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Volume 30 • Number 2 • April 2024

Review

135 Comprehensive approach to controlling chronic hepatitis B in China
Shan Shan, Xinyan Zhao, and Jidong Jia

Editorial

144 Cardiovascular risk of tenofovir disoproxil fumarate or tenofovir alafenamide in patients with chronic hepatitis B: More questions than an answer
Pin-Nan Cheng and Ming-Lung Yu

147 Toward hepatitis C virus elimination using artificial intelligence
Moon Haeng Hur and Jeong-Hoon Lee

150 Starting the journey: Understanding the roles of complement proteins in liver diseases through mendelian randomization
Mohammad Saeid Rezaee-Zavareh, Naomy Kim, and Ju Dong Yang

154 Enhancing off-nucleos(t)ide analogue outcome predictions in chronic hepatitis B with time-varying hepatitis B core-related antigen
Chen-Te Huang and Tai-Chung Tseng

157 Evaluation of the histological scoring systems of autoimmune hepatitis: A significant step towards the optimization of clinical diagnosis
Atsumasa Komori

160 Exploring the prognostic value of ultra-low-pass whole-genome sequencing of circulating tumor DNA in hepatocellular carcinoma
Ji Eun Han and Hyo Jung Cho

164 Linvencovir: Paving the way for functional cure in hepatitis B
Jiwon Yang and Jonggi Choi

166 JCAD, a new potential therapeutic target in cholestatic liver disease
Byoung Kuk Jang

168 Clinical impact of five cardiometabolic risk factors in metabolic dysfunction-associated steatotic liver disease (MASLD): Insights into regional and ethnic differences
Joo Hyun Oh and Dae Won Jun

171 Steatotic liver disease: Know your enemies
Lung-Yi Mak
174 The gene expression signature of metabolic dysfunction-associated steatotic liver disease from a multiomics perspective
Carlos Jose Pirola and Silvia Sookoian

Original Articles

177 Prognostic value of ultra-low-pass whole-genome sequencing of circulating tumor DNA in hepatocellular carcinoma under systemic treatment
Miguel Sogbe, Idoia Bilbao, Francesco P. Marchese, Jon Zazpe, Annarosaria De Vito, Marta Pozuelo, Delia D’Avola, Mercedes Iriarraidaregui, Carmen Berasain, Maria Arechederra, Josepmaria Argemi, and Bruno Sangro

191 Efficacy, safety, and pharmacokinetics of capsid assembly modulator linvencorvir plus standard of care in chronic hepatitis B patients
Jinlin Hou, Edward Gane, Rozalina Balabanska, WenHong Zhang, Jiming Zhang, Tien Huey Lim, Qing Xie, Chau-Ting Yeh, Sheng-Shun Yang, Xieer Liang, Piyawat Komolmit, Apinya Leerapun, Zenghui Xue, Ethan Chen, Yuchen Zhang, Qiaoqiao Xie, Ting-Tsung Chang, Tsung-Hui Hu, Seng Gee Lim, Wan-Long Chuang, Barbara Leggett, Qingyan Bo, Yue Zhou, Miriam Triyatni, Wen Zhang, and Man-Fung Yuen

206 JCAD deficiency attenuates activation of hepatic stellate cells and cholestatic fibrosis
Li Xie, Hui Chen, Li Zhang, Yue Ma, Yuan Zhou, Yong-Yu Yang, Chang Liu, Yu-Li Wang, Ya-Jun Yan, Jia Ding, Xiao Teng, Qiang Yang, Xiu-Ping Liu, and Jian Wu

225 Prognosis of biopsy-confirmed metabolic dysfunction-associated steatotic liver disease: A sub-analysis of the CLIONE study

235 Global incidence of adverse clinical events in non-alcoholic fatty liver disease: A systematic review and meta-analysis
Michael H. Le, David M. Le, Thomas C. Baez, Hansen Dang, Vy H. Nguyen, KeeSeok Lee, Christopher D. Stave, Takanori Ito, Yuankai Wu, Yee Hui Yeo, Fanpu Ji, Ramsey Cheung, and Mindie H. Nguyen

