td>Event</td>
<td>574</td>
<td>159</td>
<td>172</td>
<td>1,012</td>
</tr>
<tr>
<td>Crude rate/100,000 PY</td>
<td>3.3</td>
<td>7.3</td>
<td>8.0</td>
<td>15.1</td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.42 (1.19–1.70)</td>
<td>1.86 (1.57–2.21)</td>
<td>2.92 (2.64–3.24)</td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.40 (1.17–1.68)</td>
<td>1.88 (1.57–2.25)</td>
<td>2.91 (2.55–3.33)</td>
</tr>
<tr>
<td>aHR (95% CI)*</td>
<td>1.00 (reference)</td>
<td>1.48 (1.23–1.77)</td>
<td>2.04 (1.71–2.45)</td>
<td>3.21 (2.80–3.69)</td>
</tr>
</tbody>
</table>

aHRs calculated using the Cox proportional hazards model. Steatotic liver disease defined as fatty liver index≥60. MASLD, metabolic dysfunction-associated steatotic liver disease; PY, person-year; aHR, adjusted hazard ratio; CI, confidence interval. *Adjusted for age and sex. †Further adjusted for household income, body mass index, systolic blood pressure, fasting serum glucose, cigarette smoking, exercise frequency, and Charlson comorbidity index on the basis of Model A. ‡Further adjusted for antihypertensive medication, antidiabetic medication, antidysslipidemic medication, aspirin, acetaminophen, non-steroidal anti-inflammatory drugs, and advanced fibrosis on the basis of Model B.
γ-glutamyl transpeptidase but lower high-density lipoprotein cholesterol levels in a group-dependent manner (P<0.001).

Changes in MASLD status and the risk of HCC

Overall, 4,801 new HCC cases were identified during 39,910,331 person-years of follow-up. The crude rate was approximately 2.2-, 2.3-, and 4.7-fold higher in participants with resolved, incident, and persistent MASLD than those with persistent non-MASLD. In addition, persistent MASLD had the highest risk of HCC, followed by incident MASLD and resolved MASLD, compared to persistent non-MASLD (Table 2). After adjustments were made for confounding factors, persistent MASLD (aHR, 2.94; 95% CI, 2.68–3.21; P<0.001), incident MASLD (aHR, 1.85; 95% CI, 1.63–2.10; P<0.001), and resolved MASLD (aHR, 1.33; 95% CI, 1.17–1.50; P<0.001) revealed higher risks of HCC compared to persistent non-MASLD. When setting each of resolved, incident, and persistent MASLD as a reference in the intergroup analyses with full adjustments, the risk of HCC was significantly increased in the order of resolved (lowest), incident (middle), and persistent (highest) MASLD (Supplementary Table 3).

Figure 2 shows biennial numbers of HCC events for the evolutionary changes in MASLD status. MASLD, metabolic dysfunction-associated steatotic liver disease.

Sensitivity analyses

In the sensitivity analyses, the results were similar to the primary findings (Supplementary Table 4). However, in the analysis that was carried out after excluding events that occurred within 5 years, resolved MASLD (aHR, 1.20; 95% CI, 0.99–1.46) did not have an increased risk of HCC compared to persistent non-MASLD. In the competing risk analysis, resolved, incident, and persistent MASLD were associated with higher risks of HCC compared to persistent non-MASLD (Supplementary Table 5).

Stratified analyses

The study participants were stratified by age, obesity, smoking, and CCI (Fig. 3). Consistently, the risk of HCC was highest in persistent MASLD followed by incident MASLD and resolved MASLD as compared to persistent non-MASLD regardless of stratification subgroups. While both incident and resolved MASLD generally revealed a higher risk of HCC, obese participants with resolved MASLD and incident MASLD did not significantly increase the risk of HCC compared to persistent non-MASLD.

When stratified by drug prescription, no significant interaction was found for antihypertensive medication, antidyslipidemic medication, aspirin, acetaminophen, and NSAIDs (Supplementary Table 6). On the contrary, a significant interaction effect was found for antidiabetic medication (P for interaction<0.001); in the incident MASLD and persistent MASLD groups, participants with a prescription history of antidiabetic medication had even higher risks of HCC than those without. When stratifying the participants with resolved MASLD, incident MASLD, and persistent MASLD according to the presence of advanced fibrosis (BARD≥2), those with advanced fibrosis were associated with a higher risk of HCC in the incident and persistent MASLD groups (Supplementary Table 7).

DISCUSSION

In this Korean nationwide population-based cohort study,
Figure 3. A forest plot on the impact of evolutionary changes in MASLD status on the risk of HCC stratified by risk factors for HCC. Data are adjusted hazard ratio (95% confidence interval) calculated using the Cox proportional hazards model after adjustments for baseline fatty liver index, age, sex, household income, body mass index, systolic blood pressure, fasting serum glucose, cigarette smoking, exercise frequency, CCI, antihypertensive medication, antidiabetic medication, antidysslipidemic medication, aspirin, acetaminophen, NSAIDs, and advanced fibrosis. MASLD, metabolic dysfunction-associated steatotic liver disease; CCI, Charlson comorbidity index; NSAIDs, non-steroidal anti-inflammatory drugs.
we provided robust real-world evidence that evolutionary changes in MASLD status are significantly associated with the risk of HCC. Both persistent and incident MASLD were associated with a higher risk of developing HCC than those with persistent non-MASLD. Even patients with a history of MASLD, who no longer had MASLD at the second health screening, had an increased risk of HCC. In this context, we need to develop a refined screening and surveillance program for HCC in participants with resolved or incident MASLD.

NAFLD has now emerged as the leading cause of liver-related morbidity worldwide. NAFLD-related cirrhosis is deemed an evident risk factor for HCC. However, even NAFLD patients without evidence of cirrhosis may occasionally develop HCC, and the TERT promoter mutation is most commonly observed in NAFLD-related HCC, implicating that such a population at risk of HCC may be distinct from other high-risk groups for HCC such as viral hepatitis or liver cirrhosis. Furthermore, genetic mutations are increasingly accumulated in both cancerous and noncancerous tissues of the liver obtained from patients with NAFLD regardless of fibrosis progression. In the current study, participants with resolved MASLD retained a higher risk of HCC compared to those with persistent non-MASLD, suggesting that those with resolved MASLD may be at the residual risk of HCC despite lifestyle modifications or therapeutic interventions.

Current guidelines suffer from limited data regarding the selection of the target population with NAFLD to undergo HCC surveillance and the optimal frequency of monitoring HCC among NAFLD patients without NASH or cirrhosis. Therefore, the current MASLD definition, accompanied by steatotic liver disease and cardiometabolic risk factors, may provide additional information. In the current study, participants with resolved MASLD had a lower risk of HCC compared to those with persistent MASLD, and individuals with persistent non-MASLD had a lower risk of HCC compared to those with incident MASLD. In this context, we should provide more intensive care for patients with MASLD in order to reduce the risk of HCC as secondary prevention. Importantly, it is more cost-effective and easily attainable to prevent incident MASLD through preemptive measures, such as physical activity and dietary intervention, ultimately lowering the risk of HCC as primary prevention.

The underlying mechanisms of HCC arising in liver cirrhosis are characterized by repeated hepatocellular death and subsequent regeneration, accompanied by continual cell growth and proliferation that favor the ultimate development of HCC. However, the mechanism of NAFLD-related hepatocarcinogenesis is more likely associated with the pathogenesis of hepatic steatosis per se than that of advanced fibrosis or cirrhosis alone, which requires further elucidation. To date, NAFLD-related hepatocarcinogenesis may be driven by adipose tissue-derived inflammation, changes in hormones, lipotoxicity, oxidative stress, gut dysbiosis, and genetic factors, which are of increasing importance. As an advanced status of NAFLD, NASH-related HCC currently lacks a cost-effective surveillance program for HCC, leading to the diagnosis of HCC at an advanced stage, and is considered less responsive to immunotherapy targeted at programmed death-1. In addition, the defenses against ferroptosis were found to be ultimately surpassed by lipid peroxidation in MASLD and metabolic dysfunction-associated steatohepatitis, as indicated by the existence of ferroptosis executors in hepatocytes and sinusoidal cells, the decrease in polyunsaturated fatty acids in membrane phospholipids, the increase in lysophosphatidylcholine, and the elevation in breakdown products of ferroptosis, thus generating a proferroptotic environment that contributes to the development of HCC. Therefore, preventive strategies stratified according to the changes in MASLD status may be a novel approach to lower the disease burden of MASLD-related HCC.

The strength of the current study is to reveal the association of the evolutionary change in MASLD with HCC risk in adults using a nationwide population-based database with repeated measurements for the evaluation of the change in MASLD status. Ascertainment of an HCC event was made by the medical claims record, which was collected at a nationwide level, and its accuracy is deemed high along with the critical condition code for cancer. However, the current study has several limitations. First, the evolutionary change in MASLD status was defined using the FLI. There is a chance of misclassification when diagnosing MASLD using noninvasive surrogate markers. The NHIS database lacks information on the results of liver biopsy and imaging tests, such as ultrasound and computed tomography. Therefore, further studies with histological examination or imaging modalities are warranted to evaluate the evolutionary
change in MASLD status in relation to the risk of HCC. Second, additional information on potential confounders, such as dietary habits and genetic variants, was not available in the NHIS database. Third, the health screening data had neither information on the presence of liver fibrosis nor platelet counts that are required for the calculation of the fibrosis-4 index, which led us to adopt the BARD score for the operational definition of advanced fibrosis. The occurrence of HCC was followed up through the ICD-10 code for HCC in the NHIS database, thus there might be interval censoring. Lastly, the generalizability of our results is limited to Korean adults who received health screening from the NHIS. More evidence from other ethnic or multi-ethnic population-based cohorts is needed.

Given the apparent association of the evolutionary change in MASLD with the risk of HCC, a refined HCC surveillance program incorporating the evolutionary changes in MASLD may aid in reducing the future risk of HCC. Especially, patients with resolved MASLD still have a residual risk of HCC, thus requiring optimal screening and preventive strategies against HCC.

Authors’ contribution
Seogsong Jeong (Conceptualization: Equal; Data curation: Lead; Formal analysis: Lead; Methodology: Equal; Visualization: Lead; Writing – original draft: Lead; Writing – review & editing: Lead). Yun Hwan Oh (Investigation: Supporting; Methodology: Equal; Writing – review & editing: Supporting). Joseph C Ahn (Investigation: Supporting; Methodology: Equal; Writing – review & editing: Supporting). Seulggie Choi (Data curation: Supporting; Formal analysis: Supporting; Investigation: Supporting; Writing – review & editing: Supporting). Sun Jae Park (Data curation: Supporting; Formal analysis: Supporting; Investigation: Supporting; Writing – review & editing: Supporting). Hye Jun Kim (Data curation: Supporting; Investigation: Supporting; Writing – review & editing: Supporting). Gyeongsil Lee (Investigation: Supporting; Methodology: Equal; Writing – review & editing: Supporting). Joung Sik Son (Investigation: Supporting; Methodology: Equal; Writing – review & editing: Supporting). Heejoon Jang (Investigation: Supporting; Methodology: Equal; Funding acquisition: Supporting; Writing – review & editing: Supporting). Dong Hyeon Lee (Investigation: Supporting; Methodology: Equal; Funding acquisition: Supporting; Writing – review & editing: Supporting). Meng Sha (Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Equal). Lei Chen (Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Equal). Won Kim (Conceptualization: Equal; Investigation: Equal; Methodology: Equal; Supervision: Lead; Funding acquisition: Lead; Writing – review & editing: Lead). Sang Min Park (Conceptualization: Equal; Investigation: Equal; Methodology: Equal; Supervision: Lead; Funding acquisition: Lead; Writing – review & editing: Lead).

Acknowledgements
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Conflicts of Interest
The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL
Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES


Extrahepatic malignancies and antiviral drugs for chronic hepatitis B: A nationwide cohort study

Moon Haeng Hur, Dong Hyeon Lee, Jeong-Hoon Lee, Mi-Sook Kim, Jeayeon Park, Hyunjae Shin, Sung Won Chung, Hee Jin Cho, Min Kyung Park, Heejoong Jang, Yun Bin Lee, Su Jong Yu, Sang Hyub Lee, Yong Jin Jung, Yoon Jun Kim, and Jung-Hwan Yoon

1Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine; 2Department of Internal Medicine, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, Korea; 3Genome Insight Inc., San Diego, CA, USA; 4Medical Research Collaborating Center, Seoul National University Hospital, Seoul, Korea

Graphical Abstract

Tenofovir disoproxil fumarate is associated with a lower risk of extrahepatic malignancy than entecavir in patients with chronic hepatitis B: A Korean nationwide cohort study

Key Findings
• EHM incidence differed significantly between within 3 years and beyond 3 years in both groups: the incidence increased in the ETV group but decreased in the TDF group.
• During the first 3 years, there was no difference in EHM risk between groups in the propensity score-matched cohort (SHR=1.01, 95% CI=0.86-1.17, P=0.84).
• After year 3, TDF was associated with a significantly lower EHM incidence, compared to ETV (SHR=0.70, 95% CI=0.60-0.81, P<0.01).
• The antitumor effects of TDF appeared to be greater than those of ETV.

Study Highlights
• In both the ETV and TDF groups, EHM incidence changed significantly between within 3 years and beyond 3 years: the incidence increased in the ETV group, whereas it decreased in the TDF group.
• During the first 3 years, the incidence of EHM was comparable between the two groups. After year 3, however, TDF was associated with a significantly lower risk of EHM than ETV.
• Regarding intrahepatic malignancy, the superiority of TDF over ETV was observed both before year 3 and after year 3, with the latter being more prominent.
INTRODUCTION

Chronic hepatitis B (CHB) infection is the most prevalent chronic viral infection worldwide, affecting more than 250 million people and accounting for approximately 45% of hepatocellular carcinoma (HCC) cases.\(^1,2\) Entecavir (ETV) and tenofovir disoproxil fumarate (TDF) have been the most commonly used nucleos(t)ide-analogues (NAs) for CHB patients and both are currently recommended as first-line antivirals because of their high potency and genetic barrier against the development of NA resistance.\(^3-5\)

It remains controversial which of the two antivirals, ETV or TDF, is superior for the prevention of HCC in CHB patients.\(^6\) While some cohort studies and meta-analyses have shown a lower risk of HCC in CHB patients treated with TDF,\(^7,8\) other studies have demonstrated no significant differences between the two antivirals.\(^9,10\) However, no study has proved the superiority of ETV over TDF. It has been

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**Background/Aims:** Chronic hepatitis B (CHB) is related to an increased risk of extrahepatic malignancy (EHM), and antiviral treatment is associated with an incidence of EHM comparable to controls. We compared the risks of EHM and intrahepatic malignancy (IHM) between entecavir (ETV) and tenofovir disoproxil fumarate (TDF) treatment.

**Methods:** Using data from the National Health Insurance Service of Korea, this nationwide cohort study included treatment-naïve CHB patients who initiated ETV (n=24,287) or TDF (n=29,199) therapy between 2012 and 2014. The primary outcome was the development of any primary EHM. Secondary outcomes included overall IHM development. E-value was calculated to assess the robustness of results to unmeasured confounders.

**Results:** The median follow-up duration was 5.9 years, and all baseline characteristics were well balanced after propensity score matching. EHM incidence rate differed significantly between within versus beyond 3 years in both groups (P<0.01, Davies test). During the first 3 years, EHM risk was comparable in the propensity score-matched cohort (5.88 versus 5.84/1,000 person-years; subdistribution hazard ratio [SHR]=1.01, 95% confidence interval [CI]=0.88–1.17, P=0.84). After year 3, however, TDF was associated with a significantly lower EHM incidence compared to ETV (4.92 versus 6.91/1,000 person-years; SHR=0.70, 95% CI=0.60–0.81, P<0.01; E-value for SHR=2.21). Regarding IHM, the superiority of TDF over ETV was maintained both within (17.58 versus 20.19/1,000 person-years; SHR=0.88, 95% CI=0.81–0.95, P<0.01) and after year 3 (11.45 versus 16.20/1,000 person-years; SHR=0.68, 95% CI=0.62–0.75, P<0.01; E-value for SHR=2.30).

**Conclusions:** TDF was associated with approximately 30% lower risks of both EHM and IHM than ETV in CHB patients after 3 years of antiviral therapy. (Clin Mol Hepatol 2024;30:500-514)

**Keywords:** Non-liver cancer; Hepatitis B virus; Antiviral treatment; Tenofovir; Entecavir

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**Corresponding author :** Jeong-Hoon Lee
Department of Internal Medicine, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea
Tel: +82-2-2072-2228, Fax: +82-2-743-6701, E-mail: pindra@empal.com
https://orcid.org/0000-0002-0315-2080

*These two authors equally contributed to this study as co-first authors.

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**Abbreviations:**
CHB, chronic hepatitis B; EHM, extrahepatic malignancy; IHM, intrahepatic malignancy; ETV, entecavir; TDF, tenofovir disoproxil fumarate; SHR, subdistribution hazard ratio; CI, confidence interval; HCC, hepatocellular carcinoma; NAs, nucleos(t)ide-analogues; IPTW, inverse probability of treatment weighting; PSM, propensity score matching; HBV, hepatitis B virus
suggested that more effective and earlier viral suppression with TDF may lead to better outcomes. Some researchers suspect that the protumor or carcinogenic effects of ETV reported in a preclinical animal model might be responsible for the inferiority of ETV, but none of these effects have not been confirmed in humans.

Meanwhile, our group recently reported that patients with CHB have a higher risk of developing a primary extrahepatic malignancy (EHM) than controls. Furthermore, we demonstrated that complete viral suppression with long-term NA treatment was associated with a lower risk of EHM among CHB patients. There has been no study comparing ETV and TDF in terms of EHM prevention. Therefore, we aimed to compare the risk of EHM as well as intrahepatic malignancy (IHM) between patients treated with ETV and those treated with TDF; the results may reflect the antitumor or protumor effects of each antiviral.

MATERIALS AND METHODS

Data source

We established a retrospective cohort using nationwide claims in the National Health Insurance Service (NHIS) database of South Korea. NHIS is a health insurance policy that covers 97% of South Koreans; its utility for research purposes has been well-established. The NHIS database uses the tenth revision of the International Classification of Diseases (ICD-10). The Institutional Review Boards of both the NHIS (No. NHIS-2021-1-804) and SMG–SNU Boramae Medical Center (No. 07-2019-23) approved this study. The requirement for informed consent was waived due to the retrospective nature of this study and because patient data within the NHIS database is coded anonymously.

Study populations and variables

The study cohort originally included 178,937 patients with CHB who initiated treatment with ETV or TDF between January 1, 2012 and December 31, 2014. The cohort entry date and index date were defined as the first day of NA treatment and the 90th day after initiating NA therapy, respectively. After applying exclusion criteria, the final study population included 53,486 patients with CHB (24,287 received ETV [ETV group] and 29,199 received TDF [TDF group]; Fig. 1). For each subject, we obtained demographic information, comorbidity data, and NA data, including the type and cumulative defined daily dose of antiviral used. The presence of liver cirrhosis and/or decompensation was identified using ICD-10 diagnosis codes, NHIS classification codes for specific procedures (e.g., abdominal paracentesis and endoscopic treatment of esophageal or gastric varices), and relevant prescriptions. Supplementary Table 1 shows the diagnosis, procedural, and prescription codes used in this study. In the subset of individuals who received health check-ups provided by the NHIS, anthropometric data, blood test results, and health-related behaviors (smoking, alcohol intake, and physical activity) were also collected. Further details regarding the study population are described in the Supplementary Methods.

Outcomes

The primary outcome was the development of any EHM. EHMs were defined according to ICD-10 codes for non-liver cancers, as well as cancer-specific insurance claim codes. Only the first diagnosed malignancy after the index date was counted as an event. Death and a new IHM were considered competing events. Secondary outcomes were the development of specific EHMs (the 10 most prevalent EHMs in South Korea) and overall IHM. The date of the first claim with the ICD-10 code was considered the date of cancer diagnosis. Further information is provided in the Supplementary Methods.

Statistical analysis

To compare categorical and continuous variables, the standardized difference was measured between the two groups. Propensity score matching (PSM) and inverse probability of treatment weighting (IPTW) were employed to balance the ETV and TDF groups; propensity scores were calculated using all covariates. Study subjects were followed from the index date to the date of EHM diagnosis, date of any competing event, or cut-off date (December 31, 2019), whichever occurred first. The log-log plot and Schoenfeld residual test were utilized to validate the proportional hazards assumption inside the Cox model. If the proportional hazards assumption was not satisfied, an ex-
tended Cox model with Heaviside functions was used. Cumulative incidence of EHM was derived using the cumulative incidence function, and cumulative incidence curves were compared using the Gray test. Applying segmented linear regression, the cumulative incidence was fitted as a piecewise linear function, and change in slope was assessed using the Davies test. To estimate the effect of variables on the cumulative incidence, while taking competing events into account, we used the Fine–Gray model to calculate the subdistribution hazard ratio (SHR). \( P \)-value for interaction \((P_{\text{interaction}})\) was calculated to assess whether NA therapy had differential impacts on EHMs according to subgroups. Various sensitivity analyses were performed to confirm the robustness of our findings (see Supplementary Methods). E-value was calculated to estimate the magnitude of an unadjusted confounding variable needed to mitigate the association between antiviral treatment and the incidence of EHM or IHM.\(^{21}\) All statistical analyses were performed using SAS Enterprise Guide 7.1 (SAS Institute Inc, Cary, NC, USA) and R 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria). \( P \)-values were derived from two-tailed tests, with values <0.05 considered statistically significant.

**RESULTS**

**Baseline characteristics**

Table 1 shows the baseline characteristics of the ETV and TDF groups. Although the healthcare level differed slightly between groups in the crude population, all variables, including age, sex, and coexisting medical conditions, were well balanced after PSM or IPTW. Baseline

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**Figure 1.** Patient flow diagram. Specific diagnostic and procedural codes are presented in Supplementary Table 1. CHB, chronic hepatitis B; ETV, entecavir; HCV, hepatitis C virus; HDV, hepatitis D virus; HIV, human immunodeficiency virus; NA, nucleos(t)ide-analogue; NHIS, National Health Insurance Service; TDF, tenofovir disoproxil fumarate.
Table 1. Baseline characteristics of the study cohort before and after propensity score matching or inverse probability of treatment weighting

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<td>(n=24,287)</td>
<td>(n=28,559)</td>
</tr>
<tr>
<td></td>
<td>5,124 (17.6)</td>
<td>4,376 (18.0)</td>
<td>5,112 (17.9)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>5,480 (22.6)</td>
<td>5,478 (22.6)</td>
<td>5,164 (21.8)</td>
</tr>
<tr>
<td></td>
<td>(n=24,287)</td>
<td>(n=24,287)</td>
<td>(n=23,664)</td>
</tr>
<tr>
<td></td>
<td>6,323 (21.7)</td>
<td>5,314 (21.9)</td>
<td>6,241 (21.8)</td>
</tr>
<tr>
<td>CCI§ point</td>
<td>1.2±1.4</td>
<td>1.2±1.4</td>
<td>1.2±1.2</td>
</tr>
<tr>
<td></td>
<td>(n=24,287)</td>
<td>(n=24,287)</td>
<td>(n=23,664)</td>
</tr>
<tr>
<td></td>
<td>1.2±1.3</td>
<td>1.2±1.3</td>
<td>1.2±1.2</td>
</tr>
</tbody>
</table>

Data are expressed as number (%) or mean±standard deviation.
CCI, Charlson Comorbidity Index; ETV, entecavir; TDF, tenofovir disoproxil fumarate.

* Propensity scores were computed using following variables: age, sex, socioeconomic status, level of healthcare, cirrhosis, decompensated cirrhosis, ascites, varices, diabetes mellitus, hypertension, and Charlson Comorbidity Index.

† High, middle, and low socioeconomic statuses indicate socioeconomic status within the ≥75th, 25th–75th, and <25th percentiles, respectively.

‡ Patients with a special occupation such as military personnel or shipping labor union.

§ Charlson Comorbidity Index was based on data from 1 year before the cohort entry date.
characteristics of the NHIS Health Check-Up Database subcohort are summarized in Supplementary Table 2. Additional anthropometric, habitual, and laboratory variables were well balanced. Distributions of propensity scores for both the entire study population and the NHIS Health Check-Up Database subcohort showed good concordance between treatment groups, without extreme values (Supplementary Fig. 1).

**Incidence of primary extrahepatic malignancies**

Median follow-up durations of the ETV and TDF groups were 6.3 years (interquartile range [IQR]=3.7–7.2 years) and 5.7 years (IQR=5.1–6.4 years), respectively (Table 2). After PSM, there were 822 (3.4%) EHMs in the ETV group and 706 (2.9%) EHMs in the TDF group.

Figure 2 shows the cumulative incidence of primary EHM after PSM. During the entire study period, TDF was associated with a lower risk of EHM than ETV ($P=0.001$ by Gray test). Differences in risk of EHM between groups became more prominent after 3 years from the index date. The incidence rate of EHM decreased in the TDF group but increased in the ETV group after year 3 ($P<0.01$ by Davies test; Supplementary Fig. 2). In the TDF group, the crude incidence of EHM decreased from 5.88/1,000 person-years in the first 3 years to 4.92/1,000 person-years after year 3 (Table 2). Conversely, in the ETV group, the incidence of EHM increased from 5.84/1,000 person-years in the first 3 years to 6.91/1,000 person-years after year 3.

![Graph](attachment:graph.png)

**Figure 2.** Cumulative incidence of extrahepatic malignancies in propensity score-matched cohort. Analysis was performed after propensity score matching. Intrahepatic malignancy development and death were treated as competing events. ETV, entecavir; TDF, tenofovir disoproxil fumarate.

---

**Table 2.** Clinical outcomes in propensity score-matched cohort of chronic hepatitis B patients treated with entecavir or tenofovir disoproxil fumarate

<table>
<thead>
<tr>
<th>cDDDs (per patient per year)</th>
<th>Events, no. (%)</th>
<th>Median follow-up year (IQR)</th>
<th>Crude incidence of extrahepatic malignancy, per 1000 person-year</th>
<th>Within 3 years After 3 years</th>
<th>SHR (95% CI)</th>
<th>P-value</th>
<th>SHR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETV</td>
<td>288.4</td>
<td>63 (2.2–7.2)</td>
<td>5.84 (5.38–6.46)</td>
<td>5.84 (5.28–6.46)</td>
<td>6.91 (6.30–7.58)</td>
<td>0.89</td>
<td>0.70 (0.60–0.81)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TDF</td>
<td>293.4</td>
<td>822 (3.4%)</td>
<td>5.75 (5.33–6.48)</td>
<td>5.88 (5.33–6.48)</td>
<td>4.92 (4.40–5.57)</td>
<td>1.01</td>
<td>0.98 (0.88–1.17)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

**cDDDs,** cumulative defined daily doses; CI, confidence interval; ETV, entecavir; IQR, interquartile range; NA, nucleos(t)ide analogue; SHR, subdistribution hazard ratio; TDF, tenofovir disoproxil fumarate.
According to the Schoenfeld residual test, the Cox proportional hazards assumption was satisfied if the study period was divided as within 3 years and after 3 years from the index date ($P=0.82$) but not if the entire study period was considered ($P<0.01$). The log-log plot reproduced these results (Supplementary Fig. 3). Thus, we stratified the study duration into within the first 3 years and beyond the first 3 years in all analyses and applied the extended Cox model.

EHM incidence within the first 3 years did not differ between antivirals (TDF vs. ETV: SHR=1.01, 95% confidence interval [CI]=0.88–1.17, $P=0.84$), whereas the risk of EHM was significantly lower in the TDF group than in the ETV group after year 3 (SHR=0.70, 95% CI=0.60–0.81, $P<0.01$). Similar results were obtained in most subgroup analyses (age, socioeconomic status, and other medical conditions; all $P_{interaction}>0.05$ except sex (Fig. 3). E-value analysis showed that an unexplained confounder would need to be associated with both NA type and EHM incidence at a risk ratio of 2.21 to mitigate the relationship between these variables and make the SHR=1, while controlling for other covariates in our model (Supplementary Table 3).

**Sensitivity analyses**

Various sensitivity analyses showed similar results (Table 3 and Supplementary Results). The main result was maintained in the study population balanced using IPTW (SHR=0.70, 95% CI=0.61–0.81, $P<0.01$). Supplementary

![Figure 3](https://doi.org/10.3350/cmh.2024.0055)  
*Figure 3.* Risk of extrahepatic malignancy in the propensity score-matched cohort according to prespecified subgroups. SHR, subdistribution hazard ratio; CI, confidence interval; ETV, entecavir; $P_{interaction}$, $P$-value for interaction; TDF, tenofovir disoproxil fumarate. *High, middle, and low socioeconomic statuses indicate socioeconomic status within the ≥75th, 25th–75th, and <25th percentiles, respectively.*
Figure 4 presents the cumulative incidence of primary EHM in both treatment groups before and after IPTW. Similar to the analysis of the study population balanced using PSM, the difference in EHM incidence between groups became more apparent after year 3. The superiority of TDF over ETV was also reproduced when analyzing the NHIS Health Check-Up Database subcohort (SHR=0.68, 95% CI=0.57–0.83, \( P<0.01 \)). In this subcohort, the cumulative incidence of EHM differed significantly between groups (Supplementary Fig. 4). In subgroup analyses of the NHIS Health Check-Up Database subcohort, most subgroups had a \( P_{interaction} >0.05 \), except for sex and presence of cirrhosis subgroups (Supplementary Fig. 5).

**Incidence of specific extrahepatic malignancies**

Supplementary Table 4 shows the incidence of specific EHM and IHM after PSM. After year 3, TDF was associated with a significantly lower risk of stomach cancer (SHR=0.57, 95% CI=0.38–0.86, \( P=0.01 \)), breast cancer (SHR=0.53, 95% CI=0.33–0.85, \( P=0.01 \)), and non-Hodgkin lymphoma (SHR=0.34, 95% CI=0.15–0.78, \( P=0.01 \)) than ETV. Within the first 3 years, TDF was associated with a higher incidence of breast cancer than ETV (SHR=1.74, 95% CI=1.05–2.89, \( P=0.03 \)).