247 Identification of signature gene set as highly accurate determination of metabolic dysfunction-associated steatotic liver disease progression
Sumin Oh, Yang-Hyun Baek, Sungju Jung, Sumin Yoon, Byeonggeun Kang, Su-hyang Han, Gaeul Park, Je Yeong Ko, Sang-Young Han, Jin-Sook Jeong, Jin-Han Cho, Young-Hoon Roh, Sung-Wook Lee, Gi-Bok Choi, Yong Sun Lee, Won Kim, Rho Hyun Seong, Jong Hoon Park, Yeon-Su Lee, and Kyung Hyun Yoo
**Letter to the Editor**

263 Changing from NAFLD to MASLD: Similar prognosis of patients with HCC under atezolizumab/bevacizumab treatment between NAFLD and MASLD  
Hiroyuki Suzuki, Shigeo Shimose, Hideki Iwamoto, Takashi Niizeki, and Takumi Kawaguchi

266 Similar respiratory function including chronic obstructive pulmonary disease between non-alcoholic fatty liver disease and metabolic dysfunction-associated steatotic liver disease  
Tsubasa Tsutsumi, Dan Nakano, Machiko Kawaguchi, Hirokazu Takahashi, and Takumi Kawaguchi

269 Letter regarding "Hepatitis B core-related antigen dynamics and risk of subsequent clinical relapses after nucleos(t)ide analog cessation"  
Yun-Fan Liaw

**Correspondence**

272 Letter: Cardiovascular risk of tenofovir disoproxil fumarate or tenofovir alafenamide fumarate in patients with chronic hepatitis B: More questions than an answer – author’s reply  
Hyeyeon Hong and Jonggi Choi

274 Correspondence on Letter regarding "Toward hepatitis C virus elimination using artificial intelligence"  
Ming-Ying Lu and Ming-Lung Yu

276 Optimizing off-treatment outcome predictions: The potential of time-varying HBcrAg and the need for more research  
Ying-Nan Tsai, Jia-Ling Wu, and Yao-Chun Hsu

279 Both liver parenchymal and non-parenchymal cells express JCAD protein under various circumstances  
Li Xie, Li Zhang, Hui Chen, Yong-Yu Yang, and Jian Wu

281 Correspondence on Letter regarding "Prognosis of biopsy-confirmed MASLD: A sub-analysis of the CLIONE study."  
Hideki Fujii, Michihiro Iwaki, and Yoshihiro Kamada

284 In response to: Steatotic liver disease—know your enemies  
Michael H. Le, Linda Henry, and Mindie H. Nguyen

287 Correspondence on Editorial regarding "Identification of signature gene set as highly accurate determination of MASLD progression"  
Sungju Jung, Sumin Yoon, Jong Hoon Park, Yeon-Su Lee, and Kyung Hyun Yoo
291  Reply to: “Evaluation of the histological scoring systems of autoimmune hepatitis: A significant step towards the optimization of clinical diagnosis”
Haeryoung Kim and Sook-Hyang Jeong

293  Harnessing hepatitis B core-related antigen measurement to optimize posttreatment monitoring
Ying-Nan Tsai, Jia-Ling Wu, and Yao-Chun Hsu

Reply to Correspondence
297  Correspondence on Letter regarding “Both liver parenchymal and non-parenchymal cells express JCAD proteins under various circumstances”
Byoung Kuk Jang

Snapshot
299  Immunopathogenesis of liver fibrosis in steatotic liver disease
Chaerin Woo and Won-Il Jeong
Comprehensive approach to controlling chronic hepatitis B in China

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Hepatitis B virus (HBV) infection was highly endemic in China, where the prevalence of HBsAg was 9.7% in 1992. Comprehensive strategies, including universal infant hepatitis B vaccination with emphasis on timely birth-dose and 3-dose coverage, dramatically reduced the mother-to-infant transmission and early childhood acquisition of HBV, resulting in estimated HBsAg prevalence rates of 5.6% and 0.1% in the general population and among children aged <5 years in 2022, respectively. Clinical guidelines on the prevention and treatment of chronic hepatitis B have been periodically updated based on emerging evidence from clinical research. The continuously improved reimbursement policy and the massively reduced price of antiviral drugs through government negotiation and central procurement have increased treatment accessibility and affordability. However, due to the low rates of diagnosis and treatment, China still faces a large challenge in achieving the 2030 goal of lowering HBV-related mortality by 65%. A public health approach involving concerted efforts from the government, medical community, industry, and society as a whole would be necessary to increase the uptake of HBV tests and treatment to achieve the global goal of eliminating viral hepatitis as a public health threat by 2030.

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Keywords: Hepatitis B; Prevention; Treatment; Health insurance reimbursement; China

INTRODUCTION

Chronic hepatitis B (CHB) poses a tremendous public health burden worldwide. Progression to cirrhosis or hepatocellular carcinoma (HCC) occurs in approximately 15–40% of untreated people with chronic infection of hepatitis B virus (HBV). The World Health Organization (WHO) estimated that the global seroprevalence of HBsAg was 3.8%, with 296 million people being chronically infected with HBV and nearly 820,000 people dying due to HBV-related disease in 2019.

China bears a heavy CHB burden and has one-third of the world’s infected population. To combat this infectious disease, China initiated universal infant hepatitis B vaccination (HepB) in 1992. With the increasing coverage rates of the full 3-dose regimen and a timely birth dose (TBD) of HepB, the prevalence of HBsAg has steadily declined in recent decades. However, the HBV-related disease burden is still high due to the enormous population and large number of people who have already been chronically infected with HBV. In this review, we describe the comprehensive approach to the control of CHB in China.
UNIVERSAL HepB VACCINATION IN INFANTS WITH EMPHASIS ON A TIMELY TBD

Since mother-to-child transmission (MTCT) and early childhood HBV infection play major roles in the high prevalence of hepatitis B surface antigen (HBsAg), China initiated a universal infant HepB immunization program in 1992, which recommends TBD within 24 hours of birth and second and third doses at one month and six months after birth, respectively (Fig. 1).6,7

In 2002, China integrated HepB vaccination into the Expanded Program on Immunization (EPI) and started to offer free HepB vaccines to all newborns, with the family paying only a small amount of the injection fee. In 2005, the government began to provide completely free HepB vaccination services to all newborns. The China Global Alliance for Vaccines and Immunization Project was conducted from 2002 to 2009 and delivered HepB to approximately 68 million children in hard-to-reach populations.7,8

In addition, special projects were conducted in the resource-constrained western provinces from 2005 to 2009; these projects provided funding for training health care workers and subsidies for incentivizing baby delivery in facilities. These efforts increased the facility delivery rate (in township or county-level hospitals) from 43% to 97%, thereby increasing the rate of TBD of HepB from 82% to 88% in the resource-constrained western provinces.9

Owing to these efforts, three-dose HepB coverage tripled from 30.0% in 1992 to 99.6% in 2015, and the TBD rate increased from 22.2% to 95.6% during the same period.10 As the coverage of HepB has steadily improved, disparities in the rates of 3-dose coverage and TBD coverage across regions and urban/rural areas were also eliminated by 2014.6 As a result, the prevalence rate of HBsAg among children under five years dramatically declined from 9.67% in 1992 to only 0.32% in 2014.6 The results of the new nationwide seroprevalence survey conducted in 2020 have not been officially published. However, a modeling study estimated that the HBsAg prevalence in China was 5.6% in the general population and 0.1% among children <5 years of age in 2022 (Fig. 2).5,6,8,11

PREVENTION OF MTCT THROUGH A MATER-NAL-INFANT SERVICE PROGRAM

There is still a large population of HBsAg-positive women of child-bearing age in China. Indeed, it was reported that there were approximately one million infants were born to HBsAg-positive mothers in 2015.12 Consequently, a nonnegligible proportion of infants still acquire HBV infection from HBsAg-positive mothers, despite receiving a TBD and three doses of HepB. To further reduce the MTCT rate of HBV, a birth dose of hepatitis B immunoglobulin (HBIG) is administered to infants born to HBsAg-positive mothers.13 In 2011,
China launched a program of Triple Elimination of MTCT for human immunodeficiency virus (HIV), syphilis, and HBV, which provides complementary screening for HBsAg to all pregnant women and free HBIG to neonates born to HBsAg-positive mothers (Fig. 1).13,15

Finally, starting oral antiviral therapy in the second or third trimester of pregnancy in women with high HBV DNA levels could reduce the MTCT of HBV, as demonstrated by observational studies and a randomized controlled trial.16 Later, this approach was recommended by the WHO guidelines for the prevention of MTCT of HBV. A real-world study (Shield Project Stage 1) showed that with comprehensive management, including passive and active immunoprophylaxis of infants and antiviral prophylaxis of mothers, the overall rate of HBV MTCT decreased to 0.9%.17