**Incidence of overall intrahepatic malignancies**

Supplementary Figure 6 depicts the cumulative incidence of primary IHM in the entire population and the NHIS Health Check-Up Database subcohort after PSM. Similar to EHM, differences in IHM risk between groups became more prominent after approximately 3 years from the index date. However, unlike EHMs, the annual incidence rate of IHM decreased in the ETV group as well as the TDF group after year 3 (\( P<0.01 \) by Davies test; Supplementary Fig. 7). In the ETV group, the incidence of IHM decreased from 20.19/1,000 person-years to 16.20/1,000 person-years after year 3 (Supplementary Table 4). In the TDF group, the crude incidence of IHM decreased from 17.58/1,000 person-years in the first 3 years to 11.45/1,000 person-years after year 3. In terms of IHM incidence, the superiority of TDF over ETV was confirmed both within 3 years (SHR=0.88, 95% CI=0.81–0.95, \( P<0.01 \)) and after 3 years (SHR=0.68, 95% CI=0.62–0.75, \( P<0.01 \)), with the differ-
<table>
<thead>
<tr>
<th>cDDDs (per patient per year)</th>
<th>Events, no. (%)</th>
<th>Median follow-up, year (IQR)</th>
<th>Crude incidence of extrahepatic malignancy, per 1000 person-year</th>
<th>Within 3 years</th>
<th>After 3 years</th>
<th>SHR (95% CI)</th>
<th>P-value</th>
<th>aSHR (95% CI)</th>
<th>P-value</th>
<th>SHR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDF</td>
<td>293.3</td>
<td>591 (2.5%)</td>
<td>5.8 (5.1–6.4)</td>
<td>4.23 (3.77–4.74)</td>
<td>4.92 (4.40–5.51)</td>
<td>1.00 (0.84–1.18)</td>
<td>0.97</td>
<td>0.68 (0.59–0.79)</td>
<td>&lt;0.01</td>
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</tr>
<tr>
<td>ETV</td>
<td>288.4</td>
<td>822 (3.4%)</td>
<td>6.3 (3.7–7.2)</td>
<td>5.84 (5.28–6.46)</td>
<td>6.91 (6.30–7.58)</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDF</td>
<td>293.4</td>
<td>706 (2.9%)</td>
<td>5.7 (5.1–6.4)</td>
<td>5.88 (5.33–6.48)</td>
<td>4.92 (4.40–5.51)</td>
<td>1.01 (0.88–1.16)</td>
<td>0.90</td>
<td>0.69 (0.60–0.81)</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETV</td>
<td>288.3</td>
<td>794 (3.4%)</td>
<td>6.4 (3.7–7.2)</td>
<td>5.78 (5.21–6.41)</td>
<td>6.80 (6.19–7.47)</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
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</tr>
<tr>
<td>TDF</td>
<td>293.9</td>
<td>812 (2.8%)</td>
<td>5.7 (5.1–6.4)</td>
<td>5.62 (5.13–6.16)</td>
<td>4.92 (4.43–5.46)</td>
<td>0.98 (0.85–1.12)</td>
<td>0.76</td>
<td>0.70 (0.61–0.81)</td>
<td>&lt;0.01</td>
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<tr>
<td>ETV</td>
<td>296.5</td>
<td>525 (3.4%)</td>
<td>6.3 (3.8–7.2)</td>
<td>6.05 (5.34–6.85)</td>
<td>6.78 (6.03–7.62)</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDF</td>
<td>299.5</td>
<td>427 (2.8%)</td>
<td>5.8 (5.1–6.4)</td>
<td>5.58 (4.92–6.33)</td>
<td>4.73 (4.09–5.47)</td>
<td>0.93 (0.78–1.11)</td>
<td>0.41</td>
<td>0.68 (0.57–0.83)</td>
<td>&lt;0.01</td>
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<tr>
<td>ETV</td>
<td>289.8</td>
<td>822 (3.2%)</td>
<td>6.5 (3.4–7.4)</td>
<td>5.08 (4.57–5.65)</td>
<td>6.66 (6.09–7.28)</td>
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</tr>
<tr>
<td>TDF</td>
<td>295.0</td>
<td>829 (2.7%)</td>
<td>6.0 (5.3–6.6)</td>
<td>4.99 (4.54–5.49)</td>
<td>4.83 (4.38–5.33)</td>
<td>0.98 (0.85–1.13)</td>
<td>0.83</td>
<td>0.98 (0.85–1.13)</td>
<td>0.79 (0.62–0.82)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>ETV</td>
<td>288.4</td>
<td>822 (3.4%)</td>
<td>6.3 (3.7–7.2)</td>
<td>5.84 (5.28–6.46)</td>
<td>6.91 (6.30–7.58)</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
<td>[reference]</td>
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<td></td>
</tr>
<tr>
<td>TDF</td>
<td>294.0</td>
<td>829 (2.8%)</td>
<td>5.8 (5.1–6.4)</td>
<td>5.59 (5.10–6.12)</td>
<td>4.94 (4.46–5.48)</td>
<td>0.96 (0.84–1.11)</td>
<td>0.61</td>
<td>0.70 (0.61–0.81)</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different statistical approaches

Model 2A: Cause-specific analysis

| ETV | 288.4 | 822 (3.4%) | 6.3 (3.7–7.2) | 5.84 (5.28–6.46) | 6.91 (6.30–7.58) | [reference] | [reference] |
| TDF | 293.4 | 706 (2.9%) | 5.7 (5.1–6.4) | 5.88 (5.33–6.48) | 4.92 (4.40–5.51) | 1.01 (0.88–1.16) | 0.90 | 0.69 (0.60–0.81) | <0.01 |

Model 2B: After IPTW

| ETV | 288.3 | 794 (3.4%) | 6.4 (3.7–7.2) | 5.78 (5.21–6.41) | 6.80 (6.19–7.47) | [reference] | [reference] |
| TDF | 293.9 | 812 (2.8%) | 5.7 (5.1–6.4) | 5.62 (5.13–6.16) | 4.92 (4.43–5.46) | 0.98 (0.85–1.12) | 0.76 | 0.70 (0.61–0.81) | <0.01 |

Model 3: NHIS Health Check-Up Database

| ETV | 296.5 | 525 (3.4%) | 6.3 (3.8–7.2) | 6.05 (5.34–6.85) | 6.78 (6.03–7.62) | [reference] | [reference] |
| TDF | 299.5 | 427 (2.8%) | 5.8 (5.1–6.4) | 5.58 (4.92–6.33) | 4.73 (4.09–5.47) | 0.93 (0.78–1.11) | 0.41 | 0.68 (0.57–0.83) | <0.01 |

Model 4: Without window period (including events within initial 3 months)

| ETV | 289.8 | 822 (3.2%) | 6.5 (3.4–7.4) | 5.08 (4.57–5.65) | 6.66 (6.09–7.28) | [reference] | [reference] | [reference] | [reference] |
| TDF | 295.0 | 829 (2.7%) | 6.0 (5.3–6.6) | 4.99 (4.54–5.49) | 4.83 (4.38–5.33) | 0.98 (0.85–1.13) | 0.83 | 0.98 (0.85–1.13) | 0.79 (0.62–0.82) | <0.01 |

Model 5: Crude population

| ETV | 288.4 | 822 (3.4%) | 6.3 (3.7–7.2) | 5.84 (5.28–6.46) | 6.91 (6.30–7.58) | [reference] | [reference] | [reference] | [reference] |
| TDF | 294.0 | 829 (2.8%) | 5.8 (5.1–6.4) | 5.59 (5.10–6.12) | 4.94 (4.46–5.48) | 0.96 (0.84–1.11) | 0.61 | 0.70 (0.61–0.81) | <0.01 |
DISCUSSION

In this nationwide cohort study of CHB patients treated with TDF had a 30% lower risk of EHM than those treated with ETV after 3 years of antiviral therapy. During the first 3 years, the incidence of EHM did not differ between ETV and TDF groups. After year 3, however, EHM risk differed significantly between groups as the incidence of EHM accelerated in the ETV group but decelerated in the TDF group. These findings collectively suggest the superiority of TDF over ETV in terms of both EHM and IHM prevention.

Superior virologic response of TDF compared to ETV might be responsible for the outcomes of this study. Although head-to-head clinical trials are limited, prior studies showed that TDF suppressed viral RNA, DNA, more potently than ETV, and this suppression was associated with a reduced risk of HCC. Recent studies have reported associations between hepatitis B virus (HBV) infection and the development of EHM, and chronic inflammation in HBV-infected extrahepatic tissues. Moreover, interferon lambda 3 induced by nucleotide analogues (e.g., TDF and tenofovir disoproxil fumarate), but not by nucleoside analogues (e.g., ETV and lamivudine), may also contribute to the antitumor effects of TDF. Interferon lambda exhibited potent antitumor effects in animal malignancy models, and this could provide another explanation for the superiority of TDF over ETV.

In this study, we used several statistical strategies to overcome the limitations of a retrospective design. The E-value represents the minimum strength of association that an unmeasured confounder must have with both the treatment and outcome to completely explain away a given association. According to the E-values for the IHM incidence, it seems less likely that there is an unmeasured confounder that could alter the superiority of TDF (Supplementary Table 3).

Table 3. Continued

<table>
<thead>
<tr>
<th>Model 6: Treatment initiation between 2013–2014*</th>
<th>Events, no. (%)</th>
<th>Median follow-up, year (IQR)</th>
<th>Crude incidence of extrahepatic malignancy, per 1000 person-year</th>
<th>SHR (95% CI)</th>
<th>P-value</th>
<th>aSHR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETV</td>
<td>288.2</td>
<td>250</td>
<td>5.5</td>
<td>4.4–6.2</td>
<td>7.77</td>
<td>(6.54–9.23)</td>
<td>0.001</td>
</tr>
<tr>
<td>TDF</td>
<td>293.9</td>
<td>232</td>
<td>5.7</td>
<td>5.0–6.3</td>
<td>6.07</td>
<td>(5.12–7.20)</td>
<td>0.001</td>
</tr>
<tr>
<td>SHR (95% CI)</td>
<td>1.05</td>
<td>(0.82–1.34)</td>
<td>0.69</td>
<td>(0.53–0.90)</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aSHR (95% CI)</td>
<td>1.05</td>
<td>(0.82–1.34)</td>
<td>0.69</td>
<td>(0.53–0.90)</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aSHR, adjusted subdistribution hazard ratio; cDDDs, cumulative defined daily doses; CI, confidence interval; ETV, entecavir; IPTW, inverse probability of treatment weighting; IQR, interquartile range; SHR, subdistribution hazard ratio; TDF, tenofovir disoproxil fumarate.

*Propensity score-matched cohort. †Additionally adjusted for the frequency of hospital visits. ‡Further adjusted for the frequency of surveillance test (alpha-fetoprotein, abdominal ultrasonography, or contrast-enhanced computed tomography). §Hazard ratios (instead of subdistribution hazard ratios) are provided for model 2A. ‖Adjusted for the level of healthcare.
treatment–outcome association. Based on the calculated E-value for the EHM incidence, the observed SHR of 0.70 could be explained away by an unmeasured confounder that was associated with both the treatment and the outcome by a risk ratio of 2.21-fold each, above and beyond the measured confounders, which seems unfeasible. Most of the general risk factors for EHM, including age, sex, and comorbidity, were well balanced in both the entire cohort and the NHIS Health Check-Up subcohort, even before matching. In addition, the distribution of several disease-specific risk factors was similar between the two groups. For instance, Helicobacter pylori infection is a main cause of stomach cancer, which is still prevalent in Korea, and its risk factors, including elevated cholesterol, male gender, old age, and low socioeconomic status, were comparable. The risk factors for non-Hodgkin lymphoma (e.g., obesity, low physical activity, smoking, and alcohol intake) were well balanced between the two groups, and the superiority of TDF was maintained after adjusting for surveillance intensity, a crucial factor in the diagnosis of thyroid cancer. Considering the E-value for the IHM incidence, the superiority of TDF with respect to IHM risk is also unlikely to be overturned by unmeasured confounders, as baseline characteristics are evenly distributed between the two groups, including the proportion of liver cirrhosis, one of the most important risk factors for IHM. To minimize the effects of residual confounders, we also applied other statistical strategies, such as PSM, IPTW, multivariable adjustment, and various prespecified sensitivity and subgroup analyses, which produced similar results.

The lower incidence of both EHM and IHM in the TDF group compared to the ETV group became more prominent after year 3. This suggests that antitumor effects of antivirals may require a certain period of time to manifest as differences in EHM or IHM incidence. However, IHM incidence was also significantly different within the first 3 years as well, although EHM incidence was comparable between groups during this time. Since HBV is a hepatotropic virus, the viral load is much higher in the liver than in extrahepatic tissues; thus, differences in antitumor effects resulting from different efficacy of viral suppression may be more apparent when comparing the incidence of IHM, rather than EHM. The further decrease in incidence of IHM after year 3 in both groups may be attributed to regression of hepatic fibrosis induced by antiviral therapy. A previous study reported that the increase in cumulative HCC incidence decelerated after 5 years of ETV or TDF treatment, compared to the first 5 years. Furthermore, another Korean study using the NHIS database observed a similar trend of HCC incidence among ETV- or TDF-treated patients, supporting the validity of our current results.

It is notable that the absolute incidence of EHM increased in the ETV group but decreased in the TDF group after year 3. Although one may assume that this trend indicates the protumor effects of ETV, it should be interpreted with caution. Preclinical animal studies raised concerns about the potential carcinogenicity of ETV. Although two real-world retrospective studies showed that ETV at usual clinical doses does not increase cancer risk, the number of patients analyzed may have been insufficient to avoid a false-negative result, considering the low incidence of EHM. In addition, the protumor effects of ETV were possibly masked by the overall antitumor effects for both IHM and EHM. ETV can incorporate into the human genome, which may lead to carcinogenicity during subsequent replication cycles. It is possible to assume that the protumor effects of ETV became apparent after cumulative damage caused by ETV exceeded a certain threshold level (approximately 430 mg of ETV in this study). However, the doses of ETV at which its carcinogenic effects were confirmed in the animal experiments, were far higher compared to the approved dose for humans. In addition, since CHB itself increases the risk of both IHM and EHM, the true protumor effects of ETV can only be proven by comparing ETV versus no ETV over a long period time in healthy non-CHB subjects, which is unfeasible.

ETV appears to have potent antitumor effects, considering the decreasing incidence of IHM confirmed in previous studies. Therefore, in conjunction with prior evidence suggesting that both ETV and TDF treatments are beneficial in CHB patients for reducing EHM, as well as IHM, our findings can be interpreted as follows: (i) the antitumor effects of TDF are greater than those of ETV; and (ii) even if ETV has protumor effects in humans, which cannot be proven with certainty in the current setting, these effects are unlikely to be strong enough to overpower its antitumor effects associated with suppressing HBV replication. However, it may be advised that clinicians should be more suspicious of the potential protumor effects of ETV.

This study had several limitations. First, NHIS database...
does not provide detailed individual laboratory data including serum HBV DNA levels. Instead, additional data on anthropometric measurements, health-related behaviors, and blood test results were collected in more than half of the entire cohort who received medical check-up provided by NHIS and same results were maintained. Second, statistical significance was not achieved for the incidence of most individual EHM, except stomach cancer, breast cancer, and non-Hodgkin lymphoma. Despite the use of a large nationwide cohort, the low incidence of each cancer made it difficult to achieve statistical significance. However, a similar trend of higher risk with ETV was seen for most of the EHM. Regarding the differences in breast cancer before versus after year 3, a high level of estradiol may have contributed to these results; this requires additional research (see Supplementary Discussion). Third, while an association between the use of ETV or TDF and the incidence of EHM or IHM was assessed, not all aspects of the antivirals, including adverse events, were evaluated. TDF is known to have a higher risk of renal and bone toxicity compared to ETV. Although long-term use of TDF may reduce the risk of EHM, the superiority of TDF cannot be generalized to all patients, as the risks of TDF use may outweigh the benefits in patients with impaired renal function. Fourth, drug modifications or discontinuations during follow-up were not considered. We excluded patients who switched regimens within 90 days of the index date. However, cases in which ETV or TDF was subsequently discontinued or switched were not excluded. Although the number of cases that switched regimens is relatively small, and changes occur in both directions, it may have affected the results of this study as another confounder. Fifth, the results of this study may have limited generalizability, as most CHB patients in South Korea are infected with genotype C HBV. Although comparable overall virologic responses to NAs have been observed among patients with diverse HBV genotypes, further international investigations are required. Lastly, this is a retrospective study, which by its nature cannot show causality, only association. In addition, despite the use of multiple statistical strategies, it is still possible that the results were affected by unmeasured confounders as each cancer has different risk factors. Considering the low incidence of EHM in patients with CHB, a large randomized controlled trial or prospective study with long-term follow-up is not feasible, and the results of this study need to be validated at least in an independent retrospective cohort.

In conclusion, TDF was associated with approximately 30% reduced risks of both EHM and IHM than ETV after 3 years of treatment. Although the results of this study need to be validated in an independent cohort, the antitumor effects of TDF appeared to be greater than those of ETV.

Authors’ contribution

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Conflicts of Interest
Moon Haeng Hur: Nothing to declare; Dong Hyeon Lee: Nothing to declare; Jeong-Hoon Lee: Receives research grants from Yuhan Pharmaceuticals and GreenCross Cell, lecture fees from GreenCross Cell, Daewoong Pharmaceuticals, and Gilead Korea; Mi-Sook Kim: Nothing to declare; Jeayeon Park: Nothing to declare; Hyunjae Shin: Nothing to declare; Sung Won Chung: Nothing to declare; Heejoon Jang: Nothing to declare; Yun Bin
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**SUPPLEMENTARY MATERIAL**

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Macrophage ATG16L1 expression suppresses metabolic dysfunction-associated steatohepatitis progression by promoting lipophagy

Qi Wang¹²*, Qingfa Bu²³*, Zibo Xu²*, Yuan Liang², Jinren Zhou², Yufeng Pan², Haoming Zhou², and Ling Lu²³⁴

¹Department of General Surgery, First Affiliated Hospital of Anhui Medical University, Hefei, China; ²Hepatobiliary Center, The First Affiliated Hospital of Nanjing Medical University, Research Unit of Liver Transplantation and Transplant Immunology, Chinese Academy of Medical Sciences, Nanjing; ³Department of General Surgery, Nanjing BenQ Medical Center, The Affiliated BenQ Hospital of Nanjing Medical University, Nanjing; ⁴Affiliated Hospital of Xuzhou Medical University, Xuzhou, China

Graphical Abstract

Study Highlights

- ATG16L1 expression is downregulated in liver of patients with metabolic dysfunction-associated steatohepatitis.
- Macrophage-specific Atg16l1 knockout exacerbates and transgenic overexpression of Atg16l1 attenuates steatohepatitis.
- Macrophage ATG16L1 suppresses steatohepatitis progression by promoting lipophagy.
- Macrophages ATG16L1 inhibit hepatocytes steatosis and HSCs activation.
- ATG16L1 may be a promising therapeutic target for MASH management.
Background/Aims: Metabolic dysfunction-associated steatohepatitis (MASH) is an unmet clinical challenge due to the rapid increased occurrence but lacking approved drugs. Autophagy-related protein 16-like 1 (ATG16L1) plays an important role in the process of autophagy, which is indispensable for proper biogenesis of the autophagosome, but its role in modulating macrophage-related inflammation and metabolism during MASH has not been documented. Here, we aimed to elucidate the role of ATG16L1 in the progression of MASH.

Methods: Expression analysis was performed with liver samples from human and mice. MASH models were induced in myeloid-specific Atg16l1-deficient and myeloid-specific Atg16l1-overexpressed mice by high-fat and high-cholesterol diet or methionine- and choline-deficient diet to explore the function and mechanism of macrophage ATG16L1 in MASH.

Results: Macrophage-specific Atg16l1 knockout exacerbated MASH and inhibited energy expenditure, whereas macrophage-specific Atg16l1 transgenic overexpression attenuated MASH and promotes energy expenditure. Mechanistically, Atg16l1 knockout inhibited macrophage lipophagy, thereby suppressing macrophage β-oxidation and decreasing the production of 4-hydroxynonenal, which further inhibited stimulator of interferon genes (STING) carboxylation. STING palmitoylation was enhanced, STING trafficking from the endoplasmic reticulum to the Golgi was promoted, and downstream STING signaling was activated, promoting proinflammatory and profibrotic cytokines secretion, resulting in hepatic steatosis and hepatic stellate cells activation. Moreover, Atg16l1-deficiency enhanced macrophage phagosome ability but inhibited lysosome formation, engulfing mtDNA released by pyroptotic hepatocytes. Increased mtDNA promoted cGAS/STING signaling activation. Moreover, pharmacological promotion of ATG16L1 substantially blocked MASH progression.

Conclusions: ATG16L1 suppresses MASH progression by maintaining macrophage lipophagy, restraining liver inflammation, and may be a promising therapeutic target for MASH management. (Clin Mol Hepatol 2024;30:515-538)

Keywords: ATG16L1; Macrophages; Lipophagy; Metabolic dysfunction-associated steatohepatitis

Abbreviations: MASH, metabolic dysfunction-associated steatohepatitis; ATG16L1, autophagy-related protein 16-like 1; 4-HNE, 4-hydroxynonenal; HSCs, hepatic stellate cells; HFHCD, high-fat and high-cholesterol diet; MCD, methionine- and choline-deficient diet; Atg16l1ΔMf, myeloid-specific Atg16l1 knockout; Atg16l1ΔMfTmem173ΔMf, myeloid-specific Atg16l1 and Tmem173 double knockout; Atg16l1ΔMfTmem173ΔMf, myeloid-specific Atg16l1-overexpressing knockin; BMDMs, bone marrow-derived macrophages; BAT, brown adipose tissue; eWAT, epididymal white adipose tissue; ingWAT, inguinal white adipose tissue; EE, energy expenditure; CM, conditioned media; TG, hepatic triglyceride; ERGIC, endoplasmic reticulum–Golgi intermediate complex; TBK1, TANK-binding kinase 1; IFN3, interferon regulatory factor 3; ER, endoplasmic reticulum; GSDMD, Gasdermin D; mtDNA, mitochondrial DNA; STING, stimulator of interferon genes
INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) is emerging as the leading chronic liver disease worldwide.\(^1,2\) The more advanced subtype, metabolic dysfunction-associated steatohepatitis (MASH), characterized by hepatic steatosis, lobular inflammation, and ballooning with or without perisinusoidal fibrosis,\(^3\) conveys progressive liver injury that can lead to cirrhosis and hepatocellular carcinoma.\(^4\) The majority of MASLD cases are heterogeneous, asymptomatic, and not easily identifiable, even when the disease has progressed to cirrhosis.\(^5\) Currently, treatment options for MASH are extremely limited; the only proven treatments are weight loss and increased physical activity, which are difficult to sustain; there is still a lack of approved pharmacotherapies for this medical condition.\(^5,6\)

Thus, more studies are needed to identify key molecular regulators of MASH progression and to lay the foundation for developing effective therapeutic treatments.

Autophagy is a conserved catabolic pathway in eukaryotic cells that is essential for survival, development, and homeostasis, in which the lysosome is the end point for components to be phagocytosed.\(^7-9\) Autophagy in hepatic macrophages protects the liver against fibrosis,\(^10,11\) especially during experimental alcoholic injury or MASH.\(^12\) One of the main functions of autophagy is to regulate cellular metabolism and energy through lipophagy, mitophagy, refueling of the amino acid pool, or degradation of proteins involved in glucose metabolism.\(^13,14\) ATG16L1 plays an important role in the process of autophagy and mediates the conjugation of phosphatidylethanolamine to the ubiquitin-like molecule LC3—a necessary step for proper biogenesis of the autophagosome and for subsequent events in which substrates are degraded by the lysosome.\(^15\) A study indicated that disrupted ATG16L1 activity in hepatocytes impaired hepatocyte lipophagy during hepatic steatosis.\(^16\) Nevertheless, the specific role of ATG16L1 in macrophages in regulating hepatic inflammation, fibrosis, and lipid metabolism disturbance during MASH remains unclear.

Therefore, our study aimed to elucidate the role of macrophage ATG16L1 in regulating hepatic inflammation, fibrosis, and lipid metabolism disturbance during MASH. By investigating the expression and function of ATG16L1 in macrophages, we sought to uncover novel insights into the pathogenesis of MASH and to explore potential genetic-based strategies for its treatment. The elucidation of the precise mechanisms by which ATG16L1 influences MASH progression is of paramount importance, as it may influence the development of targeted therapies that address the underlying molecular dysregulation. This, in turn, holds the potential to significantly improve outcomes for MASH patients, inhibiting the progression of MASH to advanced stages such as cirrhosis and hepatocellular carcinoma. Furthermore, our findings may contribute to a broader understanding of autophagy-mediated processes in liver health and disease, providing a basis for future research endeavors aiming at unraveling the intricate interplay between cellular pathways and disease pathogenesis.

MATERIALS AND METHODS

Human liver samples

Human liver samples were collected from 39 patients with MASH (39 frozen tissues for mRNA expression detection and 6 paraffin-embedded slides for protein expression detection), and 31 patients without hepatic steatosis (patients who presented with hepatic hemangioma at the First Affiliated Hospital of Nanjing Medical University) were used as controls (Supplementary Table 1). Individuals in the control group had no history of diabetes, alcohol use, viral hepatitis, or other liver diseases. All liver specimens were assessed independently by two experienced pathologists blinded to the NAFLD activity score (NAS) clinical data, which is defined as the sum of steatosis, inflammation, and hepatocyte ballooning scores. Patients with an NAS ≥5 were considered likely to have MASH. The clinical characteristics of the patients with MASH are listed in Supplementary Table 1. The study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (approval number: 2020-SRFA-288). Informed consent for tissue analysis was obtained before liver biopsy or surgery. All experiments were performed in accordance with government policies and the Declaration of Helsinki and Istanbul.

Animals and treatments

Wild-type (WT), FloxP-Atg16l1 (Atg16l1\(^{fl/fl}\)), Lyz2-Cre At-
Bone marrow cells were isolated from the femurs and tibias of male Atg16l1 ΔM, Lyz2-Cre Atg16l1 and Lyz2-Cre Tmem173-double knockout (Atg16l1 ΔM Tmem173 ΔM), and myeloid-specific Atg16l1-overexpressing-knockin (Atg16l1 ΔE) male mice, 6–8 weeks old, on a C57BL/6 background were used in the experiments (Supplementary Table 2). Mice were randomly assigned to receive a normal chow diet (NCD), a high-fat and high-cholesterol diet (HF-HCD) (Research Diets, Inc., New Brunswick, CA, USA) for 16 weeks (n=6–8 per group), or a methionine- and choline-deficient (MCD) diet (Research Diets, Inc.) for 6 weeks (n=6–8 per group). Male wild-type C57BL/6 mice (6–8 weeks old) were administered the ATG16L1 enhancer pertinoin (0.03% for 16 consecutive weeks or 6 consecutive weeks) in the diet,17 or vehicle (saline) for 16 weeks of HF-HCD feeding or for 6 weeks of MCD feeding (n=6–8 per group). All the mice were housed under specific pathogen-free, ventilated, thermostatic conditions with a 12-hours light/dark cycle at 24°C. Food intake was measured over the indicated period. The mice were fasted at the end of the experiments. Finally, serum and tissues were harvested as described in our previous study.18 The livers and adipose tissues were rapidly excised and weighed. All animal studies were performed according to the guidelines of the Institutional Animal Use and the Animal Experimentation Ethics Committee of The First Affiliated Hospital of Nanjing Medical University.

Cell culture and treatment

Primary hepatocytes were isolated from male Atg16l1 ΔM or WT mice as previously described.19 The liver macrophage pellet was resuspended and cultured. Cells were treated with a mixture of palmitic acid (PA; 0.5 mM; Sigma-Aldrich) and oleic acid (OA; 1.0 mM; Sigma-Aldrich) coupled with bovine serum albumin (BSA; Sigma-Aldrich) for the indicated periods. BSA was used as a vehicle control. Bone marrow cells were isolated from the femurs and tibias of male Atg16l1 ΔM, Atg16l1 ΔE, and Atg16l1 ΔM mice. The bone marrow was flushed with DMEM loaded into a 1 mL syringe. Bone marrow cells were cultured at a concentration of 3x10⁵/mL in DMEM supplemented with 10% FBS and 20% L929 conditioned medium to differentiate them into bone marrow-derived macrophages (BMDMs). On day 7, all adherent cells differentiated into mature macrophages.

For coculture studies, primary hepatocytes from male Atg16l1 ΔM mice and LPS-primed (10 ng/mL of LPS for 4 hours) macrophages20 from the bone marrow of male Atg16l1 ΔM, Atg16l1 ΔE, or Atg16l1 ΔM mice were seeded in a coculture chamber (Corning Inc., Corning, NY, USA). The coculture system was supplemented with palmitic acid and oleic acid media. Mouse TNF-α-neutralizing mAb (10 ng/mL) (CST, Danvers, MA, USA), MR16-1 (12.5 ng/mL) (Chugai Pharmaceutical, Shizuoka, Japan), IL-1RA (12.5 ng/mL) (MedChemExpress, Princeton, NJ, USA), TNF-α (10 ng/mL), IL-6 (12.5 ng/mL), or IL-1β (12.5 ng/mL) (Novus Biologicals, Littleton, CO, USA) was added to the coculture system for 24 hours, and the hepatocytes were subsequently analyzed for fat accumulation and inflammatory response indices. LPS-primed (10 ng/mL LPS for 4 hours) BMDMs were incubated with hepatocyte-CM for 48 hours and subjected to RNA sequencing analysis. Primary mouse HSCs were isolated as previously described.21 Briefly, primary hepatic stellate cells (HSCs) were isolated from the livers of mice. After portal vein intubation, the livers were perfused in situ with Ca²⁺-free Hank’s balanced saline solution (HBSS) at 37°C for 15 minutes and subsequently perfused with the solution containing 0.05% collagenase and Ca²⁺ for 15 minutes at a flow rate of 10 mL/min. The perfused livers were minced, filtered through a 70 M cell strainer (BD Bioscience), and centrifuged at 50 × g for 3 minutes. The supernatant was further centrifuged at 500 × g for 10 minutes, resuspended in Ficoll plus Percoll (1:10, GE Healthcare), and centrifuged at 1,400 × g for 17 minutes. The HSCs were collected from the interface. Primary mouse HSCs were incubated with BMDM-CM in the absence or presence of TGF-β1 (8 ng/mL for 24 hours) and analyzed for HSC activation status.

Histological examination

H&E-stained liver sections were assessed by two experienced liver pathologists who were blinded to experimental grouping, and each liver specimen was evaluated for the presence or absence of MASH according to histopathology and for the NAFLD activity score (NAS), defined as the sum of steatosis, inflammation, and hepatocyte ballooning scores. Patients with an NAS of ³5 were considered likely...
to have MASH.\textsuperscript{3,22} For oil red O staining, primary hepatocytes and frozen liver sections (10 μm) were rinsed with 60% isopropanol and stained with oil red O solution (Sigma–Aldrich) for 15 minutes. Afterward, the sections were rinsed again with 60% isopropanol, and the nuclei were stained with hematoxylin before microscopic analysis.

Biochemical assays

The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using an automated chemical analyzer (Olympus, Tokyo, Japan). Hepatic triglyceride (TG) levels were determined with a Wako E-test triglyceride kit (Wako Pure Chemical Industries, Osaka, Japan). Cellular TG synthesis was determined using standard methods.\textsuperscript{23} Briefly, cellular lipids were extracted with hexane/isopropanol (3:2). Lipids were then dried with nitrogen gas, redissolved in chloroform, and resolved by thin-layer chromatography using successive solvent systems containing chloroform, acetone, methanol, acetic acid, and water at a volumetric ratio of 10:4:2:2:1, as well as hexane, methanol, and acetic acid at a volumetric ratio of 80:20:1. Phosphor images were obtained with a Storm Gel and Blot Imaging System (GE Healthcare).

Fatty acid β-oxidation assay

The rates of fatty acid β-oxidation were determined using a modification of a previously used method\textsuperscript{24} that measured the rate of carbon dioxide production. Carbon dioxide trapped for 1 hour at 37°C from stimulated cells was released onto filter paper soaked in 100 mM sodium hydroxide. The rate of β-oxidation was calculated as the amount of trapped carbon dioxide in relative units produced per mg protein per hour.

Immunohistochemical and immunofluorescence staining

Murine liver and adipose tissue immunohistochemistry was performed to detect F4/80 expression in paraffin-embedded liver biopsies using an anti-F4/80 antibody (#ab70076, CST). Immunohistochemistry of the murine liver was performed to detect GSDMD expression in paraffin-embedded human liver biopsies using an anti-GSDMD antibody (#AF4012, Affinity). In brief, liver sections were preblocked for 10 minutes after deparaffinization. The slides were then heated in an autoclave with sodium citrate for antigen retrieval, covered in 1% hydrogen peroxide to eliminate endogenous peroxidase activity, and blocked with 2% goat serum. The slides were then incubated with primary antibodies at 4°C overnight. Biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA) was used as the secondary antibody, followed by incubation with immunoperoxidase (ABC Kit; Vector) according to the manufacturer's protocol. ATG16L1 and CD68 expression in human liver tissues were determined by immunofluorescence with an anti-rabbit ATG16L1 mAb (#8089, CST) and an anti-mouse CD68 mAb (ab955, Abcam), followed by incubation with secondary goat anti-rabbit IgG (ab150088, Abcam) or goat anti-mouse IgG (ab150117, Abcam). The cells were fixed using 3% paraformaldehyde and subsequently blocked and incubated with primary and corresponding Cy5- and Texas Red-conjugated secondary antibodies. Lipid droplets (LDs) were stained by incubating cells with BODIPY 493/503 (D2191, Invitrogen) for 30 minutes. The cells were then fixed and processed for immunofluorescence as previously described.\textsuperscript{25} STING, α-SMA, LAMP1, LC3B, and GM130 expression in cells was determined by immunofluorescence using an anti-rabbit STING pAb (PA5-116052, Invitrogen), an anti-rabbit α-SMA mAb (#19245, CST), an anti-rabbit LAMP1 mAb (#99437, CST), an anti-rabbit LC3B mAb (#43566, CST), and an anti-mouse GM130 mAb (sc-55591, Santa Cruz Biotechnology), followed by incubation with secondary goat anti-rabbit IgG (ab150088, Abcam) or anti-mouse IgG (ab150117, Abcam). DAPI was used to stain the nuclei. The slides were washed twice with PBS and observed via confocal microscopy (Zeiss, Oberkochen, Germany) according to the manufacturer's protocol.

Cell transfection

A c-Jun adenoviral vector was constructed by GenePharma (Shanghai, China) to overexpress c-Jun in primary macrophages isolated from bone marrow. Targeted cells (2×10\textsuperscript{5}) were infected with 1×10\textsuperscript{6} adenovirus-transducing units in the presence of 1 mg/mL polybrene. Total RNA or proteins were prepared 48 hours after transfection. Negative control adenoviral plasmid vectors were generated and

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designated “Ad-Con.” Transfection of cells was performed using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions, and the transfection efficiency was validated by qPCR.

Reagents

The STING inhibitor C-176, the STING activator DMXAA, the JNK activator anisomycin, the JNK inhibitor SP600125, the autophagy inhibitor 3-methyladenine (3-MA), the lipid peroxidation end product 4-hydroxynonenal (4-HNE), the β-oxidation inhibitor etomoxir, the ATG16L1 enhancer peretinoin were purchased from MedChemExpress, and the lysosomal acid lipase (LIPA) inhibitor lalistat was purchased from Enamine. All mice were euthanized for further analysis after being fed a HFHCD for 16 weeks or an MCD for 6 weeks.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total mRNA was isolated from liver samples or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using a high-capacity cDNA reverse transcription kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s protocols. The mRNA expression levels were quantified by quantitative PCR using SYBR Green (Roche). The expression level of each cDNA relative to that of the β-actin endogenous control was determined using the 2−ΔΔCt method. Quantitative real-time PCR was repeated three times for each sample. The primers used in our study are listed in Supplementary Table 3.

mtDNA isolation and quantification

DNA was extracted from 200 μL of cell culture supernatant from untreated and treated primary murine hepatocytes using a QiAmp DNA Mini Kit (QIAGEN, Duesseldorf, Germany) according to the manufacturer’s instructions. Real-time polymerase chain reaction (qPCR) was performed for the quantification of mtDNA. mtDNA was quantified using mouse mt-ATP6 primers/TaqMan 5’ FAM-3’ MGB probes (Bio-Rad, Hercules, CA, USA). The mitochondrial lysates of primary hepatocytes were used to isolate mtDNA using a mitochondrial DNA isolation kit (Abcam) according to the manufacturer’s instructions. Total DNA was isolated using a DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer’s instructions. RT-qPCR analysis was performed using a sequence detection system (ABI Prism 7000; Applied Biosystems, Foster City, CA, USA) with a SYBR Green 1-step kit (Invitrogen). To evaluate the possibility of BMDMs engulfing mtDNA released from hepatocytes, mtDNA isolated from primary murine hepatocytes was tagged with Cy5-dCTP (Amersham Cy5-dCTP, GE Healthcare) using PCR following the manufacturer’s protocol (BioPrime DNA Labeling system, Life Technologies). The Cy5-labeled DNA PCR product was cleaned using a DNeasy Mini Spin Column (QIAGEN) before the DNA concentration was measured using a Nanodrop (Thermo Scientific).

Protein extraction and Western blotting

Whole-cell lysates were obtained as previously described.19 Proteins in tissues or cells were extracted with ice-cold lysis buffer (0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 0.1% SDS, 137 mM sodium chloride, 20 mM Tris, and pH 7.4). Proteins (20 g) were separated by 12% SDS–PAGE and transferred to PVDF nitrocellulose membranes. Western blot analysis was performed using the following antibodies: anti-ATG16L1 (#8089, CST), anti-β-actin (#4970, CST), anti-α-SMA (#19245, CST), anti-collagen I (#72026, CST), anti-TIMP1 (#8946, CST), anti-p-β-actin (Ser536) (ab76302, Abcam), anti-p65 (66535-1-Ig, Proteintech), anti-p-IkBα (#2859, CST), anti-IkBα (10268-1-AP, Proteintech), anti-TNF-α (60291-1-Ig, Proteintech), anti-IL-6 (66146-1-Ig, Proteintech), anti-cleaved caspase-1 (#89332, CST), anti-pro-caspase-1 (#24232, CST), anti-p-JNK (#9255, CST), anti-JNK (#9252, CST), anti-c-Jun (#9165, CST), anti-c-Fos (#74620, CST), anti-LC3B (#43566, CST), anti-p62 (#5114, CST), anti-GSDMD (#AF4012, Affinity), anti-rabbit IgG (#7074, CST), and anti-mouse IgG (#7076, CST). Image Lab software (National Institutes of Health) was used to quantify the protein expression, and β-actin was used as the con-
In vitro carbonylation

STING protein was expressed using a TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer’s instructions. \(^{26}\) STING protein was incubated with 100 μM FeSO\(_4\)/25 mM ascorbate and 25 mM H\(_2\)O\(_2\) in 100 mM KCl, 100 mM MgCl\(_2\), and 50 mM HEPES (pH 7.2), and the reaction proceeded for 5 hours. \(^{27}\) To remove excess low-molecular-weight compounds, proteins were concentrated with an Amicon Ultra 10 kDa filter (Millipore). STING carbonylation was evaluated using OxyBlot technology.

Validation of protein palmitoylation by click chemistry

BMDMs were incubated with 100 μM palmitic acid probes (Invitrogen C10265) for 4 hours at 37°C. The cells were washed twice in PBS and then lysed on ice in 100 μL of 50 mM Tris, pH 8.0, containing 0.4% SDS and protease inhibitors, and the lysate was incubated with 1 mM CuSO\(_4\), 100 μM TBTA ligands, 100 μM biotin-alkyne, and 1 mM tris(2-carboxyethyl) phosphate for 1 hour at 25°C. After precipitation, protein complexes were enriched by adding streptavidin beads for 3 hours. Samples were washed three times with PBS, and SDS loading buffer was used to elute proteins at 95°C for 20 minutes, after which SDS–PAGE was used to separate the proteins.

DNA pull-down assay

An in vitro pull-down assay was performed to detect the binding of c-Jun and c-Fos to \(Tgfb1\). BMDMs were lysed in NP-40 lysis buffer. Lysates were incubated with biotin-c-Jun or c-Fos (BioLog Life Science Institute) at 4°C for 4 hours and then incubated with streptavidin beads at 4°C for 4 hours. The beads were washed four times with lysis buffer and analyzed for \(Tgfb1\) binding by immunoblotting.

Chromatin immunoprecipitation (ChIP) assays

The ChIP assay was performed using a Simplechip Enzymatic Chromatin IP Kit (Cell Signaling Technology, Billerica, MA, USA) according to the manufacturer’s instructions. Briefly, the cells were cross-linked with 37% formaldehyde, and the nuclei were subsequently extracted. After fragmentation by sonication and enzymatic digestion, the DNA–protein complex was subjected to immunoprecipitation with 8 μg of anti-c-Jun antibody (Cell Signaling Technology) or rabbit IgG as a control. Purified ChIP DNA was amplified by real-time quantitative PCR. The primers for the AP-1 region of the \(Tgfb1\) promoter were as follows: forward, 5’-GAAGGGAGAGATGGCTCCACTGGG-3’; reverse, 5’-CTCCTCCTCAGGACTTTT-3’.

Luciferase assays

A \(Tgfb1\) promoter luciferase reporter plasmid (\(Tgfb1\) luciferase) was constructed using a pGL3 luciferase vector (Promega, WI) according to the manufacturer’s instructions. BMDMs were transfected with the pGL3-\(Tgfb1\)-luciferase vector. After transfection for 6 h, the cells were washed and transfected with Ad-c-Jun or Ad-Con. After 48 h, the cells were lysed with passive lysis buffer, and transcriptional activity was measured using a luciferase assay system (Promega) according to the manufacturer’s instructions.

Indirect calorimetry

\(VO_2\), \(VCO_2\), the respiratory exchange ratio (RER), and locomotor activity were assessed using an eight-chamber Oxymax system (Columbus Instruments). \(^3\) Mice were placed in the chambers at 23°C with free access to food and water and acclimated for more than 50 minutes before measurement. Energy expenditure (EE) was calculated as (3.815+1.232×RER)×\(VO_2\)/lean mass.

Electron microscopy

Primary hepatocytes were immersed in fixative (2.5% glutaraldehyde) and stored overnight at 4°C. The samples were rinsed in the same buffer and postfixed for 1 h in 1% osmium tetroxide and 1% potassium ferrocyanide in 0.1 M cacodylate buffer to enhance membrane staining. The cells were then rinsed in distilled water, dehydrated in acetone at a low temperature to preserve lipids, and embedded in epoxy resin. Contrasted ultrathin sections (70 nm) were ana-
lyzed under a transmission electron microscope (Hitachi, Tokyo, Japan).

**RNA sequencing and analysis**

LPS-primed Atg16l1fl/fl or Atg16l1ΔMϕ BMDMs were incubated with Atg16l1fl/fl primary hepatocyte-conditioned media (CM) for 48 h, and Atg16l1fl/fl or Atg16l1ΔMϕ BMDMs were subsequently subjected to RNA sequencing analysis (n=3/group). Total RNA was extracted from cells using TRIzol reagent (Invitrogen). cDNA libraries were constructed for each pooled RNA sample using the NEBNext Ultra™ RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) according to the manufacturer’s instructions. Pathway analysis was used to determine the pathways significantly associated with DEGs according to the KEGG database.

**Enzyme-linked immunosorbent assay (ELISA)**

The serum and media levels of the cytokines TNF-α, IL-6, IL-1β (Thermo Fisher Scientific), and 4-HNE (MyBioSource) were determined using ELISA kits according to the manufacturer’s instructions.

**Statistical analysis**

All the data were analyzed using unpaired Student’s t tests or one-way ANOVA accompanied by Bonferroni post hoc t tests. Statistical significance was set at P≤0.05 (two-tailed). Statistical analysis was performed using GraphPad Prism Version 7.0 software (GraphPad Software, San Diego, CA, USA).

**RESULTS**

ATG16L1 expression is downregulated in livers of patients with MASH, and macrophage deficiency of ATG16L1 expression exacerbates steatohepatitis development. To investigate the involvement of ATG16L1 in MASH progression, we examined ATG16L1 expression in liver tissues from 39 patients with MASH and 31 without steatosis (Supplementary Table 1). The liver tissues were subjected to H&E and oil red O staining (Fig. 1A). The results showed that ATG16L1 gene expression and ATG16L1 protein expression were significantly lower in MASH patients than in normal controls (Fig. 1B, C). Furthermore, hepatic ATG16L1 expression levels were negatively correlated with the levels of serum transaminases (ALT and AST) and hepatic inflammation indicators (TNFA, IL6, and IL1B) (Fig. 1D, E). Dual-immunofluorescence staining of liver sections with the human macrophage marker CD68 showed that ATG16L1 expression was markedly inhibited in macrophages in the livers of patients with MASH (Fig. 1F). To further confirm these findings, murine MASH models were generated via HFHCD feeding for 16 weeks or MCD feeding for 6 weeks (Supplementary Fig. 1A–D). Analysis revealed that hepatic Atg16l1 gene expression and ATG16L1 protein expression were significantly lower in MASH mice than in normal controls (Fig. 1G, H). Because macrophage infiltration is implicated in this process, we assessed the effect of macrophage ATG16L1 expression on the progression of steatohepatitis. We generated Lyz2-Cre Atg16l1-knockout (Atg16l1ΔMϕ) mice. The overall expression levels of ATG16L1 in liver tissues or hepatocytes from Atg16l1ΔMϕ and Atg16l1fl/fl mice showed no significant difference. BMDMs isolated from Atg16l1ΔMϕ mice showed ATG16L1 knockout compared to those isolated from Atg16l1fl/fl mice (Supplementary Fig. 1E). Atg16l1ΔMϕ mice and Atg16l1fl/fl mice were bred and fed an NCD or an HFHCD for 16 weeks. The results demonstrated that macrophage Atg16l1 knockout increased proinflammatory gene expression and macrophage infiltration (Supplementary Fig. 1F, G). Histopathology of liver sections demonstrated that Atg16l1ΔMϕ mice had exacerbated injury and significantly more steatosis and inflammation than did Atg16l1fl/fl mice fed an HFHCD or MCD (Fig. 1I). Accordingly, the serum ALT and hepatic triglyceride (TG) levels were significantly greater in the Atg16l1ΔMϕ mice than in the Atg16l1fl/fl mice (Fig. 1J). Moreover, Atg16l1ΔMϕ mice exhibited significantly exacerbated liver fibrosis, as indicated by increased Sirius red and α-SMA staining (Fig. 1J); increased expression levels of the profibrotic genes Acta2, Col1a1, and Timp1; and increased protein expression levels of α-SMA, collagen-I, and TIMP-1 (Supplementary Fig. 1H, I). The weights of tissues including livers, brown adipose tissue (BAT), epididymal WAT (eWAT), and inguinal white adipose tissue (iWAT) of the Atg16l1ΔMϕ mice fed an HFHCD were greater than those of the Atg16l1fl/fl mice fed the same diet.
Figure 1. ATG16L1 expression is downregulated in livers of MASH patients, and macrophage Atg16l1 knockout exacerbates the development of experimental steatohepatitis. (A) Representative H&E staining and oil red O staining (400×) of human liver tissues; n=6/group. (B) ATG16L1 mRNA expression levels in liver tissues from patients with MASH (n=39) and without MASH (n=31). (C) ATG16L1 protein expression levels in human liver tissues. (D, E) Graphs showing the correlation between the mRNA expression levels of ATG16L1 and the serum levels of ALT, AST and hepatic TNFA, IL6, and IL1B in MASH patients (n=39). (F) Dual immunofluorescence staining for CD68 and ATG16L1 (400×) expression in MASH mice; n=6/group. (H) Hepatic ATG16L1 protein expression levels in MASH mice. (I) Representative hepatic H&E staining, oil red O staining, Sirius Red staining, and α-SMA immunohistochemical analysis in MASH mice. (J) NAS, serum ALT levels, and hepatic TG levels in MASH mice; n=6/group. MASH, metabolic dysfunction-associated steatohepatitis; ATG16L1, autophagy-related protein 16-like 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NAS, NAFLD activity score; TG, hepatic triglyceride. The data are expressed as the mean±SD. *p<0.05 (unpaired t test or ANOVA). **p<0.01.

(Supplementary Fig. 1J–L). Furthermore, compared with the Atg16li+/− mice fed the same diet, the Atg16li−/− mice fed an HFHCD exhibited a significant increase in visceral adipose tissue inflammation (Supplementary Fig. 1M).

Average food intake and EE were also measured in our study based on the methods of previous studies.28,29 The body weights of the NCD-fed mice did not significantly differ between the two groups, but a marked increase in body weight was observed in the HFHCD-fed Atg16li−/− mice compared with the Atg16li+/− mice (Supplementary Fig. 2A). HFHCD-fed Atg16li−/− mice consumed significantly less food than Atg16li+/− mice (Supplementary Fig. 2B). NCD-fed Atg16li−/− mice also consumed significantly less food than Atg16li+/− mice (Supplementary Fig. 2C).
Figure 2. Knockout of macrophage Atg16l1 expression induces type I interferon signaling and inflammatory responses. (A) Schematic diagram showing that primary hepatocytes isolated from Atg16l1fl/fl mice with or without PAOA stimulation were cocultured with LPS-primed primary BMDMs from Atg16l1fl/fl and Atg16l1ΔM66 mice. (B) Volcano plot of all DEGs in cocultured Atg16l1fl/fl and Atg16l1ΔM66 BMDMs. (C) Heatmap showing the clustering of 19 upregulated genes affecting the IFN-I response. (D) STRING-based network analysis of genes detected in (C), revealing a strong contribution of an IFN-I-related signature. (E) The expression of genes detected in (C). (F) KEGG pathway enrichment analysis of the pathways associated with genes whose expression was upregulated in stimulated Atg16l1fl/fl BMDMs compared with that in Atg16l1fl/fl BMDMs. (G) The proinflammatory protein expression levels in stimulated Atg16l1fl/fl and Atg16l1ΔM66 BMDMs. (H) Heatmap showing the clustering of 65 upregulated genes that affect the phagosome signaling pathway. (I) The gene expression levels of Marco, Ms1, and Cd14. (J) Heatmap showing that Tfeb expression was markedly lower in stimulated Atg16l1fl/fl BMDMs than in Atg16l1fl/fl BMDMs. (K) The gene expression level of Tfeb in stimulated Atg16l1fl/fl and Atg16l1ΔM66 BMDMs. (L) KEGG pathway enrichment analysis of the pathways associated with genes whose expression was downregulated in stimulated Atg16l1fl/fl BMDMs compared with that in Atg16l1fl/fl BMDMs. (M) Heatmap showing the clustering of 46 downregulated genes that affect the lysosome signaling pathway. (N) The gene expression levels of Hexa, Csb, and Gla. ATG16L1, autophagy-related protein 16-like 1; PAOA, palmitic acid- and oleic acid; BMDMs, bone marrow-derived macrophages; KEGG, Kyoto Encyclopedia of Genes and Genomes; STING, stimulator of interferon genes. The data are expressed as the mean±SD. *P<0.05 (unpaired t test or ANOVA).
Moreover, the rates of oxygen consumption (VO₂), CO₂ production (VCO₂), and EE in the Atg16l1fl/fl mice were significantly lower than in the Atg16l1ΔM mice (Supplementary Fig. 2D-F, 2H-J, and 2 L). In contrast, locomotor activity did not significantly differ between the Atg16l1ΔM and Atg16l1ΔΔM mice (Supplementary Fig. 2G, K). Collectively, these results suggest that ATG16L1 expression is significantly decreased in the liver tissues of mice with MASH and is negatively correlated with MASH development and that macrophage-specific deletion of Atg16l1 expression exacerbates experimental steatohepatitis.

Macrophage Atg16l1 knockout induces Type I interferon signaling and the inflammatory response

To further explore the role of ATG16L1 expression in MASH progression, we incubated LPS-primed Atg16l1fl/fl or Atg16l1ΔΔM bone marrow-derived macrophages (BMDMs) with Atg16l1fl/fl primary hepatocyte CM for 48 hours (Fig. 2A). Proinflammatory factors were examined, and the results showed that the gene expression and secretion of proinflammatory cytokines were markedly upregulated (Supplementary Fig. 3A, B). By assessing macrophage glycolysis and mitochondrial metabolism, we found that ATG16L1 expression could affect immune responses to MASH by regulating macrophage glycan metabolism. We measured glycolytic and mitochondrial metabolic activity using a Seahorse XFe96 analyzer. The results showed that the level of glycolysis in the Atg16l1ΔΔM BMDMs was markedly greater than that in the Atg16l1fl/fl BMDMs stimulated with CM+LPS (Supplementary Fig. 3C). Moreover, the mitochondrial metabolism level in the Atg16l1ΔΔM BMDMs was significantly lower than that in the Atg16l1fl/fl BMDMs stimulated with CM+LPS (Supplementary Fig. 3D). Next, the treated Atg16l1fl/fl or Atg16l1ΔΔM BMDMs (shown in Fig. 2A) were subjected to RNA sequencing analysis. In total, we identified 5,888 DEGs (Fig. 2B), including 3076 genes with upregulated expression and 2812 genes with downregulated expression (Atg16l1ΔΔM vs. Atg16l1fl/fl). The top 19 uniquely upregulated and downregulated genes included the IFN-I–dependent genes Oas1g, Oas3, Irgm1, and Ifit7, which are known to play a role in innate immunity and the antiviral response (Fig. 2C). Using the STRING database,30 we performed an interaction analysis of the 19 abovementioned genes, which revealed a densely connected network of known IFN-stimulated genes including the Oas1g, Ifit7, Oas2, and Isg15 genes (Fig. 2D). Moreover, qRT–PCR analysis of these 19 upregulated IFN-stimulated genes confirmed the above findings (Fig. 2E). Next, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis according to genotype and treatment, which revealed significant enrichment of upregulated genes related to NF-κB signaling and the phagosome signaling pathway (Fig. 2F). The upregulation of the proinflammatory NF-κB signaling pathway was validated by observation of increased protein expression levels of p-p65 (Ser536), p-IκBα, TNF-α, IL-6, and IL-1β (Fig. 2G). We analyzed the upregulated phagosome signaling pathway and found 65 significantly upregulated phagosome-related genes (Fig. 2H). The increased expression levels of three representative phagosome-related genes, Marco, Mrsl, and Cd14, in activated Atg16l1ΔΔM BMDMs further confirmed this result (Fig. 2I). Moreover, RNA sequencing and mRNA expression data indicated that the expression of the transcription factor EB (Tfeb), a master transcriptional regulator of autophagy and lysosomes,31 was significantly lower in activated Atg16l1ΔΔM BMDMs than in activated Atg16l1fl/fl BMDMs (Fig. 2J, K). Further analysis of the KEGG pathway enrichment analysis results also revealed significant enrichment of downregulated genes related to lysosomes (Fig. 2L). Next, we analyzed the downregulated lysosome signaling pathway and identified 46 significantly downregulated lysosome-related genes (Fig. 2M). The decreased expression levels of three representative lysosome-related genes, Hexa, Ctsb, and Gla, in activated Atg16l1ΔΔM BMDMs further validated this result (Fig. 2N). To clarify the above results, we constructed myeloid-specific Atg16l1 gene knock-in mice (Atg16l1OE). The overall expression levels of ATG16L1 in liver tissues or hepatocytes from Atg16l1OE and Atg16l1ΔΔM mice showed no significant difference. BMDMs isolated from Atg16l1OE mice showed significantly higher levels of ATG16L1 than those isolated from Atg16l1ΔΔM mice (Supplementary Fig. 3E). We incubated LPS-primed Atg16l1fl/fl or Atg16l1OE BMDMs with primary hepatocyte CM from Atg16l1fl/fl mice for 48 h (Supplementary Fig. 3F). The expression and secretion of proinflammatory factors were significantly decreased (Supplementary Fig. 3G, H). The level of glycolysis in the Atg16l1OE BMDMs was markedly lower than that in the At-
**Figure 3.** Macrophage Atg16l1 deficiency promotes the activation of the cGAS/STING pathway. (A) Schematic diagram showing that primary hepatocytes with or without PAOA stimulation isolated from Atg16l1<sup>fl/fl</sup> mice were cocultured with LPS-primed primary BMDMs from Atg16l1<sup>fl/fl</sup> and Atg16l1<sup>OE</sup> mice. (B) Immunofluorescence staining for STING expression in the bone marrow of Atg16l1<sup>OE</sup>/Atg16l1<sup>fl/fl</sup> mice. (C) The expression levels of cGAS/STING signaling markers and (D) the mRNA expression level of IFN-related genes were markedly lower in activated BMDMs treated with mtDNA+LPS. (H) The expression levels of STING signaling-related pro-caspase-1, (I) proinflammatory proteins and (J) proinflammatory proteins in (mtDNA+LPS)-stimulated BMDMs were also lower in activated Atg16l1<sup>OE</sup> BMDMs than in activated Atg16l1<sup>OE</sup> BMDMs (Supplementary Fig. 3L).

**Macrophage Atg16l1 deficiency promotes the activation of the cGAS/STING pathway**

A previous study demonstrated that cyclic GMP-AMP synthase (cGAS) synthesizes the dinucleotide secondary messenger 2′,3′-cGAMP, which then activates STING, an endoplasmic reticulum-resident adaptor, inducing its traf-
ficking to the endoplasmic reticulum–Golgi intermediate complex (ERGIC), where TANK-binding kinase 1 (TBK1) phosphorylates the IFN-1-inducing transcription factor interferon regulatory factor 3 (IRF3). Meanwhile, activated IRF3 promotes NLRP3 inflammasome expression. Our results demonstrated that the cGAS/STING signaling pathway was markedly activated in LPS-primed Atg16l1OE BMDMs compared with Atg16l1fl/fl BMDMs cultured in CM from Atg16l1fl/fl primary hepatocytes for 48 hours (Fig. 3A–C).

Our previous study revealed that mitochondrial DNA (mtDNA) released from pyroptotic hepatocytes contributes to STING activation in macrophages. In this study, we also investigated hepatocyte pyroptosis in liver tissues from MASH patients and found that hepatocyte pyroptosis was significantly greater in liver tissues from MASH patients than in those from normal controls, which was confirmed by the increase in Gasdermin D (GSDMD) activation (Supplementary Fig. 4A, B). Furthermore, we examined GSDMD expression in murine MASH model mice and found that GSDMD was significantly activated in hepatocytes isolated from MASH mice, which was reflected by increased levels of GSDMD-N fragments and increased hepatocyte death (Supplementary Fig. 4C, D). As damage-associated molecular pattern molecules (DAMPs) play an important role in the inflammatory response, we examined the levels of DAMPs in hepatocytes isolated from MASH mice. The levels of ATP and mtDNA in the supernatants of pyroptotic hepatocytes isolated from MASH mice were significantly greater than those in the supernatants of control hepatocytes (Supplementary Fig. 4E, F). Next, we isolated mtDNA from pyroptotic hepatocytes (Supplementary Fig. 4G) and stimulated WT BMDMs with isolated mtDNA tagged with Cy5. mtDNA-Cy5 was engulfed by BMDMs at 12 h postcoculture (Supplementary Fig. 4H). Furthermore, CM from palmitic acid- and oleic acid (PAOA)-stimulated hepatocytes was also used to coculture LPS-primed BMDMs with or without the mtDNA scavenger ethidium bromide (EthBr) (Supplementary Fig. 4I). We found that the expression of proinflammatory genes (Tnfa, Il6, Il1b, Ifnb1, and Tgfb1) was significantly decreased in activated BMDMs with mtDNA depletion (Supplementary Fig. 4J–L). Next, mtDNA was isolated from pyroptotic hepatocytes and used to stimulate Atg16l1OE, Atg16l1AMO or Atg16l1OE BMDMs. mtDNA-Cy5 was more engulfed by the Atg16l1AMO BMDMs and less engulfed by the Atg16l1OE BMDMs at 12 hours postcoculture with mtDNA-Cy5 than by the Atg16l1fl/fl BMDMs (Supplementary Fig. 4M).