To standardize the clinical management of preventing MTCT of HBV and further reduce HBV infection among infants, the China Foundation for Hepatitis Prevention and Control published an expert-recommended algorithm for the prevention of MTCT of HBV in 2022.13 This updated algorithm suggests tenofovir disoproxil fumarate (TDF) administration to pregnant women starting at 28 weeks of gestation after informed consent; tenofovir alafenamide fumarate (TAF) could also be used for pregnant women who have or are at high risk for osteoporosis or renal function issues.13

PREVENTION OF HBV TRANSMISSION BY PROMOTING SAFE INJECTION PRACTICES AND BLOOD PRODUCTS

In China, horizontal transmission also plays a role in HBV infection, which is acquired later in life, although it is estimated that up to half of chronic HBV infections occur via MTCT or horizontal transmission in early childhood.18 The elucidation of factors associated with chronic HBV infection may help inform targeted screening in the general population. Indeed, a meta-analysis demonstrated that a set of factors, including middle age, male sex, being married, rural residence, lower educational level, smoking, having an HBsAg-positive household contact or family history of HBV, and history of surgery or blood transfusion, was significantly associated with higher HBsAg prevalence.19 Since 1998, China has banned paid blood donors and has conducted stringent screenings of volunteer blood donors, with all donated blood being tested for HBV DNA since 2015.6

Injection safety has also improved in the last two decades. The government issued a rule banning the reuse of nonsterilizable medical devices in 2000, and the Chinese Medical Association published clinical guidelines for injections and other skin-piercing procedures in 2005. In 2007, auto-disposable syringes became available for vaccine injections. By 2010, all reusable injection equipment was eliminated in China.20,21 Harm reduction interventions for people who inject drugs (PWID) have also been integrated into comprehensive HBV

![Figure 2. Increasing HepB coverage and decreasing HBsAg prevalence in China. HepB, hepatitis B vaccination; HBsAg, hepatitis B surface antigen.](http://www.e-cmh.org)
prevention strategies in China. A systematic review of studies published from 2008–2017 revealed that the pooled HBsAg prevalence was 19.6% (95% confidence interval 13.7–25.5%) for PWID in China, with the highest occurring in South China (25.3%, 14.6–36.0%), followed by Central China (20.8%, 17.4–24.1%), and the lowest occurring in North China (15.9%, 12.8–19.4%) (Table 1). A study showed that the annual number of needles and syringes distributed was estimated to be 208 per PWID, and the rate of safe injection use among PWID was 86.5% in 2015.

**CLINICAL RESEARCH PROVIDES HIGH-QUALITY EVIDENCE FOR CHB GUIDELINES**

In the past two decades, there has been steady growth in hepatology research and publications from China. The continuous support of the National Natural Science Foundation of China (NSFC) has been instrumental to such progress. From 1986 to 2017, the NSFC provided a total of 3.67 billion RMB (Chinese Yuan) to 8,587 liver research projects, with HBV projects accounting for 12.2% of the total number of projects (1,044/8,587). In addition, since 2008, major research programs on HIV, viral hepatitis, and tuberculosis have been initiated in China, which has supported hundreds of research projects that yielded high-quality clinical evidence on HBV prevention and treatment.

The guidelines on the prevention and treatment of CHB were first jointly published by the Chinese Society of Hepatology and the Chinese Society of Infectious Diseases in 2005 and updated in 2010, 2015, 2019, and 2022 (Fig. 3). Nation-wide educational activities have been conducted to promote the implementation of the HBV guidelines, especially in rural and resource-constrained areas.

The newly updated CHB guidelines recommend treating all patients with viremia, together with elevated alanine aminotransferase levels, or any evidence of cirrhosis/advanced liver fibrosis (by invasive or noninvasive measurements), age ≥30 years, or family history of HBV-related cirrhosis or HCC. The current Chinese guidelines recommend first-line nucleotide analogs (NAs), including entecavir (ETV), TDF, TAF, tenofovir amibenamide and pegylated interferon alpha (PEG-IFNα), for patients with CHB. PEG-IFN-α is now classified as Category B (partially reimbursable) in the reimbursement list of basic social medical insurance in China, yet the exact proportion of reimbursement differs across cities/provinces, depending on their local policies, which are mainly determined by economic status.