To explore the key role of mtDNA in regulating the ATG16L1-mediated macrophage immune response, we cocultured BMDMs from Atg16l1fl/fl or Atg16l1AMO mice with mtDNA isolated from pyroptotic hepatocytes (Fig. 3D). The results suggested that the cGAS/STING signaling pathway was markedly activated (Fig. 3E–G) in LPS-primed Atg16l1AMO BMDMs compared with Atg16l1fl/fl BMDMs stimulated with mtDNA. Next, we treated activated Atg16l1AMO BMDMs with the STING inhibitor C-176 to clarify the important role of STING signaling in ATG16L1-mediated macrophage inflammation. The results showed that the downstream signaling activation of STING was markedly decreased (Fig. 3H–J). Subsequently, we treated Atg16l1AMO Tmem173AMO BMDMs with mtDNA to clarify the important role of STING signaling in ATG16L1-mediated macrophage inflammation. STING signaling and inflammation were significantly suppressed in the Atg16l1AMO Tmem173AMO BMDMs compared with the Atg16l1AMO BMDMs (Fig. 3K, L). Furthermore, we cocultured Atg16l1OE BMDMs with CM from PAOA-stimulated hepatocytes or mtDNA from pyroptotic hepatocytes and found that the STING signaling pathway was markedly inhibited in the Atg16l1OE BMDMs compared with the Atg16l1AMO BMDMs (Supplementary Fig. 5A–E). Next, we treated activated Atg16l1OE BMDMs with the STING activator DMXAA. The results indicated that STING signaling was upregulated in the opposite manner in the Atg16l1OE BMDMs treated with DMXAA (Supplementary Fig. 5F–H). To explore the role of STING in MASH, we constructed MASH model Atg16l1AMO Tmem173AMO mice. As a result, attenuated MASH was found in Atg16l1AMO Tmem173AMO mice compared with that in Atg16l1AMO mice (Supplementary Fig. 6A–F). Furthermore, we also assessed the body weight and energy expenditure of HFHCD-fed Atg16l1AMO Tmem173AMO mice compared to Atg16l1AMO mice. The results indicated that HFHCD-fed Atg16l1AMO Tmem173AMO mice exhibited decreased body weight compared to Atg16l1AMO mice, along with increased energy expenditure (Supplementary Fig. 6G, H).
Figure 4. Macrophage Atg16l1 deficiency induces hepatocyte lipid accumulation and HSC activation. (A) Schematic diagram showing the co-culture of primary hepatocytes from Atg16l1ΔM mice with LPS-primed primary BMDMs from Atg16l1ΔM, Atg16l1ΔOE and Atg16l1ΔOE mice with or without anti-TNF-α, MR16-1, IL-1α, TNF-α, IL-6 or IL-1B and PAOA treatment. (B) Lipid accumulation determined by oil red O staining and (C) Tnfa mRNA expression levels in primary Atg16l1ΔM hepatocytes cocultured with Atg16l1ΔM and Atg16l1ΔMOE macrophages in PAOA medium with or without anti-TNF-α, MR16-1, or IL-1RA. (D) The protein expression levels of p-JNK, JNK, c-Jun, c-Fos and (E) Tgfb1 mRNA in LPS-primed Atg16l1ΔM and Atg16l1ΔOE macrophages cultured with CM from PAOA-stimulated Atg16l1ΔM hepatocytes. (F) DNA pull-down assay. (G) ChiP assay. (H) Dual-luciferase reporter assay. (I) The protein expression levels of p-JNK, JNK, c-Jun, c-Fos and (J) Tgfb1 mRNA in LPS-primed Atg16l1ΔM macrophages treated with CM from PAOA-stimulated Atg16l1ΔM hepatocytes with or without stimulation by the STING inhibitor C176. (K) The protein expression levels of p-JNK, JNK, c-Jun, c-Fos and (L) Tgfb1 mRNA in LPS-primed Atg16l1ΔM macrophages treated with CM from PAOA-stimulated Atg16l1ΔM hepatocytes treated with C176 with or without the JNK activator anisomycin. (M) Primary hepatocyte-conditioned media was transferred to LPS-primed Atg16l1ΔM, Atg16l1ΔOE or Atg16l1ΔOE BMDMs for 48 hours, and BMDM-CM was then transferred to primary mouse HSCs in the absence or presence of TGF-β1 (8 ng/ml for 24 hours). (N) Immunofluorescence staining for α-SMA (green) and (O) the Acta2, Col1a1, and Timp1 genes in treated primary mouse HSCs. Scale bar, 50 μm. (P) α-SMA (green) immunofluorescence staining and (Q) Acta2, Col1a1, and Timp1 gene expression in treated primary mouse HSCs. Scale bar, 50 μm. ATG16L1, autophagy-related protein 16-like 1; HSCs, hepatic stellate cells; BMDMs, bone marrow-derived macrophages; PAOA, palmitic acid- and oleic acid; STING, stimulator of interferon genes. The data are expressed as the mean±SD. *P<0.05, **P<0.01 (unpaired t test or ANOVA). ***P<0.001.
Knockout of macrophage Atg16l1 expression promotes hepatocyte lipid accumulation and HSC activation

Next, we determined whether ATG16L1-mediated macrophage inflammation affects lipid metabolism in hepatocytes. We assessed the ability of macrophage-derived TNF-α, IL-6, and IL-1β to induce steatohepatitis and the effectiveness of neutralizing antibodies against these cytokines against steatohepatitis. We used a coculture system of LPS-primed Atg16l1$^{ΔM}$, Atg16l1$^{OE}$, or Atg16l1$^{fl/fl}$ macrophages with primary Atg16l1$^{fl/fl}$ hepatocytes (Fig. 4A). The neutralizing antibodies against TNF-α, IL-6, or IL-1β, including anti-TNF-α, MR16-1, and IL-1RA, decreased hepatocyte lipid accumulation and inflammation (Fig. 4B, C) in the Atg16l1$^{fl/fl}$ hepatocytes cocultured with LPS-primed Atg16l1$^{ΔM}$ macrophages, whereas administration of TNF-α, IL-6, or IL-1β increased lipid deposition (Supplementary Fig. 7A) and inflammatory responses (Supplementary Fig. 7B) in the Atg16l1$^{fl/fl}$ hepatocytes cocultured with LPS-primed Atg16l1$^{OE}$ macrophages.

A previous study showed that JNK1 activation in macrophages could promote TGF-β1 expression and affect HSC activation. The activation of hepatic JNK1, c-Jun, and AP-1 signaling occurs in parallel with the development of steatohepatitis in MASH mice. We determined the phosphorylation levels of JNK, c-Jun, and c-Fos and found that JNK, c-Jun, and c-Fos phosphorylation was significantly greater in Atg16l1$^{ΔM}$ macrophages than in Atg16l1$^{fl/fl}$ macrophages (Fig. 4D). The expression of Tgfb1 in Atg16l1$^{ΔM}$ macrophages was also greater than that in Atg16l1$^{fl/fl}$ macrophages (Fig. 4E). We performed sequence alignment analysis of the Tgfb1 gene and identified an AP-1 binding site to which c-Jun binds in the promoter region of Tgfb1 (Fig. 4F). Based on this information, we performed a DNA pull-down assay to determine whether LPS treatment might alter the association of AP-1 with the Tgfb1 promoter. We observed that LPS treatment increased the binding of c-Jun and c-Fos to the AP-1 binding site in the Tgfb1 promoter region (Fig. 4F). Moreover, a chromatin immunoprecipitation (ChIP)-qPCR assay revealed the enrichment of c-Jun at the Tgfb1 promoter region in response to LPS treatment (Fig. 4G). Next, mutation of the AP-1 binding sites to which c-Jun binds in the Tgfb1 promoter region abrogated the increase in Tgfb1 promoter activity triggered by c-Jun overexpression (Fig. 4H). We treated activated Atg16l1$^{ΔM}$ BMDMs with the STING inhibitor C-176 and found that the protein expression levels of phosphorylated JNK, c-Jun, and c-Fos and the mRNA expression level of Tgfb1 were significantly decreased (Fig. 4I, J), whereas the protein expression levels of phosphorylated JNK, c-Jun, and c-Fos and the mRNA expression level of Tgfb1 were significantly increased when C-176-treated Atg16l1$^{ΔM}$ BMDMs were stimulated with the JNK activator anisomycin (Fig. 4K, L). To confirm the above results, we determined the protein expression levels of phosphorylated JNK, c-Jun, and c-Fos and the mRNA expression level of Tgfb1 in Atg16l1$^{OE}$ BMDMs. The results showed that Atg16l1 overexpression markedly reduced the expression levels of phosphorylated JNK, c-Jun, and c-Fos and the mRNA expression of Tgfb1 ( Supplementary Fig. 7C, D).

Next, we treated activated Atg16l1$^{OE}$ BMDMs with the STING activator DMXAA and found that the protein levels of phosphorylated JNK, c-Jun, and c-Fos significantly increased (Supplementary Fig. 7E), whereas the protein expression levels of phosphorylated JNK, c-Jun, and c-Fos significantly decreased when DMXAA-treated Atg16l1$^{ΔM}$ BMDMs were stimulated with the JNK inhibitor SP600125 (Supplementary Fig. 7F). To validate whether macrophage ATG16L1 expression affects HSC activation, we incubated primary mouse HSCs in the absence or presence of TGF-β1 (8 ng/mL) with CM from Atg16l1$^{ΔM}$, Atg16l1$^{OE}$ or Atg16l1$^{fl/fl}$ BMDMs treated with primary hepatocyte CM from Atg16l1$^{fl/fl}$ mice for 24 hours (Fig. 4M). α-SMA immunofluorescence and Acta2, Coll1a1, and Timp1 gene expression in HSCs indicated that macrophage Atg16l1 knockout markedly promoted the activation of HSCs, while Atg16l1 overexpression significantly suppressed HSC activation (Fig. 4N–Q).

Depletion of macrophage Atg16l1 expression suppresses lipophagy and promotes STING signaling activation

Next, we explored the specific mechanisms underlying the ATG16L1-mediated regulation of the STING signaling pathway in MASH. In yeast and mammalian cells, ATG16L1 is responsible for proper subcellular localization of the autophagy machinery. The decreased protein expression level of LC3B and increased protein expression level of

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Figure 5. Macrophage Atg16l1 depletion suppresses lipophagy and promotes STING signaling activation. (A) LC3B and P62 protein levels and (B) TG levels in LPS-primed Atg16l1fl/fl and Atg16l1OE macrophages cultured with CM from PAOA-stimulated Atg16l1fl/fl hepatocytes. (C) BODIPY 493/503 staining of treated macrophages. Scale bar, 50 μm. (D) Colocalization of BODIPY 493/503 (green) staining with LAMP1 (red) expression and (E) BODIPY 493/503 (green) staining with LC3 (red) expression in Atg16l1fl/fl, Atg16l1OE, and Atg16l1OE macrophages treated with CM from PAOA-stimulated Atg16l1fl/fl hepatocytes. Scale bar, 20 μm. (F) The levels of β-oxidation and (G) the levels of 4-HNE in Atg16l1fl/fl, Atg16l1OE, and Atg16l1OE macrophages treated with CM from PAOA-stimulated Atg16l1fl/fl hepatocytes with or without the autophagy inhibitor 3-MA and the LIPA inhibitor lalistat. (H) Ifnb1 mRNA expression in LPS-primed WT macrophages treated with CM from PAOA-stimulated WT hepatocytes with or without 4-HNE. (I) Ifnb1 mRNA expression in LPS-primed WT macrophages treated with CM from PAOA-stimulated WT hepatocytes with or without 4-HNE and DMXAA. (J) 4-HNE levels in activated WT macrophages treated with the β-oxidation inhibitor etomoxir. (K) Ifnb1 mRNA expression in LPS-primed WT macrophages treated with CM from PAOA-stimulated WT hepatocytes with or without 4-HNE and etomoxir. (L) Immunoblot analysis of STING carbonylation by selective labeling with m-APA in WT BMDMs treated with 4-HNE (6.4 μM) or (M) in activated Atg16l1ΔM, Atg16l1fl/fl, and Atg16l1fl/fl BMDMs. (N) Immunoblot analysis of STING palmitoylation by click chemistry in WT BMDMs pretreated with 4-HNE following DMXAA stimulation or (O) in activated Atg16l1ΔM, Atg16l1fl/fl, and Atg16l1fl/fl BMDMs. (P) Confocal analysis of the colocalization of STING (green) expression and expression of the cis-Golgi protein GM130 (red) in LPS-primed Atg16l1fl/fl, Atg16l1fl/fl, and Atg16l1fl/fl BMDMs with or without CM from PAOA-stimulated Atg16l1fl/fl hepatocytes. Scale bar, 10 μm. ATG16L1, autophagy-related protein 16-like 1; STING, stimulator of interferon genes; PAOA, palmitic acid- and oleic acid; CM, conditioned media. The data are expressed as the mean±SD. *P<0.05, **P<0.01 (unpaired t test or ANOVA).
of autophagosomes, which deliver cytoplasmic material to lysosomes.\textsuperscript{42} To confirm that lysosomes regulate intracellular lipid levels, the effect of lysosomal hydrolysis on lipid stores was examined. Dual immunofluorescence studies revealed increased colocalization of LDs with lysosome-associated membrane protein 1 (LAMP1) in LPS-primed and CM-treated Atg16l1\textsuperscript{fl/fl} BMDMs; Atg16l1 deletion inhibited lysosomal hydrolysis of lipid stores, whereas Atg16l1 overexpression promoted lysosomal hydrolysis of lipid stores (Fig. 5D). Furthermore, LD colocalization with the autophagosome marker LC3 demonstrated a direct association between LDs and autophagosomes, supporting a constitutive function for autophagy in regulating LDs (Fig. 5E). Electron microscopy was used to verify LD degradation by autophagic lysosomes (Supplementary Fig. 8D). A previous study indicated that the inhibition of autophagy decreases TG β-oxidation.\textsuperscript{38} Our study also demonstrated that Atg16l1 knockout decreased macrophage β-oxidation, whereas Atg16l1 overexpression increased β-oxidation (Fig. 5F). A recent study demonstrated that lipid peroxidation promoted the production of 4-hydroxynonenal (4-HNE), which is the major end product of lipid peroxidation. Increased 4-HNE production enhanced STING carboxylation, resulting in the inhibition of STING trafficking from the endoplasmic reticulum to the Golgi apparatus and the suppression of STING activation.\textsuperscript{43} We also examined cellular 4-HNE levels and revealed that Atg16l1 knockout decreased 4-HNE production, whereas Atg16l1 overexpression increased 4-HNE production (Fig. 5G). Another study showed that peroxisomal β-oxidation acted as a sensor for intracellular fatty acid levels and regulated lipolysis.\textsuperscript{44} Our study demonstrated that the presence of 4-HNE suppressed the mRNA expression of \textit{I\textit{I}b\textit{b}1} (Fig. 5H, I). Moreover, the inhibition of lipophagy and β-oxidation decreased the production of 4-HNE and increased the mRNA expression of \textit{I\textit{I}b\textit{b}1} (Fig. 5J, K). In addition, 4-HNE induced STING carbonylation (Fig. 5L and Supplementary Fig. 8E), Atg16l1 knockout inhibited STING carbonylation, and Atg16l1 overexpression promoted STING carbonylation (Fig. 5M). Study has indicated that the palmitoylation of STING was crucial for the IFN I response in macrophages, but it didn’t affect the translocation of STING.\textsuperscript{45} Our results indicated that 4-HNE blocked STING palmitoylation (Fig. 5N), Atg16l1 knockout promoted STING palmitoylation, and Atg16l1 overexpression inhibited STING palmitoylation (Fig. 5O). The trafficking of STING from the ER to the Golgi apparatus is a crucial step for STING activation and subsequent IRF3 activation.\textsuperscript{32} To investigate STING trafficking from the ER to the Golgi apparatus in the context of differential ATG16L1 expression, we costained for STING and the Golgi marker GM130 in LPS-primed and CM-treated Atg16l1\textsuperscript{fl/fl}, LPS-primed and CM-treated Atg16l1\textsuperscript{ AMC}, and LPS-primed and CM-treated Atg16l1\textsuperscript{CE} BMDMs (Fig. 5P).
macrophage ATG16L1 depletion promoted STING localization to the Golgi apparatus, whereas ATG16L1 overexpression inhibited STING localization to the Golgi apparatus. Furthermore, compared to Atg16l1fl/fl MASH mice, liver tissues of Atg16l1ΔMϕ MASH mice exhibited impaired autophagy and activation of the STING signaling pathway, indicated by upregulation of protein expression levels of P62, cGAS, Pal-STING, P-TBK1, and P-IRF3, along with downregulation of protein expression levels of LC3B and Carbonyl-STING (Supplementary Fig. 8F).
Macrophage Atg16l1 overexpression ameliorates the progression of experimental steatohepatitis

Next, Atg16l1^{OE} mice and Atg16l1^{fl/fl} mice were bred and fed an HFHCD for 16 weeks or an MCD for 6 weeks. Macrophage Atg16l1 overexpression decreased proinflammatory gene expression (Fig. 6A) and macrophage infiltration (Fig. 6B). Histopathology of liver sections demonstrated that Atg16l1^{OE} mice had improved liver histology and less steatosis and inflammation than Atg16l1^{fl/fl} mice (Fig. 6C). Accordingly, the levels of serum ALT and hepatic TG were significantly lower in the Atg16l1^{OE} mice than in the Atg16l1^{fl/fl} mice (Fig. 6D). Moreover, Atg16l1^{OE} mice exhibited significantly mitigated liver fibrosis, as indicated by decreased Sirius red and α-SMA staining (Fig. 6C); decreased expression levels of the profibrotic genes Acta2, Col1a1 and Timp1; and decreased protein expression levels of α-SMA, collagen-I, and TIMP-1 (Fig. 6E, F). Moreover, compared to Atg16l1^{fl/fl} mice, liver tissues of Atg16l1^{OE} mice exhibited enhanced autophagy and suppression of the STING signaling pathway, indicated by decreased protein expression levels of P62, cGAS, Pal-STING, P-TBK1, and P-IRF3, along with increased protein expression levels of LC3B and Carboxy-STING (Supplementary Fig. 8G). The weights of tissues, including livers, BAT, eWAT and ingWAT, of the Atg16l1^{OE} mice fed an HFHCD were lower than those of the Atg16l1^{fl/fl} mice fed the same diet (Fig. 6G, H).

The body weights and fat masses of the NCD-fed mice were not significantly different between the two genotypes, but we observed a marked decrease in weight gain driven by fat mass in the HFHCD-fed Atg16l1^{OE} mice (Supplementary Fig. 9A). Furthermore, there was no significant difference between the amount of food consumed by HFHCD-fed Atg16l1^{OE} mice and that consumed by Atg16l1^{fl/fl} mice (Supplementary Fig. 9B). Similarly, there was no significant difference between the amount of food consumed by the NCD-fed Atg16l1^{OE} mice and that consumed by the Atg16l1^{fl/fl} mice (Supplementary Fig. 9C). Nevertheless, the rates of VO_{2}, VCO_{2}, and EE in the Atg16l1^{OE} mice were significantly greater than those in the Atg16l1^{fl/fl} mice (Supplementary Fig. 9D-F, 9H-J, 9L). In contrast, locomotor activity did not significantly differ between the Atg16l1^{OE} and Atg16l1^{fl/fl} mice (Supplementary Fig. 9G, K). Collectively, these results suggest that Atg16l1 overexpression in macrophages ameliorates steatohepatitis in mice.

Pharmacological enhancement of ATG16L1 expression prevents MASH development

Finally, we explored the effects of pharmacologically induced ATG16L1 overexpression on steatohepatitis progres-
Figure 7. Pharmacological enhancement of ATG16L1 expression prevents MASH development. The protein expression levels of STING signaling components in activated WT BMDMs treated with CM from PAOA-stimulated hepatocytes with or without the ATG16L1 enhancer peretinoin following DMXAA stimulation. (B) The protein levels of p-JNK, JNK, c-Jun, and c-Fos in activated WT BMDMs treated with CM. (C) The proinflammatory protein expression levels, (D) proinflammatory gene expression levels, and (E) Ifnb1 and (F) Tgfb1 mRNA expression in activated WT BMDMs treated with CM. (G) Schematic representation of HFHCD-fed or MCD-fed WT mice supplemented with dietary peretinoin. (H) Hepatic ATG16L1 protein expression levels. (I) Hepatic H&E staining, oil red O staining, Sirius red staining, and α-SMA immunohistochemistry. (J) NAS, serum ALT levels, and hepatic TG levels. (K) Hepatic Acta2, Col1a1, and Timp1 gene expression; n=6 mice/group. (L) Hepatic α-SMA, collagen-1, and TIMP-1 protein expression levels. (M) Hepatic Tnfα, Il6, and Il1b gene expression levels. (N) Hepatic F4/80⁺ cell immunohistochemistry. (O) Representative eWAT H&E staining and F4/80 immunohistochemistry image of HFHCD-fed WT mice treated with or without peretinoin. MASH, metabolic dysfunction-associated steatohepatitis; ATG16L1, autophagy-related protein 16-like 1; BMDMs, bone marrow-derived macrophages; STING, stimulator of interferon genes; PAOA, palmitic acid- and oleic acid; CM, conditioned media; HFHCD, high-fat and high-cholesterol diet; MCD, methionine- and choline-deficient diet; NAS, NAFLD activity score; ALT, alanine aminotransferase; TG, hepatic triglyceride; eWAT, epididymal white adipose tissue. The data are expressed as the mean±SD. *P<0.05, **P<0.01 (unpaired t test or ANOVA).
An ATG16L1 enhancer (peretinoin) was used to treat activated WT BMDMs stimulated with or without DMXAA. The results showed that increased ATG16L1 protein expression levels inhibited STING signaling pathway activation, decreased the expression of phosphorylated JNK, c-Jun, and c-Fos, and suppressed macrophage inflammation and Tgfb1 expression (Fig. 7A–F). Moreover, peretinoin was added to the diets of WT mice fed an HFD for 16 consecutive weeks or fed an MCD for 6 consecutive weeks (Fig. 7G). Peretinoin treatment increased ATG16L1 protein expression levels in liver tissues from MASH mice (Fig. 7H). Histopathology of liver sections revealed that compared with untreated mice fed the same diet, WT MASH mice treated with peretinoin had improved liver histology with markedly reduced steatosis, inflammation, and fibrosis markers (Fig. 7I). Accordingly, the levels of serum ALT and hepatic TG were significantly lower in WT MASH mice treated with peretinoin than in untreated mice fed the same diet (Fig. 7J). Similar results were found regarding the gene and protein expression levels of profibrotic markers (Fig. 7K, L). Moreover, proinflammatory gene expression and macrophage infiltration were significantly lower in WT MASH mice treated with peretinoin than in untreated mice fed the same diet (Fig. 7J). Similar results were found regarding the gene and protein expression levels of profibrotic markers (Fig. 7K, L). Moreover, proinflammatory gene expression and macrophage infiltration were significantly lower in WT MASH mice treated with peretinoin than in untreated mice fed the same diet (Fig. 7J).

To further validate the role of macrophage ATG16L1 in MASH, we also treated Atg16l1ΔMϕ mice and Atg16l1ΔMϕ mice with peretinoin in the MCD-induced MASH model. Histopathology of liver sections revealed that compared with untreated group, the MASH phenotype in Atg16l1ΔMϕ mice treated with peretinoin showed slight improvement, with mild reductions in markers of steatosis, inflammation, and fibrosis. However, it was more severe than in Atg16l1ΔMϕ mice treated with peretinoin (Supplementary Fig. 10A). We speculate that peretinoin might also partially by promoting ATG16L1 expression in hepatocytes and other cell types to ameliorate MASH, which deserved further exploration in our future study. Accordingly, serum ALT and liver TG levels in Atg16l1ΔMϕ mice treated with peretinoin were lower than untreated Atg16l1ΔMϕ mice, but higher than peretinoin-treated Atg16l1ΔMϕ mice (Supplementary Fig. 10B). Additionally, profibrotic and proinflammatory genes also exhibited similar outcomes (Supplementary Fig. 10C, D). These results demonstrate that pharmacological enhancement of ATG16L1 protein expression levels alleviates steatohepatitis and fibrosis in mice.

**DISCUSSION**

Herein, we identified macrophage ATG16L1 expression as a novel inhibitor of MASH. In vivo studies demonstrated that macrophage-specific Atg16l1 knockout exacerbates MASH, whereas transgenic overexpression of Atg16l1 attenuates MASH in mice. Moreover, we found that Atg16l1 knockout decreased EE, whereas Atg16l1 overexpression increased EE. Further experiments showed that Atg16l1 knockout suppressed macrophage lipophagy, which subsequently suppressed macrophage β-oxidation and decreased 4-HNE production. Decreased 4-HNE levels inhibited STING carbonylation, promoted STING palmitoylation and induced STING trafficking from the ER to the Golgi apparatus and the activation of downstream STING signaling, thereby increasing proinflammatory and profibrotic cytokine secretion, resulting in hepatocyte steatosis and HSC activation. Furthermore, Atg16l1 expression deficiency promoted macrophage phagocytosis but impaired lysosomal activity, increasing the amount of mtDNA released by pyroptotic hepatocytes. Increased mtDNA abundance activated macrophage cGAS/STING signaling. Pharmacological ATG16L1 overexpression alleviated MASH progression in mice. Thus, macrophage ATG16L1 represents a promising therapeutic target for MASH treatment.

In mammalian cells, ATG16L1 is responsible for proper subcellular localization of the autophagic machinery. A previous study indicated that the induction of autophagy may ameliorate hepatic steatosis, and siRNA-mediated ATG16L1 expression knockdown resulted in increased lipid accumulation. However, the specific mechanisms underlying ATG16L1-mediated regulation of MASH remain unknown. Although decreased Atg16l1 mRNA expression was found in the livers of patients with MASH based on markers of disease progression, the specific mechanisms underlying ATG16L1-mediated regulation of MASH remain unknown.

A previous study showed that hepatic macrophages might become activated due to excess lipid accumulation.
and defective lipid processing.\(^4\) Therefore, more studies are needed to explore effective methods to promote macrophage LD degradation in MASH. Another study indicated that autophagy might promote the β-oxidation of triglycerides.\(^4\) Our study revealed that impaired lipophagy in macrophages suppressed macrophage β-oxidation and decreased the production of the lipid peroxidation end product 4-HNE. Atg16l1 knockout decreased EE, whereas Atg16l1 overexpression increased EE in mice with MASH. Macrophage-specific Atg16l1 depletion exacerbates steatohepatitis development by suppressing macrophage lipophagy.

Moreover, a previous study showed that impaired ATG16L1 expression enhances monocyte phagocytosis in Crohn’s disease patients.\(^4\) Our study revealed that Atg16l1 knockout enhances macrophage phagocytosis by increasing the amount of mtDNA released by pyroptotic hepatocytes. STING is a critical integrator that acts in response to stimulation via cGAMP produced by cGAS and has a fundamental role in the induction of innate immune responses triggered by the presence of cytosolic DNA.\(^4\) STING activation is vital for host defense against viral infection and for the mediation of DNA damage-induced inflammation. In the resting state, STING localizes to the ER, and the Ca\(^{2+}\) sensor stromal interaction molecule 1 facilitates its retention to enforce immunological quiescence.\(^4\) After engagement with cGAMP or other cyclic dinucleotides, STING is activated and moves from the ER to the Golgi apparatus,\(^5\) where it induces IRF3 activation, type I IFN expression, and the inflammatory response. Thus, STING trafficking from the ER to the Golgi apparatus is a key step in signal activation. Previous studies have reported that STING expression is increased in liver tissues from MASH patients and promotes macrophage-mediated hepatic inflammation and fibrosis in mice.\(^5\) Another study indicated that lipid peroxidation led to STING carbonylation via the lipid peroxidation end product 4-HNE and inhibited STING trafficking from the ER to the Golgi complex.\(^5\) Therefore, STING signaling plays an important role in MASH development. Our study confirmed that macrophage-specific Atg16l1 depletion exacerbates MASH development by suppressing macrophage lipophagy, which subsequently suppresses macrophage β-oxidation and decreases the production of 4-HNE. Decreased 4-HNE levels inhibited STING carbonylation, enhanced STING palmitoylation, and promoted STING trafficking from the ER to the Golgi apparatus, which subsequently maintained downstream STING signaling activation.

In summary, we revealed the role of ATG16L1 expression in MASH. Macrophage-specific Atg16l1 knockout exacerbates the development of steatohepatitis by suppressing macrophage lipophagy, which subsequently suppresses macrophage β-oxidation and decreases the production of 4-HNE. Decreased 4-HNE levels enhanced STING palmitoylation, which ultimately promoted the secretion of proinflammatory and profibrotic cytokines, resulting in hepatocyte steatosis and HSC activation. Transgenic Atg16l1 overexpression in macrophages reversed the above results. These results indicate that targeting ATG16L1 expression might be promising for the management of steatohepatitis.

**Authors’ contributions**

QW, QB, ZX, HZ, and LL participated in the research design. LL and HZ supervised the study. QW, QB, ZX, HZ, and LL drafted the manuscript. QW, QB, ZX, YL, JZ, and YP conducted the experiments. All the authors have read and approved the final manuscript.

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**Conflicts of Interest**

The authors have no conflicts to disclose.

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Novel role of MHC class II transactivator in hepatitis B virus replication and viral counteraction

Mehrangiz Dezhbord1,*, Seong Ho Kim1,*, Soree Park1, Da Rae Lee1, Nayeon Kim1, Juhee Won1,2, Ah Ram Lee1, Dong-Sik Kim1, and Kyun-Hwan Kim1

1Department of Precision Medicine, Sungkyunkwan University School of Medicine, Suwon; 2Department of Pharmacology, School of Medicine, Konkuk University, Seoul; 3Department of Surgery, Division of HBP Surgery and Liver Transplantation, College of Medicine, Korea University, Seoul, Korea

Graphical Abstract

Study Highlights
- CIITA mediates the anti-HBV activity of IFN-γ.
- CIITA suppresses the expression of HNF1 and HNF4 via activation of ERK1/2 pathway.
- HBx counteracts CIITA via direct binding and inhibiting its function.
INTRODUCTION

Despite the availability of preventive vaccines, hepatitis B virus (HBV) infection remains a global health concern, affecting over 257 million individuals worldwide. Chronic hepatitis B (CHB) is associated with serious complications, such as liver failure, cirrhosis, and hepatocellular carcinoma (HCC).\(^1\) HBV contains partial double-stranded DNA that is converted into the covalently closed circular DNA (cccDNA) minichromosome, which acts as a transcription template in antigen-presenting cells.

Background/Aims: The major histocompatibility class II (MHC II) transactivator, known as CIITA, is induced by Interferon gamma (IFN-\(\gamma\)) and plays a well-established role in regulating the expression of class II MHC molecules in antigen-presenting cells.

Methods: Primary human hepatocytes (PHH) were isolated via therapeutic hepatectomy from two donors. The hepatocellular carcinoma (HCC) cell lines HepG2 and Huh7 were used for the mechanistic study, and HBV infection was performed in HepG2-NTCP cells. HBV DNA replication intermediates and secreted antigen levels were measured using Southern blotting and ELISA, respectively.

Results: We identified a non-canonical function of CIITA in the inhibition of hepatitis B virus (HBV) replication in both HCC cells and patient-derived PHH. Notably, in vivo experiments demonstrated that HBV DNA and secreted antigen levels were significantly decreased in mice injected with the CIITA construct. Mechanistically, CIITA inhibited HBV transcription and replication by suppressing the activity of HBV-specific enhancers/promoters. Indeed, CIITA exerts antiviral activity in hepatocytes through ERK1/2-mediated down-regulation of the expression of hepatitis C virus nuclear factor 1a (HNF1a) and HNF4a, which are essential factors for virus replication. In addition, silencing of CIITA significantly abolished the IFN-\(\gamma\)-mediated anti-HBV activity, suggesting that CIITA mediates the anti-HBV activity of IFN-\(\gamma\) to some extent. HBV X protein (HBx) counteracts the antiviral activity of CIITA via direct binding and impairing its function.

Conclusions: Our findings reveal a novel antiviral mechanism of CIITA that involves the modulation of the ERK pathway to restrict HBV transcription. Additionally, our results suggest the possibility of a new immune avoidance mechanism involving HBx. (Clin Mol Hepatol 2024;30:539-560)

Keywords: Hepatitis B virus; MHC class II transactivator; HBV X protein; Interferon-gamma; Hepatocyte nuclear factor

*These authors contributed equally to this work.
plate to produce viral RNAs. HBV reverse transcriptase/polymerase synthesizes viral DNA from pre-genomic RNA (pgRNA) inside newly formed nucleocapsids. Although the life cycle of HBV and innate immune response to HBV infection have been extensively studied, the network of host factors and signaling pathways that could potentially exert inhibitory effects on HBV replication is not fully understood.  

The 3.2 kb HBV genome encodes four proteins: polymerase (P), surface (S; pre-S1, pre-S2, and S), precore/core (C), and X (HBx). HBx is a key factor that promotes the transcription and replication of HBV and is known to modulate immune avoidance by regulating host antiviral signaling pathways and molecules. HBx consists of 154 amino acids, including a transactivation domain, a nuclear translocation domain, and a negative regulatory domain. The mechanism by which HBV escapes the immune system remains unclear.  

Various cytokines have been reported to inhibit HBV transcription and replication via diverse mechanisms both in vitro and in vivo. For instance, tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) are secreted by HBV-specific CD8+ T cells. TNF-α reduces HBV transcription and capsid stability, whereas IFN-γ has been reported to eliminate pgRNA-containing capsids in a HBV-infected mouse model. In addition, pegylated-IFN-α (Peg-IFN-α) is currently used to treat HBV infections along with nucleos(t)ide analogs (NAs). Although NAs target viral reverse transcriptase to inhibit viral replication, they cannot completely eradicate CHB due to the persistence of HBV cccDNA within infected hepatocytes. Moreover, a major drawback of utilizing IFN-α as a therapeutic agent is its association with multiple side effects. Therefore, currently available therapies could be combined with targeted suppression of viral RNA and cccDNA to achieve more robust therapeutic strategies. Accordingly, ongoing efforts to exploit the interplay between the virus and host factors, with a particular focus on uncovering anti-HBV host factors, are essential for obtaining a novel strategy for a functional cure.

The major histocompatibility complex (MHC) class II transactivator (CIITA) is a regulatory transcription factor, whose expression is induced by IFN-γ. It plays a central role in stimulating the immune response against infections by elevating the expression of class II MHC molecules within antigen-presenting cells (APCs). Interestingly, CIITA may be involved in antiviral defense through the intracellular regulation of other host factors required for virus replication. In addition, CIITA induction confers cellular resistance to Ebola virus and severe acute respiratory syndrome (SARS)-like coronaviruses. Moreover, there is evidence of a relationship between HBV and CIITA, and single-nucleotide polymorphisms (SNPs) in CIITA have been shown to be associated with CHB infection.  

Here, we investigated the inhibitory function of CIITA against HBV and explored its mechanism of action. We found that CIITA has an IFN-mediated anti-HBV activity, that inhibited the transcription of HBV by suppressing the main HBV enhancers/promoters. In addition, it reduced the hepatocyte nuclear factor (HNF)4α levels through extracellular signal-regulated kinase (ERK1/2) pathway which further restricts viral transcription and replication in hepatocytes. CIITA overexpression impaired HBV DNA replication in both patient-derived primary human hepatocytes (PHH) and in vivo mouse systems. Intriguingly, HBx interacted with the CIITA protein, resulting in resistance to HBV inhibition by CIITA. Hence, our investigation has unveiled a novel transcription regulator with an anti-HBV function that can be explored further for potential therapeutic strategies to combat HBV infection.

**MATERIALS AND METHODS**

**Cell culture**

The human HCC cell line HepG2 (American Type Culture Collection, ATCC no. HB-8065), Huh7 (Korean Cell Line Bank, KCLB), and HepG2-NTCP cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (PS; Gibco, Grand Island, NY, USA) at 37°C in a 5% CO₂ humidified incubator. HepAD38 was maintained by adding 0.3 μg/mL tetracycline to the same media as HepG2.

**Isolation of PHHs from patient tissues**

PHHs were obtained via therapeutic hepatectomy in donors who tested negative for hepatitis A virus (HAV), HBV,
hepatitis C virus (HCV), or hepatitis D virus (HDV). Informed consent was obtained from both patients (66-year-old male and 89-year-old male) prior to surgery, and this study was approved by the Institutional Review Board of Korea University Hospital (IRB no. ED10287). PHHs were isolated using a two-step collagenase perfusion method, as described previously.\textsuperscript{19} Isolated PHHs were seeded on collagen-coated plates (Corning, Tewksbury, MA, USA) in William’s E medium (Gibco) containing cell maintenance supplements (CM4000; Gibco), 2% FBS, and 1% penicillin/streptomycin.

**Plasmids and siRNA transfection**

HBV 1.2-mer wild type (WT) replicon (HBV 1.2 (+)) and HBx-null 1.2-mer (HBV 1.2 (-)) have been described previously.\textsuperscript{20} The pcDNA3-myc-CIITA (P#808, addgene) plasmid was purchased from Addgene (P#808). The siRNA Negative Control (AccuTarget™ Negative Control siRNA [BioRP, 20nmole] SN-1003) and the siCIITA (Sense: GGAGCUUCUUAACAGCGAU Antisense: AUCGCU-GUUAGAACGCUCC) were obtained from Bioneer (Daejeon, Korea). Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used for plasmid transfection, and Lipofectamine RNAiMax (Thermo Fisher Scientific) was used for siRNA transfection. Transfection experiments were performed according to the manufacturer’s instructions.

**HBV infection**

The HBV inoculum was prepared from the culture supernatant of HepAD38 cells according to a previously described protocol.\textsuperscript{21} HepG2-NTCP or PHH cells were seeded onto 6-well plates coated with collagen I (Gibco) and infected with the HBV inoculum in DMEM supplemented with 4% PEG 8000 (Sigma, Darmstadt, Germany) overnight. Thereafter, the cells were washed three times with PBS, maintained in DMEM containing 2.5% DMSO, and harvested at one-week post-infection.

**Southern and Northern blot analysis**

HBV DNA replication was analyzed using Southern blotting as described previously, with minor modifications.\textsuperscript{22} Three days after transfection, cells were harvested and lysed with HEPES NP-40 lysis buffer. After centrifugation, the supernatant was separated and treated with DNase I at 37°C for 20 min to remove the transected plasmid. The samples were incubated for at least 1 h on ice with a polyethylene glycol solution (PEG 8000; Merck, Darmstadt, Germany) to precipitate the HBV core particles. Proteinase K (Merck) in SDS solution was added to the samples to disrupt the capsid structure. HBV capsid-associated DNA was purified using phenol-chloroform-isoamyl alcohol (25:24:1) (Merck) before precipitation in 100% ethanol and 3 M sodium acetate. Total DNA was separated using electrophoresis on a 1% agarose gel at 90 V for 3 h and transferred to a positively charged nylon membrane (Merck). Hybridization with a digoxigenin (DIG)-labeled DNA probe was performed, and Southern blot signals were detected using a DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany). Signals were detected using ImageQuant 800 (Amersham, Buckinghamshire, UK). RNA was extracted using TRIzol reagent (Sigma) according to the manufacturer’s protocol, and 20 μg of total RNA was separated on a 1.5% formaldehyde-agarose gel in 1X MOPS buffer (Biose-sang, Seongnam, Korea). 28S and 18S rRNAs were used as controls. After electrophoresis, the protocol was performed in the same manner as that for the Southern blot analysis.

**cccDNA extraction**

Hirt DNA was extracted as described previously.\textsuperscript{23} Briefly, the cells were lysed using Hirt lysis buffer (50 mM Tris–HCl [pH 7.5], 10 mM EDTA, 150 mM NaCl, and 1% SDS). After high salt precipitation using 2.5 M KCl, the DNA was subjected to phenol-chloroform extraction followed by isopropanol precipitation. Subsequently, cccDNA was purified by ethanol precipitation and resuspended in Tris-EDTA (TE) buffer. To test the authenticity of cccDNA, a standard procedure involving boiling at 88°C and subsequent EcoRI digestion was applied.\textsuperscript{24} Phosphonoformic acid (PFA) was added to the cells to block cccDNA formation\textsuperscript{25} and then was removed after four days. The prepared cccDNA was quantified using Southern blotting.
Western blotting

To analyze the protein levels, cells were harvested using RIPA buffer containing a protease inhibitor cocktail (Merck) for 30 min on ice. The supernatant was transferred to a new e-tube after centrifugation at 13,000 RPM for 20 min. The samples were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and boiled at 95°C for 5 min, followed by chilling on ice. Then, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h at 80 V and transferred onto a polyvinylidene fluoride (PVDF) membrane using the Trans-blot Turbo RTA PVDF Transfer Kit (Bio-Rad). Membranes were washed with TBS-T (20 mM Tris, 150 mM NaCl, and 0.1% Tween 20). After blocking with 5% skim milk, membranes were shaken overnight at 4°C with a primary antibody solution containing 3% BSA. The membranes were washed three times with TBS-T and incubated with a secondary antibody solution containing 3% skim milk at room temperature for 1 h. Membranes were visualized by ECL (Abclon, Seoul, Korea) using ImageQuant 800 (Amersham). The primary antibodies used in this study are listed in Supplementary Table 1.

Enzyme-linked immunosorbent assay (ELISA)

The cell supernatants were collected to quantify the secretion levels of HBeAg and HBsAg after dilution with PBS. The secreted HBV antigen levels were measured at an optical density (OD) of 450 nm using an ELISA kit (Wantai Pharm Inc., Beijing, China) and a spectrophotometer (SpectraMax Plus 384).

Luciferase reporter assay

Two days after transfection, cells were lysed, and luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA). β-galactosidase activity was determined using a β-galactosidase enzyme assay system (Promega) to confirm the transfection yield.

Co-immunoprecipitation (Co-IP)

Cells were treated with 20 μM MG132 for 5 h before harvesting. Initially, harvested cells were lysed using Pierce™ IP Lysis Buffer (Thermo Fisher Scientific, 87787) containing protease inhibitor cocktail (Merck). Then, 20 μL lysate was transferred to new micro-tube, mixed with Laemmlli sample buffer (Bio-Rad) and retained as the input. For IP, the supernatant was incubated with a primary antibody at 4°C overnight. Protein A-agarose beads (Merck) were added, and the samples were rotated for 4 h. After centrifugation at 3,000 rpm for 1 min, the supernatant was discarded and the beads were washed with PBS three times. The immune complexes were separated using SDS-PAGE, and protein signals were examined using Western blotting.

Quantitative real-time PCR

Total cellular RNA was isolated, and CIITA, STAT-A, HNF4α, HNF1α, HNF3β, C/EBPα, GAPDH, and HBV DNA expression were analyzed using real-time PCR. Reverse transcription was performed using 2 μg of total RNA and a high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed in the QuantStudio™ 5 Real-Time PCR System using SYBR Green PCR Master Mix (Applied Biosystems). The results were expressed as the fold difference relative to the calibrator, determined using the ΔΔCT method. The specific primer sequences used for real-time PCR are listed in Supplementary Table 2.

Hydrodynamic injection in mice

Six-week-old male C57BL/6 mice were hydrodynamically injected with HBV 1.2 (+) and Myc-CIITA plasmids at a volume equivalent to 10% of the mouse body weight. All animal experiments were approved by the Animal Care Committee of Sungkyunkwan University. Four days after injection, the mice were sacrificed, and the liver tissues and serum were isolated for Southern blotting and ELISA, respectively.

Confocal microscopy

To evaluate the co-expression levels of HBV 1.2-mer and FAM-negative control siRNA (Bioneer), confocal microscopy analysis was performed. Huh7 cells grown on cover slides were incubated overnight at 4°C with HBCAg primary antibody (1:300) containing 3% BSA in PBS. Cells were
washed with PBS and incubated with secondary antibody conjugated with Alexa 568 to detect red signal. The fluorescence signals were visualized using a TCS SP8 Hyvolution confocal laser scanning microscope (Leica Microsystems CMS GmbH). The images were analyzed using LAS X software (Leica Microsystems).

**Statistical analysis**

The data were obtained from at least three independent experiments and values represent the mean±SD. Statistical significance was evaluated by one-way ANOVA in GraphPad Prism.

**RESULTS**

**CIITA suppresses HBV replication in HCC cell lines and mediates the anti-HBV activity of IFN-γ**

Initially, through new analysis of previously obtained gene expression profiles, we identified a correlation between HBV infection and CIITA gene expression. The specimens used for this analysis were obtained from HBV-negative normal liver donors (or patients who underwent liver resection for hemangioma) and donors with HBV-associated acute liver failure. While CIITA levels remained unchanged in the normal liver or non-HBV-related hemangioma patients, we observed a significant increase (between 5- to 15-fold increase) in the gene expression of CIITA in eight patients with acute liver failure caused by HBV (Fig. 1A). According to previous reports, CIITA expression is induced by IFN-γ, and IFN-γ, along with TNF-α, is associated with HBV viral clearance. Therefore, to determine whether CIITA expression is actually induced by IFN-γ in hepatocytes, HepG2, Huh7, and PHH cells were treated with different cytokines; CIITA induction levels were measured. Semi-quantitative RT-PCR results showed that CIITA expression was induced by IFN-γ in the two HCC cell lines as well as PHH (Fig. 1B-right). Interleukin-32γ (IL32) gene expression levels were measured as the positive control. In the tissues isolated from a PHH donor and Huh7 cells, CIITA mRNA levels were substantially increased, as demonstrated by real-time PCR (Fig. 1B-left). To explore the anti-HBV activity of CIITA, Huh7 cells were co-transfected with the HBV 1.2 (+) replicon and the myc-CIITA plasmid. The experimental scheme is shown in Figure 1C. As shown in Figure 1D, CIITA reduced HBV replication in a dose-dependent manner as determined by Southern blotting. Similarly, the ELISA results showed that the levels of HBeAg and HBsAg secreted in the cell supernatant significantly decreased (Fig. 1E).

After evaluating the function of the siRNA by measuring CIITA RNA levels (Fig. 1F), we silenced the expression of CIITA using 20 nM of siCIITA (Fig. 1F) and treated the Huh7 cells with IFN-γ, as shown in the scheme in (Fig. 1G). The co-transfection efficiency was examined by confocal microscopy (Supplementary Fig. 1). FN-γ-mediated reduction of HBV replication was reversed by CIITA depletion (Fig. 1H). Moreover, HBeAg and HBsAg levels in the cell supernatant were similarly restored (Fig. 1I). Collectively, these findings suggest that CIITA inhibits HBV replication and partially mediates IFN-γ-induced anti-HBV activity in HCC cells.

**CIITA exhibits antiviral activity in the HBV infection system**

Next, we validated the effects of CIITA in a biologically relevant HBV infection system by isolating PHHs from two liver tissue donors (Fig. 2A). In both donor samples, CIITA diminished HBV DNA replication levels in a dose-dependent manner, consistent with the decline in HBsAg and HBeAg levels (Fig. 2B–E). A similar experiment was conducted using HepG2-NTCP cells, which are a well-established infection model, to explore the anti-HBV effects of CIITA. In line with the results in PHHs, the intracellular capsid-associated HBV DNA and secreted antigen levels were reduced in a concentration-dependent manner (Fig. 2F–I). Collectively, these findings indicate that CIITA displays antiviral properties against HBV in an actual infection system.

**CIITA impairs HBV at the transcriptional level but does not exert regulatory control over cccDNA levels**

Considering that the conventional function of CIITA is as a major regulator of MHC class II transcription, we wondered whether CIITA inhibited the transcriptional activity of
HBV. The experimental flowchart of the mechanistic study is summarized in Supplementary Figure 2. Following the ectopic expression or knockdown of CIITA, HBV transcripts were examined using Northern blotting. CIITA overexpression decreased pgRNA, preS1/S mRNA, and X mRNA levels by more than 50% (Fig. 3A, B). In addition, after silencing CIITA, IFN-γ failed to efficiently reduce HBV RNA levels (Fig. 3C, D), suggesting that CIITA partially mediates the anti-HBV activity of IFN-γ. These results imply that CIITA induces HBV resistance by affecting viral gene expression.
and is involved in IFN-γ-mediated anti-HBV activity.

Since HBV RNA levels were significantly decreased in cells transfected with CIITA, we sought to determine whether the impact of CIITA extended to cccDNA alterations. After investigating the kinetics of cccDNA in HepAD38 cells (Fig. 3E–G), a suitable strategy for the transfection of the CIITA plasmid was employed (Fig. 3H and I, upper panel). The robust formation of cccDNA was detected at 7 d and reached a plateau at 9 d after Tet-off (Fig. 3F, G). Therefore, we harvested the cells 7 and 10 days after Tet-off to determine the impact of CIITA on cccDNA formation and destabilization. Furthermore, PFA was used to arrest HBV DNA synthesis, which allows for the time-dependent formation of cccDNA. Intriguingly, CIITA had no noticeable effect on cccDNA formation or destabilization (Fig. 3H and I bottom). We confirmed these results in an HBV infection model using HepG2-NTCP cells (Fig. 3J, K). Nonetheless, the levels of HBV cccDNA remained constant despite transient transfection with increasing doses of CIITA plasmid. These observations imply that CIITA may regulate HBV transcription rather than directly affect cccDNA abundance.

**CIITA suppresses activity of HBV enhancers and promoters**

HBV transcription is regulated by enhancers I (Enh I) and II (Enh II), the latter of which overlaps with the core promoter (Cp). Since CIITA exhibited pronounced inhibition of HBV transcription without affecting cccDNA, we examined the possible impact of CIITA on the HBV enhancer and promoter regions using a luciferase reporter assay (Fig. 4A). The results demonstrated that the concentration-dependent overexpression of CIITA caused a notable reduction in the activity of each enhancer (Fig. 4B). We reasoned that the classical function of CIITA is to regulate transcription by binding to the promoter of MHC class II genes; therefore, we determined whether CIITA directly affects specific regions of the HBV enhancer and promoter via deletion mutants. A downward trend was detected in the activity of all enhancer sites in both HepG2 and Huh7 cells transfected with the CIITA plasmid (Fig. 4C), highlighting the ability of CIITA to simultaneously suppress HBV Enh I and Enh II/Cp.
Figure 2. HBV replication is restricted by CIITA in an HBV infection system. (A) PHH cells were isolated from two patient donors and seeded in 6-well plates. HBV infection was induced with 4% PEG and 2% DMSO. The plasmid expressing Myc-CIITA and the mock vector plasmid were transfected at 2 dpi and 5 dpi, respectively. Cells and the supernatant were harvested at 7 dpi. (B, D) HBV replication was determined using Southern blot analysis. CIITA protein levels were measured using Western blot analysis of the same sample. (C, E) HBeAg and HBsAg levels in the supernatant were measured using ELISA. (F) HepG2-NTCP cells were seeded in 6-well plates and were infected using the same protocol. Myc-CIITA was transfected into the cells at 4 dpi and cells were harvested three days later. (G) HBV intracellular-capsid-associated levels were determined using Southern blotting and real-time PCR. (H) HBV-secreted antigen levels in the cell supernatants were quantified. dpi: days post-infection. HBV, hepatitis B virus; CIITA, class II transactivator; PHH, primary human hepatocytes; ELISA, enzyme-linked immunosorbent assay; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen. *P<0.05, **P<0.01.
Figure 3. CIITA suppresses HBV transcription but does not regulate HBV cccDNA levels. (A and C) Huh7 cells were co-transfected with HBV 1.2 (+), myc-CIITA, and the mock vector control (pcDNA3.1) plasmid using lipofectamine RNAiMAX reagent. (C) Cells were treated with IFN-γ for 8 h before harvesting. HBV RNA was extracted and analyzed using northern blotting. The signals were normalized to the quantity of 18S and 28S ribosomal RNA. (B and D) Relative HBV RNA levels were quantified and plotted in a graph. Data were obtained from three independent experiments (mean±SD). The statistical significance of the differences was assessed using the Student's t-test: *P<0.05; **P<0.01; ***P<0.001. (E) HepAD38 cells were seeded into a 6-well plate. Tetracycline (Tet) was removed from the media at the indicated time points. (F) HBV DNA was obtained using hirt DNA extraction. The samples shown in the left panel were electrophoresed immediately. (G) Samples were boiled at 88°C for 5 min and were incubated with EcoRI restriction enzymes at 37°C for 30 min prior to electrophoresis. (H and I) The upper panel presents the experimental scheme. (Bottom panel) HepAD38 cells were treated with 2 mM PFA between day 2 to day 6 following Tet removal. Cells were transfected with CIITA plasmid on the indicated days. Lysates from HepAD38 cells were harvested for hirt DNA extraction and Southern blotting. (J) One day after seeding, HepG2-NTCP cells were infected with HBV inoculum and were washed the next day. Plasmid transfection was performed 72 h before harvesting. (K) Samples were prepared for Southern blotting analysis of cccDNA levels. mtDNA served as the loading control. mtDNA: mitochondrial DNA; HBV, hepatitis B virus; CIITA, class II transactivator; PFA, phosphonoformic acid; cccDNA, covalently closed circular DNA.
CIITA reduces the expression of major hepatocyte nuclear factors through the ERK1/2 pathway

CIITA exhibited a broad inhibitory effect on the activity of most HBV enhancers (Fig. 4). Transcriptional regulation of HBV involves the engagement of various transcriptional regulatory factors within hepatocytes. We hypothesized that, as a master regulator of transcription, CIITA might exert control over the expression of essential liver-enriched transcription factors involved in the transcription of both Enh I and Enh II of HBV, including C/EBPα, HNF1α, HNF3β, and HNF4α (Fig. 5A). Our findings revealed a reduction in both the protein (Fig. 5B) and mRNA levels (Fig. 5C and D) of HNF1α and HNF4α in response to CIITA. However, the levels of HNF3β and C/EBPα did not significantly differ (Fig. 5B–D). These results indicated that CIITA exerts its anti-HBV effects by downregulating transcription factors that are essential components of the virus life cycle.

HNFs are regulated by diverse regulatory proteins and signaling pathways, including PGC1a, the FOXO family, and mitogen-activated protein kinase (MAPK). Consequently, to explore the mechanism by which CIITA downregulates the expression of HNF4α and HNF1α, we examined the activation of different transcription factors known to regulate HNF4α expression. Western blotting results showed that ectopic CIITA protein expression had no significant impact on PGC1α, FOXO1, FOXO3α, and FOXO4 protein levels in Huh7 cells (Fig. 5E). Therefore, we investigated the activity of MAPK signaling pathway components using a similar experiment. CIITA activated ERK1/2 by significantly increasing its phosphorylation. However, increasing dose of CIITA did not affect the phosphorylation levels of p38 and Jun N-terminal kinase (JNK) phosphorylation levels (Fig. 5F). To further determine whether the main mechanism through which CIITA suppresses HBV is by activating the ERK pathway, we treated cells with U0126, a highly selective inhibitor of the ERK pathway. As expected, phosphorylation of ERK1/2 (p-ERK1/2) was attenuated in the cells treated with U0126 (Fig. 5G, bottom). Interestingly, the antiviral effects of CIITA were largely nullified in the presence of U0126, whereas ERK1/2 levels remained unchanged (Fig. 5G and H). The reduction of HNF1α and HNF4α levels by CIITA was also restored following U0126 treatment. These observations indicate that the mechanism underlying CIITA-mediated downregulation of HBV transcription involves the induction of the ERK1/2 pathway, which eventually leads to a remarkable reduction in the levels of HNF1α and HNF4α.

**HBx protein counteracts the antiviral function of CIITA**

To further validate the effect of CIITA on HBV replication in various HCC cell lines, we conducted parallel experiments using HepG2 cells. Although the inhibitory effect of
**Figure 4.** CIITA inhibits HBV enhancer and promoter activity. (A) Schematic diagram of the WT and mutant HBV reporter plasmids used in this study. (B and C) HepG2 and Huh7 cells were seeded in a 12-well plate for 24 h, and cells were co-transfected with a set amount of the indicated enhancer reporter, control vector (pcDNA3.1), and β-galactosidase and increasing amounts of myc-CIITA. In the experiment shown in panel C, 0.5 µg of Myc-CIITA plasmid was used. The relative luciferase activity of each enhancer was determined at 48 h post-co-transfection and normalized to the expression levels of β-galactosidase. Bars represent the standard error of triplicate experiments (mean±SD). One-way ANOVA with Tukey’s multiple comparisons; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. HBV, hepatitis B virus; CIITA, class II transactivator; WT, wild type.
CIITA on HBV enhancers was consistent in HepG2 and Huh7 cells, the effect of CIITA on HBV replication was less pronounced in HepG2 cells (Fig. 6A). HBx protein expression was lower in Huh7 cells than in HepG2 cells. Although this may be predominantly dependent on cell line characteristics, the fact that HBx has been identified as a pivotal orchestrator of immune evasion mechanisms led us to wonder whether HBx counteracts CIITA antiviral activity. We found that the HBV DNA, RNA, and secreted protein levels decreased less drastically in HepG2 cells following

Figure 5. CIITA reduces the expression of major hepatocyte nuclear factors through the ERK1/2 pathway. (A) Representation of the HBV enhancers and their specific binding regions for key transcription factors. (B) Huh7 cells were co-transfected with HBV 1.2 (+), myc-CIITA, and the mock plasmid (pcDNA3.1). The protein expressions of transcription factors were determined using western blot analysis, and related protein levels were quantified by the intensity of each band and plotted. (C and D) The mRNA levels of transcription factors were determined using (C) semi-quantitative RT-PCR and (D) real-time PCR. (E–H) Huh7 cells were co-transfected with HBV 1.2 (+), myc-CIITA, and the mock vector (pcDNA3.1). (E and F) Cell lysis was performed using a lysis buffer with phosphatase inhibitors, and the protein levels of MAPK signaling regulatory factors were determined using Western blot analysis. (G) HBV 1.2 (+) and myc-CIITA were co-transfected into the cells at 1 μg. After 24 h, 10 μM U0126 was added, and, after 2 days, the cells were harvested. HBV replication intermediates were measured using Southern blot analysis and the intensity of each band was quantified, as shown in the graph. (H) HBeAg and HBsAg levels were measured using ELISA. Bars represent the standard error of triplicate values (mean±SD). (A–D) Two-way ANOVA with Tukey’s multiple comparisons; (E–H) One-way ANOVA with Tukey’s multiple comparisons; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. CIITA, class II transactivator; ERK1/2, extracellular signal-regulated kinase; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; ELISA, enzyme-linked immunosorbent assay.
CIITA treatment. Moreover, to achieve a significant reduction in HBV transcription and replication, HepG2 cells required more than double the amount of CIITA protein than Huh7 cells (1 vs. 2 μg in Huh7 and HepG2 cells, respectively) (Fig. 6A). To further test the effect of HBx on the activity of CIITA, HepG2 cells were co-transfected with wild-type HBV 1.2 (+) or HBx minus HBV 1.2 (-) plasmids, along with increasing doses of the CIITA construct. Although there was a slight reduction in the levels of HBV DNA, RNA, and secreted antigens in the presence of the HBx-expressing 1.2 (+) plasmid, cells transfected with the HBx-null 1.2 (-) replicon exhibited a more rapid reduction in the levels of HBV components (Fig. 6B–D). A similar experiment was performed using Huh7 cells, which revealed no discernible differences in the cells between the presence or absence of HBx (Fig. 6E).