Notably, over the last two decades, the application of PEG-IFN-α for CHB treatment in China has evolved considerably. Currently, the notion of highly selected patients with favorable baseline predictors (HBV DNA suppression, HBeAg seroconversion, and very low levels of HBsAg) is widely recognized in the hepatology community. PEG-IFN-α is indicated for treatment-naive patients or those already receiving NAs as monotherapy, add-on therapy, or de novo combination therapy. Treatment durations might extend beyond the standard of 48 weeks to 72, 96, or even 120 weeks.

<p>| Table 1. Estimate of the prevalence of HBsAg among PWID in China by region (2008–2017) |</p>
<table>
<thead>
<tr>
<th>Regions</th>
<th>Prevalence of HBsAg 95% UI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mainland China</td>
<td>20.2 (14.1–26.2)</td>
</tr>
<tr>
<td>North</td>
<td>15.9 (12.8–19.4)</td>
</tr>
<tr>
<td>Northeast</td>
<td>-</td>
</tr>
<tr>
<td>Northwest</td>
<td>18.0 (2.6–38.7)</td>
</tr>
<tr>
<td>Central</td>
<td>20.8 (17.4–24.1)</td>
</tr>
<tr>
<td>East</td>
<td>18.4 (0.0–37.0)</td>
</tr>
<tr>
<td>South</td>
<td>25.3 (14.6–36.0)</td>
</tr>
<tr>
<td>Southwest</td>
<td>18.3 (5.1–31.5)</td>
</tr>
<tr>
<td>China Hong Kong, Macao, and Taiwan</td>
<td>8.1 (3.3–15.9)</td>
</tr>
<tr>
<td>All of China</td>
<td>19.6 (13.7–25.5)</td>
</tr>
</tbody>
</table>

Values in parentheses are 95% uncertainty interval.
HBsAg, hepatitis B surface antigen; PWID, people who inject drugs.
in highly selected patients with favorable baseline (HBsAg <1,500 IU/mL) and on-treatment (HBsAg <200 IU/mL) features for a greater chance of HBsAg loss.38

REIMBURSEMENT POLICY AND MASSIVE PRICE REDUCTIONS FOR ANTIVIRALS

Current antiviral drugs can suppress HBV replication and reduce liver disease progression, thus improving the long-term outcomes of CHB patients.39,40 However, the high price and limited reimbursement for HBV therapy used to be major hurdles to its wide clinical use.41

To improve the accessibility of antiviral therapy for HBV, NAs and IFN-α (conventional and PEG-IFN-α) have been included on the reimbursement list since 2010. By 2017, ETV, TDF, TAF and PEG-IFN-α were all included in the updated national list of reimbursable medicines in China.42 The reimbursement criteria for antiviral therapy for CHB have evolved considerably in China. In general, the reimbursement criteria reflect the updated treatment guidelines, with only minor variation at the city or province level.

In 2019, the General Office of the State Council of the People’s Republic of China issued the National Centralized Drug Procurement (NCDP) policy to improve the accessibility and affordability of medicines.43 This was the first attempt at nationwide volume-based drug (mainly generic) procurement in the mainland of China, aiming at providing high-quality drugs at lower prices. Based on the data from the National Healthcare Security Administration, before the implementation of the NCDP policy, the average per person-year prices of the original brand names of ETV, TDF, and TAF were 650 RMB (90.90 USD), 1139 RMB (159.30 USD) and 6480 RMB (906.29 USD), respectively. These figures dramatically declined to 70 RMB (9.79 USD), 180 RMB (25.14 USD) and 360 RMB (50.35 USD) per person-year for the corresponding generic drugs after the adoption of the NCDP policy (per the People’s Bank of China, the exchange rate of RMB to USD was 7.15 to 1 on November 27, 2023). Moreover, the prices of brand name ETV, TDF, and TAF have also dramatically decreased.44 As a result, the NCDP policy in China has substantially reduced drug costs, increasing the accessibility and affordability of antiviral treatment (Fig. 3).