The influence of CIITA on HBV promoter activity was also mitigated by the HBx protein (Fig. 6F). To confirm that this counteraction was caused by HBx specifically, co-transfection of HBx-HA and Myc-CIITA plasmids was performed. Unsurprisingly, HBV replication levels were dramatically increased following the ectopic expression of HBx (Fig. 6G). Notably, HBx overexpression resulted in disappearance of the antiviral effect of CIITA, as HBV core-associated DNA levels remained the same in the presence of HBx and CIITA (Fig. 6G). Similar results were observed for HBeAg and HBsAg levels, as demonstrated by ELISA (Fig. 6H). Collectively, these findings implied that HBx interfered with the antiviral activity of CIITA.

**HBx protein interacts with CIITA**

The above data revealed an unexpected increase in CIITA protein levels in the presence of HBx (Fig. 6G). Therefore, we investigated the possible mutual effects of these two proteins by co-transfecting HBx-HA and myc-CIITA
plasmids in HepG2 cells. The presence of HBx led to a dose-dependent increase in CIITA protein levels without affecting its mRNA expression (Fig. 7A). However, the HBx protein levels remained unchanged in cells transfected with CIITA (Fig. 7B). To elucidate the interplay between an increase in CIITA protein levels and a reduction in its antiviral effect by HBx, we hypothesized that there might be a direct interaction between the two proteins, which eventually leads to stabilization or deactivation of the CIITA protein.

Accordingly, Co-IP was performed on HepG2 cells ex-
pressing myc-CIITA and HBx-HA plasmids, and protein-protein binding was confirmed using western blotting (Fig. 7C). To determine the critical region of HBx for binding to CIITA, deletion mutants of HBx were used (Fig. 7D). Remarkably, CIITA interacted with WT and mutant 1 (M1) HBx, which corresponded to the observed increase in CIITA protein levels (Fig. 7E, upper panel). Moreover, the CIITA signal was only detectable following IP against WT and M1 HBx (Fig. 7E, bottom panel). To further validate the effect of HBx mutants on HBV replication, plasmids containing HBx WT or mutant variants were introduced into cells along with the HBx-null HBV replicon (HBV 1.2 (-)). Subsequently, we assessed the anti-HBV activity of CIITA using Southern blot analysis. As depicted in Figure 7F, in the presence of WT and M1 HBx, CIITA was incapable of preventing HBV replication. Conversely, M2 and M3 HBx did not interfere with the CIITA function. This indicates that the amino acid sequence 51–154 of HBx bind to CIITA and plays a critical role in inhibiting the anti-HBV activity of CIITA.

CIITA exhibits anti-HBV activity in an in vivo mouse model

Finally, we examined the in vivo antiviral effect of CIITA using an HBV mouse hydrodynamic injection (HDI) model. Mice were injected with the full HBV genome, to establish a transient HBV transgenic infection state, along with the CIITA plasmid construct. The control group was injected with the HBV construct and the Myc plasmid. Four days after injection, serum samples were collected to measure HBeAg and HBsAg levels and liver tissues were homogenized to quantify HBV DNA levels (Fig. 8A). As depicted in Figure 8B–D, CIITA exerted substantial viral suppression in mice, as indicated by the significantly reduced HBV antigen (Fig. 8B and C) and DNA levels in mouse liver tissues (Fig. 8D, upper panel) as well as serum (Fig. 8E). Furthermore, HNF4α expression levels in liver tissues were determined using western blot analysis (Fig. 8D, bottom panel). Notably, in the mice administered the CIITA plasmid, a significant decrease in HNF4α levels was observed. In summary, these findings demonstrated that CIITA exerts an anti-HBV
Figure 7. HBx interacts with CIITA. (A and B) HepG2 cells were transfected with the Myc-CIITA and HBx-HA plasmids. Cells and supernatants were harvested after 72 h and the mRNA and protein levels of CIITA and HBx were determined using semi-quantitative RT-PCR (left panel) and western blot analysis (right panel). (C) HepG2 cells were transfected with the mock vector or HBx-HA- and myc-CIITA-encoded plasmids. At 48 h post-transfection, cell lysates were immunoprecipitated with anti-HA antibody or anti-myc antibody. CIITA and HBx proteins purified on protein A-agarose beads were measured by immunoblotting. A total of 10% of the lysate was loaded in parallel as the input. (D) (Left panel) The amino acid sequences of the HBx and HBx truncated mutants used in this study. (Right panel) The expression levels of the HBx mutants were examined using western blot. The protein levels of HBx WT and mutant constructs were visualized using an anti-HA tag antibody. β-actin was loaded as the control for each lane. (E) HBx-truncated mutants were transfected into the cells, and cell lysates were subjected to Co-IP. Protein levels were determined by SDS-PAGE following immunoblotting with specific antibodies. (F) The plasmids expressing HBx WT or HBx deletion mutants (HBx M1, M2, and M3) and CIITA were co-transfected with the HBV 1.2(-) construct as demonstrated. Southern blotting was conducted to test the intracellular capsid-associated HBV DNA levels. The expression levels of CIITA and HBx protein were visualized using anti-CIITA and anti-HA antibodies, respectively. CIITA, class II transactivator; WT, wild type.
Figure 8. Hydrodynamic injection of CIITA decreases HBV replication and antigen production in mice. (A) HBV 1.2 (+) and the CIITA plasmid were hydrodynamically injected into the tail vein of mice. After 4 days, the blood and liver tissues of mice were collected. (B and C) The titer of HBsAg and HBeAg in serum was measured using ELISA. (D) HBV DNA was isolated from the mouse liver tissues and serum. Viral gene expression and replication were examined using Southern blot and (E) quantitative real-time PCR, respectively. (D, bottom) A portion of each tissue lysate (10%) was used for protein analysis by Western blot. (F) A hypothetical mechanism of CIITA-mediated suppression of HBV through the downregulation of HNF1α and HNF4α expression. Effects of IFN-γ-induced CIITA expression on HBV replication and transcription. CIITA decreases the expression of HNF4α and HNF1α through activation of the ERK1/2 pathway, thereby inhibiting the transcription of HBV. However, HBx binds to CIITA and counteracts its antiviral activity. This illustration was created with BioRender.com. CIITA, class II transactivator; HBV, hepatitis B virus; ERK1/2, extracellular signal-regulated kinase.
effect in vivo.

**DISCUSSION**

Cytokines play an important regulatory role in HBV transcription and replication; however, the precise mechanisms underlying this regulation remain unclear. Therefore, identifying HBV-associated host factors that are influenced by cytokines can yield valuable insights into antiviral immunity as well as novel therapeutic agents. Notably, IFN-γ-activated CIITA, known for its role in MHC class II antigen presentation, has been suggested to have roles beyond its canonical function. Remarkably, a report revealed that activated CIITA, given that the 51–154 amino acid sequence of HBx, known for its regulatory influence on various host proteins, undergoes regulation through a variety of mechanisms, involving both transcriptional and post-transcriptional processes. Additionally, there is evidence of crosstalk and regulatory interactions between the ERK1/2 pathway and HNF4α/HNF1α. ERK1/2 activation can lead to post-translational modifications of these transcription factors, influencing their activity and DNA-binding properties. For example, the phosphorylation of HNF4 by ERK1/2 inhibits HNF4 transcriptional activity. MAPKs are able to downregulate the expression of HNF4α, which leads to less binding between HNF4α and the HBV promoter. Additionally, certain cytokines that activate ERK1/2 can affect the expression of HNF4α and HNF1α, potentially altering their roles in gene regulation. In this study, we identified CIITA as a novel host protein that inhibited HBV transcription by modulating ERK signaling. The data presented in this study demonstrate that CIITA, induced by IFN-γ, decreases the expression of HNF4α and HNF1α by activating the ERK1/2 pathway. Subsequently, this regulation by CIITA reduced enhancer activity, which eventually attenuated HBV transcription (Fig. 8E). The transcriptional regulatory effect of CIITA in other viruses has also been identified. For instance, CIITA blocks the replication of human T-cell leukemia virus type 2 (HTLV-2) by interfering with the function of Tax-2, a major transactivator required for enhancing virus transcription.

Several studies have identified the well-developed immune evasion strategies of HBV. In particular, HBx plays a pivotal role in evading host immunity and defense mechanisms. In this study, we propose a novel mechanism of HBV immune evasion. Our findings indicate that HBx does not inhibit CIITA expression levels but instead affects CIITA function through direct protein-protein binding (Figs. 6, 7). The interaction between HBx and CIITA increases the protein stability of CIITA but results in its dysfunction. Nevertheless, the mechanism by which HBx inhibits CIITA activity remains elusive and we suggest two possible mechanisms. First, HBx may facilitate the translocation of CIITA, given that the 51–154 amino acid sequence of HBx, which is known to interact with CIITA (Fig. 7), contains a nuclear translocation domain. Secondly, HBx may induce post-transcriptional modifications (PTMs) in CIITA, as it is known for its regulatory influence on various host proteins. Moreover, previous reports have indicated that CIITA is subject to PTMs; specifically, Lys63 ubiquitinated CIITA is concentrated in the cytoplasm. Further research may reveal the exact role of HBx in controlling the anti-HBV activi-
ty of CIITA.

As mentioned previously, the classical function of CIITA is to enhance the expression of MHC class II molecules on the surface of antigen-presenting cells (Fig. 8F). Recent studies have demonstrated that CIITA increases the expression of MHC class II molecules in hepatocytes, which act as antigen-presenting cells. Given that HBV-specific CD8+ cytotoxic T cells release IFN-γ, CIITA plays a crucial role in mediating the immune response against HBV by facilitating antigen presentation, in addition to its intracellular anti-HBV action identified here. Similarly, by targeting the transcription of CIITA, Human cytomegalovirus (HCMV) reduces the expression of MHC class II genes, thereby promoting HCMV infection in mature Langerhans cells. Taken together, these findings revealed a novel role of CIITA in regulating HBV transcription and present an immune evasion strategy against HBV (Fig. 8F). The discovery of CIITA as a suppressor of HBV replication opens up new possibilities for the development of therapeutic interventions against HBV infection. Further investigation is needed to elucidate the precise mechanisms of the interplay between CIITA and HBV and to explore the therapeutic potential of targeting CIITA in HBV infection.

Authors’ contribution

KH Kim supervised the study and was involved in study concept and data interpretation. M Dezhbord and SH Kim contributed to the study design and conduct, data acquisition and data interpretation. AR Lee and SR Park was involved in data interpretation. DR Lee, NY Kim and JH Won assisted with data acquisition. DS Kim contributed to providing study materials. M Dezhbord wrote and revised the paper. M Dezhbord and KH Kim were involved in critical revision and editing of the manuscript. All authors have seen and approved the final version of the manuscript.

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

CORRECTION

This article was corrected on July 16, 2024 due to a change in the order of the Figures.

REFERENCES

Mehrangiz Dezhbord, et al.
Novel role of CIITA on HBV replication

Bariatric intervention improves metabolic dysfunction-associated steatohepatitis in patients with obesity: A systematic review and meta-analysis

Juchul Hwang¹, Hyeyoung Hwang¹, Hyunjae Shin¹, Bo Hyun Kim¹, Seong Hee Kang², Jeong-Ju Yoo³, Mi Young Choi⁴, Dong eun Lee⁵, Dae Won Jun⁶, and Yuri Cho⁷

¹Center for Liver and Pancreatobiliary Cancer, National Cancer Center, Goyang; ²Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju; ³Department of Gastroenterology and Hepatology, Soonchunhyang University Bucheon Hospital, Bucheon; ⁴National Evidence-based Healthcare Collaborating Agency, Seoul; ⁵Biostatistics Collaboration Team, Research Institute, National Cancer Center, Goyang; ⁶Department of Internal Medicine, Hanyang University School of Medicine, Seoul, Korea

Graphical Abstract

Bariatric Intervention for Obese patients with MASH

NAS (NAFLD activity score)

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</tbody>
</table>

MRI-PDFF (after 6 month)

<table>
<thead>
<tr>
<th>Study</th>
<th>Risk of Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fujii (2014)</td>
<td>0.46</td>
</tr>
<tr>
<td>Heidtmeir (2017)</td>
<td>0.31</td>
</tr>
<tr>
<td>Liu (2018)</td>
<td>0.26</td>
</tr>
<tr>
<td>Mendilipat (2020)</td>
<td>0.32</td>
</tr>
<tr>
<td>Polder (2019)</td>
<td>0.27</td>
</tr>
<tr>
<td>Tan (2023)</td>
<td>0.33</td>
</tr>
<tr>
<td>Heterogeneity: $I^2 = 0%$, $Q = 0.417$</td>
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</tr>
</tbody>
</table>

Study Highlights

- This study assessed the changes in MRI-determined hepatic proton density fat fraction (MRI-PDFF) and NAS after bariatric intervention in patients with obesity and MASLD. We found that bariatric intervention significantly reduces BMI, intrahepatic fat, as well as NAS. Overall, our study reported that bariatric intervention could significantly improve MRI-PDFF and the histologic features of MASH in patients with obesity, and hence, could be a possible alternative treatment option for patients with MASLD who otherwise are resistant to lifestyle modification and/or medical treatment.
INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD), previously known as nonalcoholic fatty liver disease (NAFLD), includes a spectrum of liver conditions, characterized by excessive fat in the liver, without proven secondary cause of hepatic fat accumulation. Although the pathophysiology of MASLD is not completely understood due to its complex and multifactorial nature, insulin resistance is considered to be a key factor driving the development of the disease. Therefore, MASLD is thought to be a component of metabolic syndrome, which is a cluster of interconnected metabolic risk factors that increase the risk of developing type 2 diabetes mellitus (DM), hypertension, and cardiovascular disease. Besides, it can gradually progress through various liver stages, from simple steato-
sis to metabolic dysfunction-associated steatohepatitis (MASH), previously known as nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and eventually liver failure or hepatocellular carcinoma over several years or even decades. The prevalence of MASLD has risen alongside the increasing prevalence of obesity and metabolic syndrome worldwide, with significantly higher prevalence in specific population, such as approximately 50–90% in obese individuals and 47–63% in patients with type 2 DM. Considering its high prevalence and the implications in public health, addressing MASLD is of paramount importance.

The primary goal in addressing MASLD begins with lifestyle management aimed at achieving a weight loss, the only validated approach for the condition. Weight loss of more than 7–10% has been demonstrated to effectively improve liver steatosis, inflammation, and fibrosis. In addition to lifestyle modification, medication, such as GLP-1 receptor agonist or GLP-1/GIP dual receptor agonist (a novel medication yielding the most potent result in weight loss), is known to improve the histological findings in MASLD. However, apart from the cases of potential treatment failure, sustaining the reduced weight can indeed pose challenges, even with the assistance of new medications, particularly among certain populations. In such populations, surgical intervention may play a pivotal role in treating MASLD. Bariatric intervention is among the most effective weight loss interventions for morbidly obese individuals, even over long-term; it improves metabolic parameters as well as liver steatosis and inflammation. As of now, its indication includes those with BMI >35 kg/m², regardless of comorbidities, or those with BMI >30 kg/m² with the presence of MASH, type 2 DM or failure of non-surgical intervention.

Several meta-analyses have showed significant metabolic and histological improvements after bariatric surgery. However, concerns regarding the controversial results regarding the effectiveness in more advanced forms of MASLD, such as MASH, persist. Moreover, despite liver biopsy being the gold standard for diagnosis of MASLD, it is invasive and potentially life-threatening procedure, prompting the exploration of non-invasive evaluation methods for MASLD, such as magnetic resonance imaging proton-density fat fraction (MRI-PDFF). MRI-PDFF has garnered significant recognition for its diagnostic value, demonstrating high sensitivity and specificity in the assessment of MASLD. Although several studies investigating the association between bariatric intervention and MASLD, assessed by MRI-PDFF, have been published till date, no meta-analysis using this method has been conducted yet.

In this study, we conducted a systemic review to evaluate the changes in BMI, NAS, as well as intrahepatic fat composition measured by MRI-PDFF, following bariatric intervention in patients with MASLD.

**MATERIALS AND METHODS**

**Data sources and search strategy**

The protocol for this systemic review was registered with PROSPERO (International Prospective Register of Systemic Reviews, CRD42041241243). The aim of this study is to identify the effect of bariatric intervention on MASLD, determined by histologic findings and/or MRI findings. This study was conducted along with others that identified the effect of exercise intervention on MASLD, or validated the accuracy of noninvasive scoring system in assessing liver fibrosis. Study selection followed the Preferred Reporting Items for Systemic Review and Meta-analyses extension for Diagnostic Test Accuracy (PRISMA-DTA) statements. We searched the Ovid-MEDLINE, EMBASE, KMBASE, Korean Studies Information Service System (KISS), and Cochrane library, covering the period from database inception, through January 1, 1997 to October 31, 2023. We only included papers published in English. The search keywords used were: metabolic dysfunction-associated steatotic liver disease, MASLD, metabolic dysfunction-associated steatohepatitis, MASH, nonalcoholic fatty liver disease, nonalcoholic fatty liver, NAFLD, nonalcoholic steatohepatitis, NASH, obesity/obese, bariatric surgery, intragastric balloon, and gastric banding.

**Study selection and data extraction**

Two researchers (Y.C. and S.H.K.) reviewed the screened papers independently, by their titles and abstracts, in the first screening. In the secondary screening, full text of the papers that passed the first screening was examined, providing specific reasons for any exclusions. During the pro-
cess of literature selection, any discordance between the two clinical researchers was resolved through mutual consultation. In cases where a consensus could not be reached, the final decision was made by the research committee through a formal meeting. The study design, outcome measure (histology vs. MRI-PDFF), sample size, intervention, mean age, duration of follow-up, and BMI changes in each study were extracted as basal characteristics. The entire search process was administered by a professional statistician (M.Y.C. and D.L.).

**Inclusion and exclusion criteria**

The following criteria were required for studies to be selected: (1) patients who underwent bariatric surgery or intragastric balloon/gastric banding for obesity; and (2) those who were diagnosed with histologically proven MASLD or MASH designs included randomized controlled trials, cross-sectional studies, and cohort studies, both prospective and retrospective. Studies were excluded based on the following criteria: (1) case reports; (2) case series, in which less than five patients in total were involved; (3) reviews; (4) cell or animal studies; (5) studies on chronic viral hepatitis, such as hepatitis B or hepatitis C; (6) studies on human immunodeficiency virus; (7) studies on population with significant alcohol consumption; (8) studies with no histology result provided; or (9) pediatric studies.

**Outcome assessed**

Studies that reported at least one histologic or MRI variable were included in the analyses. All included studies presented baseline and follow-up BMI. Histologic variables are as follows: (1) NALFD activity score (NAS), which assesses the severity of inflammation and hepatocellular injury in liver biopsy;\(^\text{19}\) (2) Histologic features of MASH, including steatosis, lobular inflammation, ballooning degeneration, and fibrosis, and (3) worsening MASH. MRI variable includes the change in steatosis and BMI.

**Quality assessment**

We performed the quality assessment of the final selection of papers, based on key questions, using RoBANS tool for non-randomized controlled studies and Cochrane risk-of-bias tool for randomized controlled study. QUADAS-2 evaluation tool was used to evaluate the diagnostic accuracy. The following factors were checked: comparability of participants, selection of participants, confounding variables, measurement of exposure, blinding of outcome assessment, outcome evaluation, incomplete outcome data, and selective reporting. Publication bias was evaluated by a funnel plot for studies investigating populations with more than ten individuals. The certainty of the evidence was evaluated by Grading of Recommendations Assessment, Development, and Evaluation (GRADE) approach, which considers factors such as risk of bias, inconsistency of results, indirectness of evidence, imprecision, and publication bias.

**Statistical analysis**

The process of meta-analysis with paired ratio of means data and proportion data involved the estimation of Hedge’s corrected standardized mean difference, under the assumption of a random-effects model, to account for heterogeneity across the studies. The pooled effect estimates between pre- and post-operation within each study’s specific time frame were derived to measure the effect size, using Freeman-Tukey variant of the arcsine square root transformation, along with a 95% confidence interval (CI). To address the differences in follow-up periods across the selected studies, we categorized the studies according to the length of the follow-up time in further analyses. Analysis of each pooled data is shown in a forest plot. Heterogeneity of the studies was assessed by Cochrane’s Q test; \(I^2\) statistics higher than 50% was considered indicative of significant heterogeneity, and more than 75% was considered “high” heterogeneity. Next, sensitivity analyses and meta-regression were conducted to assess the influence of other factors on diagnostic accuracy across the studies with significant heterogeneity. Cochrane Review Manager version 5.4 (London, UK), R Foundation for Statistical Computing version 4.1.2 (Vienna, Austria), and GRADEpro GDT were used in the analysis, with a significance level set at a \(P\)-value less than 0.05.
RESULTS

Study characteristics

From January 1, 1997 to October 31, 2023, a total of 1,546 papers were identified through preliminary data searching using search keywords described in the Methods section. The numbers of papers from each database are as follows: Ovid-MEDLINE (n=593), KMBASE (n=5), EMBASE (n=845), KISS (n=11), and Cochrane library (n=92). After removal of duplicates (n=1,069), 246 papers were excluded based on the title only. In the first screening, two researchers independently reviewed the screened papers by their titles and abstracts (n=231), followed by exclusion of 156 papers after review. Furthermore, we thoroughly examined the full-text articles of the remaining studies (n=75), resulting in an additional exclusion of 46 studies. Finally, thirty studies were incorporated into this analysis (Fig. 1). Out of all these papers, twenty-four were evaluated through histological examination while the remaining six were assessed using MRI. General characteristics of the selected studies are presented in Table 1. A total of 3,134 patients from the studies were analyzed. It was noteworthy that only two were randomized controlled studies, whereas others employed a cross-sectional design. To address the difference in follow-up duration, we categorized the data into three time periods, as follows: 3–6 months, 12–24 months, and 36–60 months, for further analyses.

Impact of bariatric intervention on BMI and histology in MASH

A total of thirty studies showed a significant reduction in BMI except for one randomized controlled trial with small sample size. BMI was reduced by 19% at a rate of 0.81 (95% CI, 0.71–0.92) at 3–6 months, by 28% at a rate of 0.72 (95% CI, 0.68–0.76) at 12–24 months, and by 27% at a rate of 0.73 (95% CI, 0.68–0.79) at 36–60 months after...
Table 1. General characteristics of 30 studies

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Study design</th>
<th>Method</th>
<th>No. of samples</th>
<th>Intervention</th>
<th>Mean age (years)</th>
<th>Follow-up duration</th>
<th>Pre-BMI</th>
<th>Post-BMI</th>
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<tbody>
<tr>
<td>Aldoheyan et al. (2017)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>27</td>
<td>Bariatric surgery</td>
<td>35±8</td>
<td>3 months</td>
<td>44.6±7.8</td>
<td>34.2±6.3</td>
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<tr>
<td>Barker et al. (2006)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>19</td>
<td>Roux-en-Y gastric bypass (RYGBP)</td>
<td>48.6 (35–58)</td>
<td>21.4 months (13.3–31.7)</td>
<td>46.8±4.4</td>
<td>28.8±5.2</td>
</tr>
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<td>Caiazzo et al. (2014a)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>681</td>
<td>RYGBP</td>
<td>41±11.1</td>
<td>1 year</td>
<td>49.8±8.2</td>
<td>36.0±6.9</td>
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<td>Caiazzo et al. (2014b)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>555</td>
<td>Adjustable gastric banding (AGB)</td>
<td>40.3±11.4</td>
<td>1 year</td>
<td>46.8±6.5</td>
<td>39.9±6.7</td>
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<tr>
<td>Chaim et al. (2020)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>895</td>
<td>Bariatric surgery</td>
<td>39.4±10.2</td>
<td>21±22 months</td>
<td>35.9±2.8</td>
<td>25.7±3.8</td>
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<tr>
<td>Esquivel et al. (2018)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>63</td>
<td>Sleeve gastrectomy (SG)</td>
<td>40±10</td>
<td>1 year</td>
<td>44.9±5.6</td>
<td>30.5±4.2</td>
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<td>Fazel et al. (2007)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>18</td>
<td>RYGBP</td>
<td>46.6±7.3</td>
<td>2 years</td>
<td>51.7±7.4</td>
<td>31±2</td>
</tr>
<tr>
<td>Jaskiewicz et al. (2006)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>87</td>
<td>Bariatric surgery</td>
<td>40.7±10.0</td>
<td>41 months</td>
<td>46.7±8.8</td>
<td>N/A</td>
</tr>
<tr>
<td>Kral et al. (2004)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>689</td>
<td>Biliopancreatic diversion</td>
<td>36.9±9</td>
<td>41±25 months</td>
<td>47±8.4</td>
<td>31±7.9</td>
</tr>
<tr>
<td>Lassailly et al. (2020)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>180</td>
<td>Bariatric surgery</td>
<td>46.7±10.6</td>
<td>5 year</td>
<td>48.1±7.9</td>
<td>36.1±7.8</td>
</tr>
<tr>
<td>Lee et al. (2012)</td>
<td>Randomized controlled trial</td>
<td>Histology</td>
<td>8</td>
<td>Intragastric balloon</td>
<td>43±19.8</td>
<td>6 months</td>
<td>30.3±4.2</td>
<td>28.8±3.0</td>
</tr>
<tr>
<td>Liu et al. (2007)</td>
<td>Retrospective cohort</td>
<td>Histology</td>
<td>39</td>
<td>Laparoscopic RYGBP</td>
<td>41.4±9</td>
<td>18 (6–41) months</td>
<td>47.7±6.2</td>
<td>29.5±5.6</td>
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<tr>
<td>Mathurin et al. (2009)</td>
<td>Prospective cohort</td>
<td>Histology</td>
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<td>Band, bypass, RYGBP</td>
<td>41.5±9.6</td>
<td>1 year, 5 years</td>
<td>50±7.8</td>
<td>39±8.2 (1 year), 37.7±8.4 (5 years)</td>
</tr>
<tr>
<td>Mattar et al. (2005)</td>
<td>Retrospective cohort</td>
<td>Histology</td>
<td>70</td>
<td>Laparoscopic RYGBP, Laparoscopic AGB, LSG</td>
<td>49±9</td>
<td>15±9 months</td>
<td>56±11</td>
<td>39±10</td>
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<tr>
<td>Meinhardt et al. (2006)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>50</td>
<td>End-to-side JIB</td>
<td>379±7.6</td>
<td>67.0±42.8 months</td>
<td>52.8±7.5</td>
<td>35.7±7.5</td>
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<tr>
<td>Moretto et al. (2012)</td>
<td>Retrospective cohort</td>
<td>Histology</td>
<td>78</td>
<td>Gastric bypass</td>
<td>39.5±11.4</td>
<td>1 year</td>
<td>45.4±8.1</td>
<td>29.7±3.9 &amp; 29±6.5 (two groups)</td>
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<tr>
<td>Mottin et al. (2005)</td>
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<td>Histology</td>
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<td>Parker et al. (2017)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>106</td>
<td>RYGBP</td>
<td>46±11</td>
<td>N/A</td>
<td>48±8</td>
<td>N/A</td>
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<td>Russo et al. (2021)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>37</td>
<td>Biliopancreatic diversion</td>
<td>42±9</td>
<td>5 years</td>
<td>49.3±5.9</td>
<td>32.8±6.4</td>
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<td>Salman et al. (2020)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>94</td>
<td>LSG</td>
<td>41.4±7.6</td>
<td>1 year</td>
<td>44.5±5.45</td>
<td>34.23±2.66</td>
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<tr>
<td>Salman et al. (2021)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>67</td>
<td>Gastric bypass</td>
<td>44.4±5.7</td>
<td>15 months</td>
<td>44.2±4.3</td>
<td>34.4±2.7</td>
</tr>
<tr>
<td>Author (year)</td>
<td>Study design</td>
<td>Method</td>
<td>No. of samples</td>
<td>Intervention</td>
<td>Mean age (years)</td>
<td>Follow-up duration</td>
<td>Pre-BMI</td>
<td>Post-BMI</td>
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<td>Taitano et al. (2015)</td>
<td>Prospective cohort</td>
<td>Histology</td>
<td>160</td>
<td>Laparoscopic AGB or RYGBP</td>
<td>47±12</td>
<td>31±26 months</td>
<td>52±10</td>
<td>33±8</td>
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<tr>
<td>Verrasto et al. (2023a)</td>
<td>Randomized controlled trial</td>
<td>Histology</td>
<td>77</td>
<td>RYGBP</td>
<td>46.4±8.5</td>
<td>1 year</td>
<td>43.9±4.14</td>
<td>29.70±4.26</td>
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<td>Verrasto et al. (2023b)</td>
<td>Randomized controlled trial</td>
<td>Histology</td>
<td>79</td>
<td>SG</td>
<td>46.8±8.8</td>
<td>1 year</td>
<td>40.76±3.74</td>
<td>30.82±4.08</td>
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<tr>
<td>von Schönfels et al. (2018)</td>
<td>Retrospective cohort</td>
<td>Histology</td>
<td>257</td>
<td>SG or RYGBP</td>
<td>42±15</td>
<td>6 months</td>
<td>49.9±11.3</td>
<td>37±9</td>
</tr>
<tr>
<td>Folini et al. (2014)</td>
<td>Prospective cohort</td>
<td>MR PDFF</td>
<td>18</td>
<td>Intragastric balloon or gastric banding</td>
<td>43.6±12.2</td>
<td>6 months</td>
<td>42.8±7.1</td>
<td>38.2±6.19</td>
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<td>Hedderich et al. (2017)</td>
<td>Prospective cohort</td>
<td>MR PDFF</td>
<td>19</td>
<td>RYGBP + LSG</td>
<td>41.4±12.5</td>
<td>6 months</td>
<td>44.1±5.2</td>
<td>33.8±5.6</td>
</tr>
<tr>
<td>Luo et al. (2018)</td>
<td>Prospective cohort</td>
<td>MR PDFF</td>
<td>124</td>
<td>LSG, LRYGBP</td>
<td>50.9±10.8</td>
<td>6 months</td>
<td>45.3±5.9</td>
<td>34.4±5.1</td>
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<tr>
<td>Mamidipalli et al. (2020)</td>
<td>Prospective cohort</td>
<td>MR PDFF</td>
<td>54</td>
<td>RYGBP or LSG</td>
<td>52±12</td>
<td>6 months</td>
<td>42.3±5.0</td>
<td>34.3±4.7</td>
</tr>
<tr>
<td>Pooner et al. (2019)</td>
<td>Retrospective cohort</td>
<td>MR PDFF</td>
<td>50</td>
<td>Gastric bypass, sleeve, band, or plication</td>
<td>51±11.2</td>
<td>6 months</td>
<td>44.9±6.5</td>
<td>34.5±5.4</td>
</tr>
<tr>
<td>Tan et al. (2023)</td>
<td>Prospective cohort</td>
<td>MR PDFF</td>
<td>9</td>
<td>LSG</td>
<td>45.1±9.0</td>
<td>6 months</td>
<td>39.7±5.3</td>
<td>32.4±4.8</td>
</tr>
</tbody>
</table>

BMI, body mass index; RYGB, Roux-en-Y gastric bypass; LSG, laparoscopic sleeve gastrectomy; SG, sleeve gastrectomy; AGB, adjustable gastric banding.