As shown by data analysis of the China Registry of Hepatitis B (CR-HepB), the proportion of ETV and TDF usage in those who received any antiviral agents had increased from 13.5% in 2003 to 79.7% in 2016.45 Basic social medical insurance coverage of first-line medicines for antiviral treatment was associated with a substantially reduced risk of liver-related death, from 0.38% to 0.16% for patients with noncirrhotic CHB and from 4.03% to 3.39% for those with compensated cirrhosis in Beijing, China.46

CHALLENGES IN REDUCING THE HEAVY DISEASE BURDEN OF CHB

One study showed that 98.22% of hepatitis-related deaths from liver disease in China were attributed to HBV-related cirrhosis/other chronic liver diseases (26.04%) and HBV-related liver cancer (72.18%) in 2019.47 Analysis of the Global Burden of Diseases (GBD) data showed that HBV accounted for 68%
of cirrhosis cases and 65% of HCC cases in China.48,49 The main indications for liver transplantation in China are HBV-related cirrhosis, with or without acute-on-chronic liver failure (ACLF) and HCC. HBV infection was the most common cause of ACLF (66.24%), followed by alcoholic liver disease (10.56%), autoimmune liver disease (6.53%) and hepatitis C virus infection (2.25%).50 Although a favorable trend in the mortality of liver disease due to hepatitis B was observed between 1990 and 2019,51 China still faces challenges in achieving the WHO’s goal by 2030.52

The prevalence and magnitude of reduction in chronic HBV infection vary by geographical region and across other strata of the Chinese population. In the mainland of China, the highest prevalence in the latest study period was observed in the Xizang Autonomous Administrative Region (formerly known as Tibet), followed by other provinces mostly clustered in southern China (Hainan, Jiangxi, Fujian, Guangxi, Guangdong and Qinghai), whereas the lowest seroprevalence was observed in Beijing and Shanxi.53 This geographical heterogeneity in HBsAg prevalence is consistent with that observed in a recently published study among nearly 100 million pregnant women across 31 provinces in China.54 The various HBV infection rates could be explained by several factors, but the major factor may be the difference in the rates of 3-dose coverage and TBD coverage of HepB after the implementation of the universal infant HepB program. In 1999, a national EPI review showed that in Beijing, the coverage rates of TBD and three-dose HepB among 1-year-old children were 69.0% and 99.0%, respectively, whereas in Xizang, the coverage rates for 2-year-old children were only 7.8% and 2.1%, respectively.55 As mentioned above, disparities in the 3-dose coverage rate and TBD coverage rate across regions and urban/rural areas were largely eliminated by 2014.5

In 2022, it is estimated that in China, there were still 79,747,000 people living with chronic HBV infection; however, only 19,131,000 (24%) were diagnosed, and 5,076,000 of 33,874,000 (15%) who were potentially eligible for treatment were actually receiving treatment.11 Therefore, efforts should be focused on increasing the coverage of the diagnosis and treatment of hepatitis B in China.

A modeling study estimated that if we treat only the few millions of individuals with CHB who are already on treatment, HBV-related cirrhosis/HCC mortality will continue to increase over the next two decades; if we scale up test-and-treatment strategies and treat most or even all people who need treatment, HBV-associated mortality will dramatically decline, which is cost-effective or even cost-saving.56 Another modeling study also demonstrated that China would spend $55 billion USD extra money and lose 334,000 more lives by a 1-year delay in achieving the 80% treatment goal by 2030.57

**FURTHER EFFORTS TO ELIMINATE VIRAL HEPATITIS AS A PUBLIC HEALTH THREAT BY THE 2030**

A modeling study by Su and colleagues showed that universal screening for HBV among adults aged 18–70 years is cost-effective and could identify most infected individuals in China.58 Another modeling study showed that treating all HBsAg-positive individuals aged 18–80 years could facilitate the achievement of the 2030 target of a 65% reduction in HBV-related deaths and is cost-effective.59

To increase the diagnostic rate of CHB and scale up diagnosis and treatment, the following measures can be taken. First, advocacy is necessary to increase awareness of the HBV-related disease burden and convey that antiviral therapy is cost effective.57 Second, the treatment algorithm should be simplified with expanded antiviral indications as recommended in the updated guidelines.54 Third, capacity building should be strengthened by conducting continuous medical education to train local doctors in remote or economically underprivileged areas to promote the new treatment guidelines. Fourth, an HBV-specific notification and reporting system should be established to monitor the diagnosis and treatment rates and facilitate evidence-based decision-making. Finally, pilot microelimination programs should be implemented at the township, countywide, or even city/province level to explore the feasibility of large-scale testing and treatment initiatives, including capacity building, task shifting, and link-to-care models, by exploiting instant communication and social media apps.

In summary, China has adopted a comprehensive strategy involving the motivational investment of various resources and achieved great success in HBV prevention and control. However, China still faces a large challenge in reducing HBV mortality through large-scale testing and treating the large number of people who are already infected with HBV. The concerted efforts of the government, medical community, industry, civil society, and the public are key to eliminating
CHB as a public health threat by 2030 in China.