\(^{1}\) indicates different patient cohort according to surgery type.
clinical intervention (Fig. 2A). The overall NAS, before and after bariatric intervention, with the ratio of their mean values, was examined in seven studies (Supplementary Table 1). NAS was reduced by 60% at a rate of 0.40 (95% CI, 0.30–0.54) at 3–6 months, by 40% at a rate of 0.60 (95% CI, 0.40–0.89) at 12–24 months, and by 50% at a rate of 0.50 (95% CI, 0.35–0.70) at 24–60 months (Fig. 2B). In the study conducted by von Schönfels et al., post-NAS revealed a value of 0, rendering the calculation of confidence interval unattainable. The results of the histology were evaluated according to Brunt’s criteria and Kleiner score. Nineteen studies revealed that the proportion of patients with steatosis decreased by 44% at 3–6 months, 37% at 12–24 months, and 29% at 36–60 months (Fig. 2C); lobular inflammation by 33% at 3–6 months, 36% at 12–24 months, and 51% at 36–60 months (Fig. 2D); ballooning degeneration by 20% at 3–6 months, 38% at 12–24 months, and 18% at 36–60 months (Fig. 2E). Remarkably, the proportion of patients with stage 2 fibrosis or higher (≥F2), or with more advanced form of MASLD, was found to decrease by 18% at 12–24 months, and by 17% at 36–60 months after intervention, compared to that before the operation (Fig. 2F, Supplementary Table 2). Most of these analyses revealed the I² statistics exceeding 75%, signify-
Study

After 3-6 months

<table>
<thead>
<tr>
<th>Study</th>
<th>Risk of Means</th>
<th>ROM</th>
<th>95%-CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lee (2012)</td>
<td></td>
<td>0.40</td>
<td>[0.30; 0.54]</td>
</tr>
<tr>
<td>von Schonfels (2018)</td>
<td></td>
<td>0.00</td>
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<tr>
<td>Random effects model</td>
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<td>0.40</td>
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Heterogeneity: $I^2 = NA\%$, $\tau^2 = NA$, $P = 1.000$

After 12-24 months

<table>
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<th>95%-CI</th>
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<td>Caiazzo (2014a)</td>
<td>0.35</td>
<td>[0.30; 0.41]</td>
<td></td>
</tr>
<tr>
<td>Caiazzo (2014b)</td>
<td>0.65</td>
<td>[0.56; 0.75]</td>
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</tr>
<tr>
<td>Liu (2007)</td>
<td>0.40</td>
<td>[0.35; 0.47]</td>
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<tr>
<td>Mathurin (2009)</td>
<td>0.54</td>
<td>[0.46; 0.64]</td>
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<td>Salman (2020)</td>
<td>0.51</td>
<td>[0.44; 0.58]</td>
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<td>Verrasto (2023a)</td>
<td>0.99</td>
<td>[0.92; 1.08]</td>
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<td>Verrasto (2023b)</td>
<td>1.09</td>
<td>[0.93; 1.28]</td>
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<tr>
<td>Random effects model</td>
<td></td>
<td>0.60</td>
<td>[0.40; 0.89]</td>
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</tbody>
</table>

Heterogeneity: $I^2 = 98\%$, $\tau^2 = 0.1966$, $P < 0.001$

After 36-60 months

<table>
<thead>
<tr>
<th>Study</th>
<th>Risk of Means</th>
<th>ROM</th>
<th>95%-CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caiazzo (2014a)</td>
<td>0.35</td>
<td>[0.27; 0.46]</td>
<td></td>
</tr>
<tr>
<td>Caiazzo (2014b)</td>
<td>0.59</td>
<td>[0.49; 0.70]</td>
<td></td>
</tr>
<tr>
<td>Mathurin (2009)</td>
<td>0.51</td>
<td>[0.42; 0.62]</td>
<td></td>
</tr>
<tr>
<td>Russo (2021)</td>
<td>0.54</td>
<td>[0.44; 0.66]</td>
<td></td>
</tr>
<tr>
<td>Random effects model</td>
<td></td>
<td>0.50</td>
<td>[0.35; 0.70]</td>
</tr>
</tbody>
</table>

Heterogeneity: $I^2 = 71\%$, $\tau^2 = 0.0269$, $P = 0.015$

Heterogeneity: $I^2 = 96\%$, $\tau^2 = 0.1672$, $P < 0.001$

Study

After 3-6 months

<table>
<thead>
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<th>Study</th>
<th>Proportion</th>
<th>Difference</th>
<th>95%-CI</th>
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<tr>
<td>Aldoheyan (2017)</td>
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<td>-0.44</td>
<td>[-0.68; -0.21]</td>
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</table>

After 12-24 months

<table>
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<th>Study</th>
<th>Proportion</th>
<th>Difference</th>
<th>95%-CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caiazzo (2014a)</td>
<td>-0.25</td>
<td>[-0.30; -0.21]</td>
<td></td>
</tr>
<tr>
<td>Caiazzo (2014b)</td>
<td>-0.13</td>
<td>[-0.19; -0.07]</td>
<td></td>
</tr>
<tr>
<td>Esquivel (2018)</td>
<td>-0.34</td>
<td>[-0.47; -0.21]</td>
<td></td>
</tr>
<tr>
<td>Furuya (2007)</td>
<td>-0.33</td>
<td>[-0.56; -0.11]</td>
<td></td>
</tr>
<tr>
<td>Liu (2007)</td>
<td>-0.87</td>
<td>[-0.98; -0.76]</td>
<td></td>
</tr>
<tr>
<td>Mattar (2005)</td>
<td>-0.63</td>
<td>[-0.74; -0.51]</td>
<td></td>
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<tr>
<td>Salman (2020)</td>
<td>-0.10</td>
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</tr>
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<td>Chaim (2020)</td>
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<tr>
<td>Random effects model</td>
<td></td>
<td>-0.37</td>
<td>[-0.56; -0.19]</td>
</tr>
</tbody>
</table>

Heterogeneity: $I^2 = 97\%$, $\tau^2 = 0.0667$, $P < 0.001$

After 36-60 months

<table>
<thead>
<tr>
<th>Study</th>
<th>Proportion</th>
<th>Difference</th>
<th>95%-CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caiazzo (2014a)</td>
<td>-0.24</td>
<td>[-0.30; -0.19]</td>
<td></td>
</tr>
<tr>
<td>Caiazzo (2014b)</td>
<td>-0.17</td>
<td>[-0.22; -0.11]</td>
<td></td>
</tr>
<tr>
<td>Kral (2004)</td>
<td>-0.36</td>
<td>[-0.45; -0.26]</td>
<td></td>
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<tr>
<td>Lassailly (2020)</td>
<td>-0.33</td>
<td>[-0.44; -0.21]</td>
<td></td>
</tr>
<tr>
<td>Meinhart (2006)</td>
<td>-0.11</td>
<td>[-0.29; 0.07]</td>
<td></td>
</tr>
<tr>
<td>Russo (2021)</td>
<td>-0.54</td>
<td>[-0.71; -0.37]</td>
<td></td>
</tr>
<tr>
<td>Random effects model</td>
<td></td>
<td>-0.29</td>
<td>[-0.39; -0.18]</td>
</tr>
</tbody>
</table>

Heterogeneity: $I^2 = 82\%$, $\tau^2 = 0.0146$, $P < 0.001$

Heterogeneity: $I^2 = 94\%$, $\tau^2 = 0.0424$, $P < 0.001$

**Figure 2.** Continued.
ing substantial heterogeneity across the studies. Subsequent sensitivity analysis and meta-regression were conducted; however, the source of heterogeneity remained unclear (data not shown).

**Impact of bariatric intervention intrahepatic fat measured by MRI-PDFF**

Six studies showed significant reduction of intrahepatic fat in MRI-PDFF at six months after bariatric intervention (Table 2). Ratio of the means of pre-operative to post-operative MRI-PDFF was 0.28 (95% CI, 0.24–0.33), which implied that 72% of intrahepatic fat was reduced after intervention with 21% of BMI reduction (Fig. 3). We observed a borderline heterogeneity in BMI, but no heterogeneity was identified in steatosis measured by MRI-PDFF.
Figure 2. Continued.

Table 2. Ratio of means of MRI-PDFF meta-analysis of the efficacy of bariatric intervention in obese patients

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>No. of samples</th>
<th>Ratio of mean (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BMI</td>
</tr>
<tr>
<td>After 3–6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folini et al. (2014)</td>
<td>18</td>
<td>0.87 (0.79–0.97)</td>
</tr>
<tr>
<td>Hedderich et al. (2017)</td>
<td>19</td>
<td>0.77 (0.70–0.84)</td>
</tr>
<tr>
<td>Luo et al. (2018)</td>
<td>124</td>
<td>0.76 (0.73–0.79)</td>
</tr>
<tr>
<td>Mamidipalli et al. (2020)</td>
<td>54</td>
<td>0.81 (0.77–0.85)</td>
</tr>
<tr>
<td>Pooler et al. (2019)</td>
<td>50</td>
<td>0.77 (0.72–0.82)</td>
</tr>
<tr>
<td>Tan et al. (2023)</td>
<td>9</td>
<td>0.82 (0.72–0.93)</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>0.79 (0.75–0.83)</td>
</tr>
<tr>
<td>Heterogeneity - $i^2$</td>
<td></td>
<td>50.9% (0.0–80.5%)</td>
</tr>
<tr>
<td>$p$-value</td>
<td></td>
<td>0.070</td>
</tr>
</tbody>
</table>

MRI, magnetic resonance imaging; PDFF, proton density fat fraction; BMI, body mass index; CI, confidence interval.
Mortality after bariatric surgery and MASH aggravation

Two studies reported mortality rate after the bariatric surgery, with pooled mortality rate of 13% (95% CI, 0.07–0.21). The other three studies reported cases in which MASLD worsened based on liver histologic examination after the surgery, with a pooled proportion of 6% (95% CI, 0.04–0.09) (Supplementary Table 3).

Quality of the selected studies

Out of a total of thirty studies, most studies showed low risk of bias, whereas a few showed moderate/high (Supplementary Figs. 1 and 2). To visually inspect the potential publication bias, we also created funnel plots for studies investigating populations with more than ten individuals where BMI was measured within 12–24 months. We found significant publication bias (Supplementary Fig. 3). To address this bias, we applied trim and fill method, confirming the correction of publication bias in the modified result. Notably, the adjusted effect size values remained consistent with the trend observed in the analysis using raw values. For populations with a follow-up period of 12–24 months, the GRADE assessment was used to rate the quality of evidence (Supplementary Table 4).

DISCUSSION

The current study aimed to comprehensively assess the impact of bariatric intervention on hepatic steatosis and MASH, with particular focus on presenting the association using a novel measure, MRI-PDFF, which, to the best of our knowledge, has not been reported till date. MASLD is one of the most common causes of chronic liver disease worldwide. While the complete pathophysiology remains unclear, insulin resistance is considered a pivotal factor of the disease development. This condition leads to an increase in free fatty acids released from adipose tissue, which subsequently move to the liver and are taken up by hepatocytes. Moreover, insulin resistance upregulates fatty acid transport proteins, impairs mitochondrial fatty acid oxidation, and alters the adipokine profile, all of which can accelerate hepatic fat accumulation and inflammation. Hence, the primary treatment goal has been focused on improving insulin resistance by managing obesity, particularly central obesity, in MASLD. Lifestyle modification and, if indicated, medications targeting weight loss are valid options for initial management of the disease. Among the medical options, GLP-1 receptor agonist has shown superiority over other drugs in managing MASH. In case these approaches fail to achieve substantial weight loss, the consideration of bariatric intervention for obese patients would...
be justified.

However, whether the impact of weight loss extends to more advanced liver conditions, such as MASH or liver fibrosis, when inflammation in the liver progresses, has remained a long-standing controversy. This is probably due not only to the disease progressing to an irreversible stage but also to the liver's inability to tolerate the lipotoxicity arising from the massive release of free fatty acids, mainly originating from visceral fat, following rapid weight loss, which is particularly evident in the early stage of bariatric surgery. Nevertheless, several previous meta-analyses have found a favorable effect of the surgery on improving MASH. Notably, Lee et al. reported their meta-analysis, wherein histological worsening of MASLD after bariatric surgery was 12%. Our study showed, in line with the above studies, a pooled mortality rate of 13% within a pooled follow-up time of 30.8 months, and a pooled worsening MASH rate of 6%. It would be worth noting that the number of studies reporting post-surgery deaths was considerably limited. Thus, we emphasized that the potential risks associated with bariatric surgery should not be disregarded, especially in advanced liver disease.

We have presented robust evidence of the significant impact of bariatric intervention on MASH, which aligns with the findings from prior meta-analyses. Moreover, we assessed the risk of bariatric intervention, which was not routinely evaluated in prior studies, emphasizing that the potential risk should not be overlooked when considering intervention. Our approach involved not only considering the type of liver histology but also incorporating NAS, a comprehensive tool for assessing MASLD. This enabled us to evaluate the effect of bariatric intervention from multiple perspectives. Finally, we showed, for the first time, that MRI-PDFF is a reliable indicator for assessing MASLD for bariatric intervention, particularly in its early stages. While several imaging modalities have been proposed as alternatives to liver biopsy, due to its invasive nature, MRI-PDFF demonstrated excellent sensitivity and specificity in diagnosing relatively early-stage MASLD.

Our study has several limitations. First, we encountered considerable heterogeneity in most analyses, despite categorizing by follow-up period and other interventions. This result was consistent with the GRADE assessment in our post hoc analyses. This methodological limitation has been identified in previous studies as well, suggesting that differences in study design and/or population may have significantly impacted this high heterogeneity. Nonetheless, the consistent and substantial impact of bariatric intervention has been evident across various study methodologies, reducing doubts about the reliability of this study result. Second, despite our meta-analysis showing the reliability of MRI as an alternative to liver biopsy in bariatric intervention, it would be important to note that the applicability of MRI-PDFF in more advanced liver diseases, such as MASH or liver cirrhosis, still remains a subject of scrutiny. Its limitations in establishing consistent correlations of the severity of steatohepatitis with advanced liver diseases underscore the necessity for further research in this area. Third, our analysis did not include the individual biochemical measures. However, the assessment of liver function through laboratory findings was thoroughly analyzed in prior meta-analyses, prompting us to avoid redundancy. Finally, the absence of individual patient data restricted the extent of more comprehensive analyses.

In conclusion, this meta-analysis reaffirmed the efficacy of bariatric intervention in the improvement of MASH for patients with obesity and MASLD while also highlighting the robust reliability of MRI-PDFF in assessing hepatic steatosis after bariatric intervention. Our study further reported that the favorable impact of bariatric intervention on MASH patients with obesity, like significant liver fibrosis or cirrhosis, remains uncertain due to potential risks of exacerbating liver conditions.

Authors’ contribution

All authors substantially participated in the analysis, data interpretation and preparation of manuscript. JH and HH should be considered joint first author.

Acknowledgements

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Dear Editor,

Patients with steatotic liver disease (SLD) associated with cardiometabolic dysregulation exhibit a higher incidence of extrahepatic diseases, including atherosclerotic cardiovascular disease (ASCVD). In June 2023, the consensus group composed of multiple societies opted to replace the term nonalcoholic fatty liver disease (NAFLD) with metabolic dysfunction-associated steatotic liver disease (MASLD), aiming to minimize the potential for stigma and more accurately reflect its underlying pathophysiology. MASLD requires a new inclusion criterion of “the presence of at least one or more cardiometabolic risk factors,” which reflects the importance of cardiometabolic dysregulation in patients with SLD. While the use of alternative terminology can lead to occasional misunderstandings and hinder progress, it is crucial to extend investigations on NAFLD to MASLD. This will ensure that valuable research resources are effectively utilized and contribute to the advancement of knowledge in this field.

We aimed to compare the prevalence and progression of ASCVD risk in patients with NAFLD and those with MASLD in Asia. This investigation included 7,286 consecutive health check examinees who were subjected to ultrasoundography and monitored at the Saga Health and Clinical Examination Center (Saga, Japan) from January 2010 to March 2020. All the individuals were Asian. We eliminated 895 participants from our sample because of insufficient data on alcohol consumption habits (n=541), alcohol consumption ≥60 g/day (n=161), hepatitis B virus infection (n=81), and hepatitis C virus infection (n=112). The study population comprised 2,306 individuals diagnosed with SLD.

NAFLD was diagnosed in 63.4% (1,462/2,306) of the patients, including 98 who did not fulfill the cardiometabolic criteria for MASLD (Fig. 1A). These cases were classified as cryptogenic SLD, and a significant proportion (93.3%) of patients with NAFLD were also diagnosed with MASLD. Our findings align well with those of previous studies that reported that almost all NAFLD patients fulfilled the MASLD criteria.
There were no significant differences in age, sex, baseline Suita score, or Framingham risk score between the NAFLD and MASLD groups (Suita score, low/middle/high, 795/531/79 vs. 715/521/79, \( P = 0.507 \); Framingham Risk Score, low/high, 1,025/239 vs. 948/235, \( P = 0.543 \)). We defined the event as worsening of the Suita score (from low-risk \([\leq 40]\) to middle-risk \([41–55]\) or high-risk \([\geq 56]\))^9 or Framingham risk score (from low-risk \([<15]\) to high-risk \([\geq 15]\))^9 To compare the incidence of worsening ASCVD risk scores between the groups, we constructed Kaplan–Meier curves (Fig. 1B, C). The rate of five-year/ten-year cumulative incidence of worsening scores was not significantly different between patients with NAFLD and those with MASLD (Fig. 1B, C). These results indicate that the prevalence and progression of ASCVD risk are similar in patients with MASLD and NAFLD. These results were similar to previous studies that have compared the differences between MASLD and NAFLD, accentuating the augmented risk of cardiovascular diseases, including ASCVD.\(^1,10\) Although previous studies have reported from the U.S. and the EU, our report highlights similar importance in Asia.

In conclusion, data on ASCVD obtained using the term NAFLD can be extrapolated to MASLD.

**Authors’ contribution**

Hiroyuki Suzuki and Tsubasa Tsutsumi: study concept, design, and drafting; Keisuke Amano: data extraction, interpretation of data, and critical revision of the manuscript; Machiko Kawaguchi: interpretation of data, statistical analysis, and interpretation of data and critical revision of the manuscript; Takumi Kawaguchi: study concept, interpreta-

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**Abbreviations:**

SLD, steatotic liver disease; ASCVD, atherosclerotic cardiovascular disease; NAFLD, nonalcoholic fatty liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease.
tion of data and critical revision of the manuscript.

Acknowledgements

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Conflicts of Interest

Takumi Kawaguchi received lecture fees from Janssen Pharmaceutical K.K., Taisho Pharmaceutical Co., Ltd., Kowa Company, Ltd., Otsuka Pharmaceutical Co., Ltd., Eisai Co., Ltd., ASKA Pharmaceutical Co., Ltd., AbbVie GK., and EA Pharma Co., Ltd. The other authors have no conflicts of interest to declare.

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Dear Editor,

On March 14, 2024, the US Food and Drug Administration (FDA) approved resmetirom for fast-track treatment of adult patients with metabolic dysfunction-associated steatohepatitis (MASH) with fibrosis, making it the world’s first approved drug. Chronic hepatitis B (CHB) and metabolic dysfunction-associated steatotic liver disease (MASLD) are the most prevalent chronic liver diseases worldwide, and concurrent MASLD is common in patients with CHB, with a prevalence of 29.6–34.9%. When using resmetirom to treat MASH patients with CHB, it is important to consider the potential impacts of hepatic steatosis on improving functional cure of CHB.

**Hepatic steatosis is beneficial to functional cure of CHB by decreasing hepatitis B virus DNA levels and increasing HBsAg seroclearance**

Both CHB and MASLD can lead to hepatic inflammation and liver fibrosis, increasing the risk of adverse liver outcomes. However, CHB patients with concurrent steatosis tend to have lower HBV activity, including lower proportions of hepatitis B e antigen (HBeAg) positivity, lower serum HBV DNA levels, as well as higher rates of hepatitis B surface antigen (HBsAg) seroclearance, and are more likely to achieve functional cure. Studies on animals and cells show that hepatic steatosis can decrease HBV activity and induce lower levels of HBsAg, HBeAg, and HBV DNA. The molecular mechanism might be explained as liver fat activating innate immunity, disrupting the liver’s metabolic environment, and increasing liver cell apoptosis, reducing HBV survival.

**Keywords:** Resmetirom; Metabolic dysfunction-associated steatohepatitis; Hepatitis B, chronic; Functional cure
Can resmetirom potentially decrease functional cure of CHB?

In the phase 3, randomized, controlled trial of resmetirom in MASH with liver fibrosis, both the resmetirom treatment groups significantly improved hepatic steatosis and inflammation. It is unclear if resmetirom can be used to treat MASH patients with concurrent CHB. We recommend the answer is YES, because both CHB and MASLD can increase the risk of adverse liver outcomes. However, close monitoring of HBV activity is necessary as resmetirom could potentially alleviate steatosis and increase HBV activity. Further research is needed to determine if more aggressive antiviral therapy (e.g., entecavir combined with tenofovir, nucleotide analogues combined with interferon, etc.) is necessary when using resmetirom to treat MASH patients with concurrent CHB. The field remains blank, and more clinical data are needed to reveal the answers and guide drug treatment choices in this large population.

Authors’ contribution
M-H.Z. researched data for the article. All authors contributed substantially to discussion of the content. N-B.Y. wrote the article. All authors reviewed and/or edited the manuscript before submission.

Acknowledgements
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Conflicts of Interest
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REFERENCES

Abbreviations:
MASH, metabolic dysfunction-associated steatohepatitis; CHB, chronic hepatitis B; MASLD, metabolic dysfunction-associated steatotic liver disease; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen
Evaluating the therapeutic efficacy of NAD supplementation in management of metabolic dysfunction-associated steatotic liver disease: Key considerations

Xinyi Lu¹², Rui Yang², Yu Chen³, and Daozhen Chen¹²³

¹Wuxi Medical Center, Nanjing Medical University, Jiangsu, China; ²Wuxi Maternal and Child Health Hospital, Wuxi School of Medicine, Jiangnan University, Jiangsu, China; ³Department of Laboratory, Haidong Second People’s Hospital, Haidong, China

Keywords: Nicotinamide adenine dinucleotide; Energy metabolism; Metabolic dysfunction-associated steatotic liver disease; Fecal microbiota transplantation
Supplementation with nicotinamide adenine dinucleotide (NAD) precursors plays a crucial role in metabolic regulation, revealing significant therapeutic potential in clinical trials, particularly for high-risk factors associated with metabolic dysfunction-associated steatotic liver disease (MASLD), such as enhancing cardiovascular health, lowering low density lipoprotein and triglyceride levels, and boosting pancreatic cell sensitivity, however, its efficacy in treating MASLD has received varied evaluations.

Extensive research has demonstrated the effectiveness of NAD precursors in markedly improving metabolic levels in MASLD in murine models. These studies highlight a profound enhancement in metabolic health in cases of MASLD, particularly through the administration of nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN). In models of high-fat diet-induced MASLD, treatment with NR (400 mg/kg/day for one week), optimizes NAD levels in critical areas such as the liver and muscle mitochondria, leading to metabolic improvements. Results show a 40% reduction in liver triglycerides and nearly 50% decrease in fasting insulin levels, indicating a notable improvement in key metabolic parameters. Furthermore, these studies have uncovered novel mechanisms, including the activation of sirtuins pathways and the NAMPT-SIRT1-AMPK axis, which are instrumental in inducing lipolysis and mitigating liver steatosis. These innovative discoveries pave the way for new therapeutic strategies in MASLD, although their application in human clinical settings awaits further exploration.

Despite FDA approval of NAD precursors such as NMN or NR trials, their clinical efficacy in MASLD remains inconsistent. The discrepancy in clinical outcomes compared to animal studies necessitates careful consideration of the following points in current and upcoming clinical research:

1. The effectiveness of NAD metabolism on MASLD treatment is significantly influenced by various factors, such as age, gender, basal metabolism, and diet. For instance, a study by Yoshino et al., which was a 10-week randomized, placebo-controlled, double-blind trial, exemplifies the necessity of acknowledging these elements. The study focused on a homogeneous group of 25 postmenopausal women with prediabetes and obesity or overweight conditions. It was observed that NMN supplementation at a dose of 250 mg/day improved muscle insulin sensitivity by 25±7%, emphasizing the need for personalized treatment strategies.

2. The efficacy of long-term supplementation with NAD precursors may be limited due to the body’s metabolic compensation, which is influenced by the circadian regulation of NAD levels. While short-term use of 1 g NAD precursor supplements boosts metabolic levels, extended use fails to significantly increase NAD beyond baseline levels. This suggests the necessity for further modulation of NAD metabolism. In a notable study by Dellinger et al., within a 6-month prospective, randomized, double-blind, placebo-controlled clinical trial, the combined effects of NR and Pterostilbene (a SIRT1 activator) on MASLD were examined. The study demonstrated that treatment with 500 mg of NR and 100 mg of pterostilbene led to nearly a 20% decrease in alanine transaminase and gamma glutamyl transferase.

Abbreviations:
NAD, nicotinamide adenine dinucleotide; MASLD, metabolic dysfunction-associated steatotic liver disease; LDL, low density lipoprotein; NR, nicotinamide riboside; NMN, nicotinamide mononucleotide; HFD, high fat diet; ALT, alanine transaminase; GGT, gamma glutamyl transferase; FMT, fecal microbiota transplantation

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Corresponding author : Daozhen Chen
Wuxi Maternal and Child Health Hospital, Wuxi School of Medicine, Jiangnan University, Huaishu Lane, Wuxi, Jiangsu, 214002, China
Tel: +86-0510-82713324, Fax: +86-0510-82713324, E-mail: chendaozhen@163.com
https://orcid.org/0000-0003-4791-8065

Yu Chen
Wuxi Maternal and Child Health Hospital, Wuxi School of Medicine, Jiangnan University, Huaishu Lane, Wuxi, Jiangsu, 214002, China
Tel: +86-0510-82713324, Fax: +86-0510-82713324, E-mail: cy-78@hotmail.com
https://orcid.org/0000-0001-9420-1636

transferase levels over time, indicating that activating NAD enzymes may sustain NAD metabolism for long-term therapeutic benefit.

3. Fecal microbiota transplantation (FMT) also presents a promising direction, particularly for MASLD patients with gut microbiota imbalances. This imbalance affects the conversion and effectiveness of NAD precursor supplements. A clinical trial revealed that MASLD patients receiving FMT, along with diet and exercise, experienced improved gut microbiota composition and a reduction in fat attenuation in lean MASLD patients decreased to normal levels. Key bacterial strains like *Ruminococcus 2* and *Prevotella 2*, which are involved in regulating NAD, were significantly increased, along with findings from animal studies with NR supplementation. Therefore, FMT, in conjunction with diet and exercise, might improve gut microbiota in MASLD patients, potentially boosting the efficacy of NAD supplements as an auxiliary therapy.

In summary, although NAD precursor supplementation exhibits promise in treating MASLD, comprehensive understanding of its long-term effects and efficacy across diverse populations remains necessary. Based on individual metabolic profiles, strategies like activating sirtuins or FMT should be incorporated for enhanced effectiveness.

**Authors’ contribution**

Xinyi Lu and Rui Yang wrote the manuscript and revised. Daozhen Chen and Yu Chen revised the manuscript.

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Figure was created with BioRender.com.

**Conflicts of Interest**

The authors have no conflicts to disclose.

**REFERENCES**

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All investigations involving human participants must be conducted according to the ethical guidelines of the Declaration of Helsinki, and be approved by the institutional review board. For studies involving animal experimentation, author(s) must provide assurance that all the animals received humane care according to the criteria outlined in the NIH “Guide for the Care and Use of Laboratory Animals”. The author must state that the use of animals (means all mammals and birds) in the manuscript was approved by the institutional Animal Ethical Committee (AEC) in accordance to the article 14th of Korean Animal Protection Law, or equivalent, in the paper. It must be clearly stated that animal use has complied to the article 13th of Korean Animal Protection Law (The principles of animal use) and the relevant institutional policies in the manuscript. Copies of the protocol approved by institutional AEC or equivalents, must be available for review by the editor if necessary.

The corresponding author must give written assurance that neither the submitted material nor portions thereof have been published previously or are under consideration for publication elsewhere. Any material that could constitute prior or concurrent publication of similar data by any one of the authors should be submitted with the manuscript. It is assumed that the corresponding author speaks for his or her co-authors and certifies that all the listed authors meaningfully participated in the study and that they have seen and approved the final manuscript.

Authors should acknowledge any commercial affiliation or consultancy that could be constructed as potential conflicts of interest under a heading “Conflict of Interest statement” prior to the references.

The policies on the research and publication ethics not stated in this instructions, ‘Good Publication Practice Guidelines for Medical Journals (https://www.kamje.or.kr/board/view?b_name=bo_publication&bo_id=7&per_page=)’ or ‘Guidelines on good publication (http://www.publicationethics.org.uk/guidelines)’ can be applied.

Ensure correct use of the terms sex (when reporting biological factors) and gender (Identity, psychosocial or cultureral factors), and, unless inappropriate, report the sex and/or gender of study participants, the sex of animals or cells, and describe the methods used to determine sex and gender.

If the study was done involving an exclusive population, for example in only one sex, authors should justify why, except in obvious cases, (e.g., prostate cancer).

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The Journal has adopted the following policies, as specified by the International Committee of Medical Journal Editors (ICMJE), on the use of artificial intelligence (AI) in preparation of material to be submitted for publication in the Journal.

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The manuscript should be written in A4 (21×30 cm) paper in double space texts by leaving 3 cm space in the right, left, top and bottom sides at 10 point fonts.

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Original articles describing clinical and basic studies in the field of hepatology. Manuscripts are expected to be well-organized and clearly written. They should not exceed 6,000 words, including the abstract, references, tables, and figure legends. No more than 8 figures and tables, with a maximum of 6 panels per figure. It is permitted for you to submit additional methodological details, non-essential figures or portions of your manuscript as supplementary material for online publication only. References cited in the main text may not be listed in the supplementary materials. The only references be listed in the supplement are those cited exclusively in the supplement. References should not exceed a maximum of 50.

Original article must arranged as follows: (1) title page (2) abstract (250 words or less with a list of 5 or less key words), (3) introduction, (4) materials and methods (or patients and methods), (5) results, (6) discussion, (7) acknowledgements, (8) conflict of interest statement (9) references, (10) tables, and (11) figure legends.

In case of submission of original articles (not applicable for reviews, editorials, and letters), authors should summarize the contents of the article in a concise, pictorial form designed to easily understand main findings of the work described in the article. Graphical abstracts should be submitted as a separate JPG or TIFF files at the online submission step of file upload. The submission of the graphical abstract is mandatory when submitting an original article. Graphical abstracts should be provided as an image with a minimum size of 531 × 531 pixels (height × width) using a minimum resolution of 600 dpi. When submitting a larger image, please make sure to use the same ratio. Also, please note that your image will be scaled proportionally to fit in the available window, which is a rectangle with a size of 200 × 500 pixels.

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Review articles on selected topics of interest for the readers of the Clinical and Molecular Hepatology and will be solicited by the Editors. Review articles are expected to be clear, concise and updated. The maximum length is 5,000 words. The inclusion of a maximum of 8 high quality tables and/or colored figures to summarize critical points is highly desirable.
**Editorials**

This section consists of invited brief editorial comments on articles published in *the Clinical and Molecular Hepatology*. The length of an editorial should not exceed 1,500 words and 1 table or 1 figure is allowed. References should not exceed a maximum of 20.

**Letters to the editor**

Letters to the editor should be related to a recent article published in *the Clinical and Molecular Hepatology* within previous two years. Letters to the editor must be arranged as follows: (1) title page, (2) body (3) references (maximum of 15), and (4) a maximum number of 1 tables or figures is allowed. The length of a letter to the editor should not exceed 800 words, and the maximum number of authors is 6. Abstract is not required.

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The correspondence consists of replies on editorials from the authors of the original publication in *the Clinical and Molecular Hepatology*. The length of a correspondence should not exceed 1,500 words and 1 table or 1 figure is allowed. References should not exceed a maximum of 15. Correspondence letters are not usually peer reviewed, but we might invite replies from the authors of the original publication.

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Special topics should be no longer than 800 words with 10 or less references.

**Snapshot**

Snapshot consists of a large single page figure with schematic diagrams and tables that graphically summarize current knowledge about a particular subject within the field of hepatology. A detailed figure legend which includes all relevant information can be included and may be incorporated into the main figure. The figure is accompanied by a short summary article that should not exceed a maximum of 600 words. References should not exceed a maximum of 10. The snapshot should contain a descriptive title.

**1. Title page**

Provide a concise title. List the full names of all authors and their institutional affiliation. In a multi-authored work involving more than a single institution, indicate individual affiliation by means of superscript Arabic numbers. Indicate a change of address in a similar fashion. List the footnotes to the title page. Provide the contact information for the corresponding author (name, address, telephone number, fax number, e-mail address and Orcid ID), and running title (Less than 50 characters). All abbreviations should be explained in this page (e.g. AFP, alpha fetoprotein; ALT, alanine aminotransferase). *The Clinical and Molecular Hepatology* employs a system to screen plagiarism (CrossRef). When submitting your manuscript to this journal, you accept that your manuscript may be screened for plagiarism against previously published material.

**2. Abstract**

Abstract of original articles must contain 250 words or less and must be organized as follows: Background/Aims, Methods, Results, and Conclusions. Three to Five keywords should be provided at the end of the abstract.

**3. Highlight**

Authors of original articles are requested to include “Highlights” which consist of three to four sentences summarizing the originality and main findings of the article. “Highlights” should not exceed 100 words in total. Highlights must be organized in a box and placed after the end of the abstract. The authors are encouraged to include the “Highlights” with initial article submission. When submitting a revised manuscript, the submission of the “Highlights” is mandatory.
4. Introduction
Provide the minimum background information that will orient the general reader. Do not engage in a literature review.

5. Methods
Provide a level of detail such that another investigator could repeat the work. For methods that are used without significant modification, citation of the original work will suffice. Identify and provide references for all the statistical methods used.

6. Results and discussion
Present the major findings of the study in graphical form if practicable. Do not illustrate minor details if their message is adequately conveyed by simple descriptive text. Mention all the tables and figures. In the discussion, concisely present the implications of the new findings for the field as a whole, minimizing any reiteration of the results and avoid repetition of material in the introduction; keeping a close focus on the specific topic of the paper.

7. Acknowledgements
An acknowledgement of persons who made a genuine assistance and provided special reagents may be included. Grant and financial support related with the work should be specifically stated.

8. Authors' contribution
Based on the ICMJE guidelines for authorship criteria, how each author has contributed to the paper should be clarified (e.g, Conception or design of the work, Data collection, Data analysis and interpretation, Drafting the article, Critical revision of the article, and Final approval of the version to be published).

9. References
References should be numbered in the order they are cited, and the number of reference should be marked in the text by means of a superscript Arabic numerical. Only literature that is published or in press (with the name of the publication) may be numbered and listed; abstracts and letters to the editor may be cited. Cite the names of all authors when there are six or less; when seven or more list the first six followed by et al.

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Prepare tables on individual sheets of paper, double spaced and numbered consecutively with Arabic numerals in the order of their appearance in the text. The title of tables should be written concisely in clauses and phrases. The first letter of the table title starts with a capital letter. Explain all abbreviations and symbols such as *, †, ‡, §, ††, ‡‡, §§. Do not duplicate the material presented in a figure.

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Number the figures with Arabic numerals in the order they are mentioned in the text. Provide a title (this should not appear on the figure itself) and sufficient explanation to render the figure intelligible without reference to the text. For any copyrighted material, indicate that permission has been obtained (see Permissions, above). Figure legends should be typed consecutively on a separate sheet of paper.

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Position indicate current status at your affiliation; professor, fellow, resident, student, post doc.

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<th>1) General Format</th>
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<th>No</th>
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<td>[1] Did you have the title page, abstract, the text (introduction, materials and methods, results, and discussion), acknowledgements, conflict of interest statement, references, tables, and legends for figures?</td>
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<th>Yes</th>
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<td>[1] Identify the committee(s) approving the study protocol and include a statement of compliance with ethical regulations.</td>
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<td>[4] All citations in the paper have a complete and accurate reference in the reference list. The number of references in special topics should be 10 or less.</td>
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<th>4) Tables and Figures</th>
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You can trust EPCLUSA® to deliver consistent outcomes in a variety of settings1,5,k

Adverse events:
Headache, fatigue and nausea were the most common AEs associated with EPCLUSA® in clinical trials. Headache, fatigue and nausea (incidence ≥10%), as well as other AEs, were reported at a similar frequency in placebo-treated patients. Cardiac disorders, skin rashes and angioedema have been identified during post approval use of sofosbuvir. EPCLUSA® should not be administered concurrently with other medicinal products containing sofosbuvir.

Abbreviations:
AE = adverse event; CPT = Child-Pugh-Turcotte; DDI = drug-drug interaction; EASL = European Association for the Study of the Liver; GT = genotype; HBV = hepatitis B virus; HCV = hepatitis C virus; IV = intravenous; PI = protease inhibitor; RBV = ribavirin; STR = single-tablet regimen; SVR = sustained virological response at 12 weeks.

Footnotes:
a) Despite unknowns in baseline characteristics of some patients, such as: HCV genotype, fibrosis stage, former/current IV drug use, PPU use at baseline and treatment history.1,5 Hepatitis B virus (HBV) reactivation has been reported in HCV/HBV co-infected patients who were undergoing or had completed treatment with HCV direct acting antivirals, and who were not receiving HBV antiviral therapy. Test all patients for evidence of current or prior HBV infection by measuring HBsAg and anti-HBc before initiating HCV treatment with this drug. In patients with serological evidence of HBV infection, monitor for clinical and laboratory signs of hepatitis flares or HBV reactivation during HCV treatment with this drug and during post-treatment follow-up. Initiate appropriate patient management for HBV infection as clinically indicated.1,5 In Korea, Epclusa® is indicated for the treatment of adults and pediatric patients 12 years of age and older or weighing at least 30 kg with chronic hepatitis C virus (HCV) genotype 1, 2, 3, 4, 5, or 6, as a 12-week course of once-daily oral sofosbuvir-velpatasvir (400 mg/100 mg) tablets. Study drug was dispensed monthly; however, there were no on-treatment study assessments. The primary efficacy endpoint was rate of sustained virologic response (HCV RNA < 15 IU/mL) 12 weeks after treatment (SVR12), which was compared to a pre-specified performance goal of 85%. In a Phase 2 study in patients with chronic HCV and CPT-C cirrhosis, EPCLUSA®+RBV® for 12 weeks led to a 78% SVR12 rate. Treatment was well tolerated, with observed AEs consistent with expectations for a patient population with advanced liver disease.1 In a large cohort international real-world study, all patients with unknown genotype, unknown fibrosis score and unknown treatment history were cured with EPCLUSA® for 12 weeks.1 In patients who inject drugs.1,5bConsistent outcomes defined as SVR12 between 91% and 100% in all patient subpopulations.1,5b


Footnotes:
1) Pregnancy: If this drug is administered with ribavirin, the combination regimen is contraindicated in pregnant women. Sofosbuvir is pregnancy Category B and ribavirin is pregnancy Category D. Sofosbuvir is contraindicated in women of childbearing age who are or may become pregnant. Sofosbuvir must be used with effective contraception to prevent pregnancy in women of childbearing age. Ribavirin may cause fetal harm when administered to a pregnant woman. Sofosbuvir must not be administered to a breastfeeding woman. This drug is not recommended for use in women of childbearing age who are or may become pregnant. Sofosbuvir must be used with effective contraception to prevent pregnancy in women of childbearing age. Ribavirin is contraindicated in breastfeeding women. Sofosbuvir must not be administered to a breastfeeding woman. If this drug is used together with ribavirin, special care of a usual lumbar (e.g. drug monitoring) at bass faster pace of female patient is the follows patients of case patient. 2) Lactation: It is not known whether the components of this drug are present in human breast milk, affect human breast milk, or are excreted in human milk. The effect of this drug on milk production has not been studied. Sofosbuvir and velpatasvir should not be used in breastfeeding women. 3) Drug Interactions: For patients for whom the drug is required for patients with contraindications to sofosbuvir, alternative medicines such as sofosbuvir must be used. If the drug is used together with ribavirin, special care of a usual lumbar (e.g. drug monitoring) for female patient is the follows patients of case patient. 4) Use in Specific Populations: 1) Safety and effectiveness of this drug have not been established in pediatric patients 12 years of age and older or weighing at least 30 kg or in pediatric patients weighing less than 30 kg. Clinical and hepatic laboratory monitoring (including direct bilirubin), as clinically indicated, is recommended for patients with mild, moderate, or severe hepatic impairment (Child-Pugh Class A, B, or C). Clinical and hepatic laboratory monitoring (including direct bilirubin), as clinically indicated, is recommended for patients with decompensated cirrhosis receiving treatment with this drug and ribavirin.13 On-treatment monitoring may be required for patients with comorbidities or on certain comedications. Please refer to prescribing information for further information. 7) In a phase 3 study conducted at 16 sites in India, 129 adult patients with chronic HCV infection of any genotype initiated 12 weeks of once-daily sofosbuvir-velpatasvir (400-100 mg). Study drug was dispensed monthly; however, there were no on-treatment study assessments. The primary efficacy endpoint was rate of sustained virologic response (HCV RNA < 15 IU/mL) 12 weeks after treatment (SVR12), which was compared to a pre-specified performance goal of 85%. 8) In a Phase 2 study in patients with chronic HCV and CPT-C cirrhosis, EPCLUSA®+RBV® for 12 weeks led to a 78% SVR12 rate. Treatment was well tolerated, with observed AEs consistent with expectations for a patient population with advanced liver disease. 9) In a large cohort international real-world study, all patients with unknown genotype, unknown fibrosis score and unknown treatment history were cured with EPCLUSA® for 12 weeks. In patients who inject drugs. Consistent outcomes defined as SVR12 between 91% and 100% in all patient subpopulations.
Our heartfelt wish for curing HBV, we present Vemlia.

Comparable antiviral efficacy vs. TDF\(^1\)

Improved safety profile in renal and bone parameters\(^2\)

Increased affordability with lower price, 2,474/tablet\(^{3*}\)

Improved patients' compliance with daily pill bottle\(^4\)

---

3. The data above are clinical data conducted with Tenofovir alafenamide hemifumarate.
6. *895 won lower price than Original drug (June 2023)
**Remarkable Response**

The ORR was more than three times higher with lenvatinib versus control group. Based on the masked IIR according to mRECIST, about 41% of patients showed $\geq 30\%$ decrease in tumor size.

**40.6\%**

**Response Rate**
(Masked IIR according to mRECIST)

---

**Table:**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lenvatinib (n=478)</th>
<th>Sorafenib (n=476)</th>
<th>ORR (95% CI)</th>
<th>P value</th>
</tr>
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<tr>
<td>Objective response</td>
<td>115 (24.1%, 20.2-27.9)</td>
<td>44 (9.2%, 6.6-11.8)</td>
<td>3.13 (2.13-4.56)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Masked independent imaging response</td>
<td>194 (40.6%, 36.2-45.6)</td>
<td>59 (12.4%, 9.4-15.4)</td>
<td>5.01 (3.59-7.01)</td>
<td>&lt;0.0001</td>
</tr>
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mRECIST: modified Response Evaluation Criteria in Solid Tumors; IIR: Independent Imaging review; ORR: Objective Response Rate; CI: Confidence interval; 95\%: presented as 95\% confidence interval; OR: Odds ratio; 95\% CI: Confidence interval

---

*ORR is one of the secondary endpoints and this is the result of the post-hoc exploratory tumour assessments using mRECIST by masked central independent imaging review. For more information, please refer to the full text of the article (Loiolo M. et al., 2018).*

**Image:**

- Lenvatinib is depicted in green and sorafenib in red. The graph shows the percentage of patients with a reduction in tumor size compared to the control group.

---

**Notes:**

- Lenvatinib treatment is expected to yield a greater objective response rate compared to sorafenib treatment.
- The ORR was statistically significant with lenvatinib.
- The masked independent imaging review was conducted by a central, independent imaging review committee.

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**References:**


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**Eisai Korea Inc.**

EISAI KOREA 10F Revensani, 6, Bongeunsa-ro 86-1, Gangnam-gu, Seoul, 06163, Korea TEL: 02-3461-5500
Liver Fibrosis Single Biomarker

**M2BPGi** (Mac-2 Binding Protein Glycosylation isomer)

**M2BPGi** forms in blood when hepatic fibrosis occurs

**Collect blood** for M2BPGi test

**Measure with full automated system**

![](sysmex.png)

- The only single biomarker that is approved reimbursement (Code: D1980)
- Pick up only 10μl of serum
- Test time 17min
- Included in the KASL clinical practical guidelines for managing NAFLD and CHB

**Subject & Utility of M2BPGi Test**

**Diabetes:** There is a high possibility of advanced hepatic fibrosis with an abnormal M2BPGi level (>1.0).¹

**NAFLD patients:** Serum M2BPGi could serve as a reliable biomarker for diagnosing advanced fibrosis and cirrhosis.²

**Liver fibrosis risk population:** Serum M2BPGi has proven to be a dependable, non-invasive surrogate marker for predicting advanced fibrosis.³

**CHB patients receiving long-term antiviral treatment:** The serum M2BPGi level functions as an independent predictor of HCC and complements the stratification of HCC risks.⁴

**CHB with oral antiviral therapy:** A baseline M2BPGi level above 1.73 consistently demonstrated predictive value for higher HCC risk.⁴

**TACE treatment for HCC:** The combination of M2BPGi and up-to-seven criteria could serve as a surrogate marker for predicting CP grade deterioration.⁵

**CHB:** The M2BPGi level can predict HCC development independently.

References


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www.sysmex.co.kr
 Quickly† deliver cure‡ with 8-week MAVIRET so your patients§ can move forward free from HCV.

*Free from HCV with cure. † For GT 1-4 treatment-naive, non-cirrhotic and compensated-cirrhotic patients, 8-week MAVIRET versus 12-week MAVIRET.
‡Sustained virologic response (SVR12), defined as HCV RNA less than the lower limit of quantification at 12 weeks after the end of treatment. GT 1-4 treatment-naive, non-cirrhotic and compensated-cirrhotic patients. MAVIRET is not indicated in decompensated cirrhosis.
§MAVIRET is contraindicated in patients with moderate or severe hepatic impairment (Child-Pugh B or C).

MAVIRET is indicated for the treatment of chronic hepatitis C virus (HCV) infection in adult and adolescent patients over 12 years of age.

References 1. MAVIRET™ Product Information Revised from 16th Feb 2021.
2. Product name/Maviret? Tab. Dose and quantity/Glumetrex 100.2mg, pibrentasvir 40.2mg.
3. Importer/Distributor/Achilles Korea, 6th Floor, Sapphire Building, No. 2, Seongdong-dong, Gangnam-gu, Seoul, Korea, Tel (02) 3429-9100, www.abbvie.co.kr
4. For the latest and full product information, please refer to the QR code or product information.
5. To report adverse events and side effects, call Konka Institute of Drug Safety & Risk Management (1644-4223 or 1644-3330, www.drugsafe.or.kr)

KR-MAVA-040213 / Mar 2024

abbvie
NEXT PIECE FOR BEST PEACE

Experience a better tomorrow with VEMILINO

VEMILINO, Effective for early stage and impaired renal function or decreased bone mineral density of hepatitis B patients.
Confidence for NAFLD treatment
Evidenced by numerous clinical results

GODEX® cap.

- Restoration of Hepatic Mitochondrial Dysfunction by Carnitine Complex
- Rapid Normalization of ALT Level
- Improving effect for NAFLD as Evidenced by CT scans

Product Information

- Description: Reddish brown colored hard gelatin capsule containing yellowish brown colored powder
- Composition: Each capsule contains Carnitine Orotate 150mg (73.8mg as orotic acid, 76.2mg as carnitine), Liver Extract Antitoxic fraction 12.5mg, Adenine HCl 2.5mg, Pyridoxine HCl 25mg, Riboflavin 0.5mg, Cyanocobalamin 0.125mg, Bp-hydroxymethyl dicarboxylate 25mg
- Indication: 1) General therapeutics for the following hepatic disease - Acute, Subacute and Chronic Hepatitis, Hepatic cirrhosis, Fatty liver, Drug or chemical induced hepatitis 2) Acute, chronic hepatitis involving high transaminase value
- Dosage & Administration: Usually, each time 2 capsules, 2~3 times a day as adult dosage. Dosage unit can be changeable depending on symptom or age of patient. Special caution: 1) Severe state of chronic hepatitis 2) Severe state of hepatic cirrhosis 3) General caution: 1) Rarely skin rash can be represented, in this case general antihistamin therapy will be required. 2) In severe case, sometimes intermittent jaundice can be occur in this case, discontinue administration for awhile and other adjuvant therapy for jaundice shall be required. 3) Rarely nausea, gastric discomfortness can be represented 4) Rarely itching or redness can be occur, in this case, discontinue administration and follow physician’s instruction. Insurance Code: 6939000080
- Packing Unit: 100, 300 caps. (bottle)/ 100 caps. (PTP)
- Storage: Keep closed container, room temperature (1~30°C) in dry place. Expiry: 60 months from Manufacturing date.

Diagnostic Codes

- B15-19: Viral hepatitis
- K70.0: Alcoholic fatty liver
- K71.0: Toxic liver disease
- K73.0: Chronic persistent hepatitis, NEC
- K74.0: Hepatic fibrosis
- K75.8: Other specified inflammatory liver disease
- K77.0: Liver disorders in disease classified elsewhere

*Celltrion Pharm* Domestic Business Division

1/F, West Building of IT Venture Tower, 135, Jungdan-ro, Songpa gu, Seoul, South Korea

Service Center: +82-80-850-1611~2

www.celltrion.com
LIVER LEADERSHIP
Unfinished journey in viral hepatitis

2022
EPCLUSA®

2022
VOSEVI®

2017
VEMLIDY®

2015
HARVONI®

2015
SOVALDI®

2011
VIREAD®

* 처방하시기 전에 반드시 허가사항 전문을 확인하여 주시기 바랍니다.
최신 허가사항은 QR 코드를 통해 확인하실 수 있으며, 길리어드 사이언스코리아 홈페이지(www.gilead.co.kr) 또는 의약품통합정보 시스템 (http://onedrug.mfds.go.kr)에서 확인하실 수 있습니다.

길리어드 사이언스 코리아(유)
서울특별시 중구 을지로5길 26 센터원빌딩 서관 15층
(대표전화: 02-6030-3300, 제품관련문의: 0079-814-800-9172 (수신자 부담))
VEMLIDY-for the flow of life with chronic hepatitis B

평생 관리해야 하는 만성 B형 간염1, 환자의 생애주기를 고려한다면2, Vemlidy®로 시작해 주세요.1,3,4


베믈리디®정 (테노포비르알라페나미드헤미푸마르산염)

QR 코드를 스캔하여 Vemlidy®PI 확인하십시오.
The new wave of GERD Treatment, P-CAB

FEXUCLUE
Fexuprazan hydrochloride

- Excellent nocturnal symptom control: Longest half-life
- Significantly improved chronic cough of EE
- Take once a day regardless of meal
- Full and fast onset of effect with the first dose
- Less affected by CYP2C19
- Low potential of DDI individual variations

HCC anticancer cellular Immunotherapeutics

Immuncell-LC
Anticancer cellular Immunotherapeutics
Recognize & Kill Cancer Cells

Reduction in Recurrence Risk
37%

Reduction in Mortality Risk
79%

- Product
  Immuncell-LC (Autologous Blood-Derived Lymphocytes)

- Active Pharmaceutical Ingredient (200ml)
  Number | Ingredient | Dosage | Unit | Specification
  1 | Autologous Blood-Derived Lymphocytes | 1.0x10^9~2.0x10^10 cells | Special Specification

- Physical
  A cloudy fluid of pale-yellow color contained in an opaque polyethylene or polyvinyl chloride bag

- Efficacy/Effect
  Adjuvant therapy in patients with tumor removal after hepatocellular carcinoma resection (surgery, radiofrequency ablation, percutaneous ethanol injection)

- Dose/Dosage
  Gently shake the bag 3-4 times before administration to ensure that the cells are fully suspended in the solvent. Administer the product intravenously with 22G or smaller needle within 1 hour. A single dose is 200 ml containing 1.0 x 10^9~2.0 x 10^10 cells, and the numbers and intervals of administration are as follows: 4 times, once a week 4 times, once every two weeks 4 times, once every four weeks 4 times, once every eight weeks 16 times in total

- Package Unit
  1 pack/box (200mL/pack)

- Reimbursement Information
  not reimbursed (676600031)

- Storage Method
  Store in a sealed container, at 2-25 ℃
  Shelf Life: 36 hours from the manufacturing date (36 hours from the time of manufacture)
Obtained ‘Exclusive Marketing Rights’!
First Generic of Sorafenib

Soranib was officially approved by MFDS on October 29th, 2020.

Treatment of hepatocellular carcinoma, thyroid carcinoma and renal cell carcinoma

Soranib Tab. 200mg on Market! (since December 1st, 2020.)
(Sorafenib tosylate(Micronized))

1. Obtained ‘exclusive marketing rights’
   by demonstrating bioequivalence to the original product
2. Accumulated more than 10 years of experience in prescribing Sorafenib
3. The First-generic to ease the burden of medication cost
4. Improved patient convenience by redesigning the package

Hanmi Pharm.
Korea’s first COMBIGEL type of Product for a treatment of mixed dyslipidemia
SK Albumin (Inj) 5%/20%

Human serum albumin

- Maintenance of Intravascular pressure
- Acid-base balance
- Drug transport
- Transport of ions, fatty acids, bilirubin and hormones

Summary of Prescribing information

[PRODUCT NAME]: SK Albumin 5% / 20% 

[CONTENT]: Each 100 mL contains 5 g and 20 g of Human Serum Albumin as active ingredient, for 5% [Inj] and 20% [Inj], respectively

[INDICATION AND USAGE]: Hypoalbuminemia caused by albumin loss (burn, nephrotic syndrome, etc.) and dysfunction of albumin synthesis (liver cirrhosis, etc.), hemorrhagic shock

[DOSAGE AND ADMINISTRATION]: 1-5% [Inj]: 500 mL, equivalent to human serum albumin 25 g should be administered by intravenous drip infusion or by slow direct intravenous injection. The recommended infusion rate is 2-4 mL/min. The dosage may be adjusted according to body weight, age and symptoms. 2-20% [Inj]: 125-375 mL, equivalent to human serum albumin 25-75 g should be administered by intravenous drip infusion or by slow direct intravenous injection. The recommended infusion rate is 2-4 mL/min. It may be diluted with 5% glucose when necessary. The dosage may be adjusted according to body weight, age and symptoms.

[CONTRAINDICATION]: Patients with a history of hypersensitivity reactions to this drug and its components.

[MANUFACTURER]: SK Plasma Co., Ltd. (13494) 310 Pangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

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[CONTRAINDICATION]: Patients with a history of hypersensitivity reactions to this drug and its components.

[MANUFACTURER]: SK Plasma Co., Ltd. (13494) 310 Pangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

* For the details, you are recommended to check on prescribing information. The latest approved label is available on the website following. http://drug.mnd.go.kr

Ramnos® not only strengthens intestinal health and immunity, but also improves atopic symptoms.
The next generation VCTE™ technology

FibroScan® by echosens

The non-invasive gold standard solution for comprehensive management of liver health

NEW

Guided VCTE
Seamless liver health assessment for all

AASLD  ADA  AHA  APASL  EASL
AACE  AGA  AISF  Baveno VII  NICE Guidance
**Antiviral effect of Besivo**
- Besivo has antiviral efficacy comparable to that of TDF after 48 weeks of treatment, with durable effects for 192 weeks.

**Tolerance of Besivo**
- Besivo had no drug-resistance mutation for 192 weeks.

**Safety data of Besivo**
- Besivo has a better safety profile than TDF*, in terms of bone and renal outcomes.

**Histological effect of Besivo**
- Besivo showed a significantly higher proportion of patients with improved histological scores** than TDF.

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**Indication and Usage**
- Treatment of chronic hepatitis B in adults

**DOSAGE AND ADMINISTRATION**
- One tablet containing 150 mg besifovir dipivoxil once daily orally with or without food in adults. When taking this medicine, take 660 mg of L-Carnitine together to prevent a decrease in serum L-Carnitine level. Patients with nephropathy. Patients with mild renal impairment: dose adjustment is not required. Patients with moderate, severe renal impairment: It is recommended to administer one tablet once every two days for moderate symptoms and one tablet every four days for severe symptoms are recommended. Patients with end-stage renal disease: administration of this drug is not recommended because there is no treatment experience.
CAUTION: The law restricts these devices to sale by or on the order of a physician. Indications, contraindications, warnings and instructions for use can be found in the product labelling supplied with each device. Products shown for INFORMATION purposes only and may not be approved or for sale in certain countries. This material not intended for use in France. 2022 Copyright © Boston Scientific Corporation or its affiliates. All rights reserved. PI-1429402.

WE 胃 KHA DO IT

Prokinetics in Functional Dysepsia proved in NEJM¹

Motility improvement from stomach to large intestine through peripheral D₂ Anagonist and AChE inhibitor mode of action²³


KHA is JW Pharmaceutical's digestive organ drug brand

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For more information on the product, please refer to the product manual for the headquarters website: www.jwpharm.co.kr
Novo Nordisk at a glance

Novo Nordisk is a leading global healthcare company, founded in 1923 and headquartered in Denmark.

- Products marketed in 170 countries
- Among the world’s 10 largest pharmaceutical companies measured by market cap
- Supply of nearly 50% of the world’s insulin
- Over 30M people use our diabetes care products

Novo Nordisk is dedicated to helping address the unmet needs of people.