Authors’ contribution
Drafting of the manuscript: Shan Shan and Xinyan Zhao; Critical revision and finalization of the manuscript: Jidong Jia.

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Conflicts of Interest
The authors have no conflicts to disclose.

REFERENCES


Cardiovascular risk of tenofovir disoproxil fumarate or tenofovir alafenamide in patients with chronic hepatitis B: More questions than an answer

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\textbf{Keywords:} TDF; TAF; HBV; SDG 3; Good health and well-being

Hepatitis B virus (HBV) infection has been associated with lower cardiometabolic risks and even a protective factor for major adverse cardiovascular events (MACE) when compared with hepatitis C virus infected patients or controls.\textsuperscript{1,2} Antiviral treatment also provides a lower risk of MACE than those without antiviral treatment in patients with chronic hepatitis B.\textsuperscript{3} As the lipid-lowering effect has been unexpectedly and uniquely observed in patients treated with tenofovir disoproxil fumarate (TDF) but not in those treated with tenofovir alafenamide (TAF), their impact on long-term MACE has been raised. A recent retrospective study by Hong et al. investigated the risk of MACE in patients with chronic hepatitis B (CHB) treated by TDF or TAF. After propensity score matching to control confounding factors, a comparable risk of long-term MACE between TDF- and TAF-treated patients was observed despite different lipid profiles between the two groups.\textsuperscript{4} This study provides important information to some degree to answer the uncertain association of TDF or TAF with cardiovascular outcomes. However, unresolved issues remain and need to be investigated.

In Hong’s study, significantly lower lipid profiles were observed in TDF-treated patients rather than in TAF-treated patients,\textsuperscript{5} which was consistent with a previous study that showed TDF reduced lipid profiles in HBV patients, when compared to TAF.\textsuperscript{6} A recent study further demonstrated that the lipid profiles greatly increased in HBV patients switching from TDF to TAF, but not in patients switching from entecavir to TAF.\textsuperscript{7} Meanwhile, the presence of metabolic traits, including gain of body weight, worsening of insulin resistance, and
a trend towards increased atherosclerotic cardiovascular disease scores, may possibly predispose to cardiovascular diseases in TDF-switch patients. These results indicated that TDF and TAF may exert effects on metabolic risk factors other than lipid profiles. In contrast to the lipid-lowering effects of TDF, TAF monotherapy could greatly increase lipid profiles when compared with entecavir monotherapy. In patients with human immunodeficiency virus infection, the TAF-containing regimen did show an increase in lipids compared to the non-TAF-containing regimen. The two phase 3 studies also showed progressively increased low-density lipoprotein and triglycerides, but decreased high-density lipoprotein during 5-year TAF treatment for CHB patients. These results may imply that TAF alone may induce changes in lipid profiles, increasing the risk of MACE. Therefore, the investigations on mechanisms of lipid changes associated with TDF or TAF treatment are important not only to clarify the mechanisms of changes in lipid profiles but also to understand the detailed changes in lipid components that are beneficial or harmful to the occurrence of MACE. From these points of view, more studies addressing the association between TDF/TAF and MACE are needed.

The established common risk factors for MACE include hypertension, diabetes, obesity, hyperlipidemia, tobacco smoking, a sedentary lifestyle, and a lack of adequate physical activities. As expected, Hong’s study showed that active smoking and a history of cardiovascular events were the two independent factors associated with an increased risk of MACE. As the HBV infection has been known not to be a risk factor for MACE, the association between long-term TDF/TAF treatment and changes in lipid profiles may influence or add impacts on cardiovascular risk for certain degree, if any, but not serve as one of the major determinants. For long-term outcomes in CHB patients, liver-related complications remain the main causes of morbidity and mortality. In addition to chronic viral hepatitis, metabolic derangements also contribute to the progression of liver diseases. Traditional risk factors for developing MACE still play a central and important role. Furthermore, HBV infection has been reported to be inversely associated with hepatic steatosis. As MACE and metabolic dysfunction-associated fatty disease (MAFLD) share common risk factors, cardiovascular disease is the leading cause of mortality in patients with MAFLD. Patients with simultaneous CHB and MAFLD tend to have accelerated progression of liver disease, exhibit more liver-related complications, and have a higher death rate than patients with CHB alone or MAFLD alone. In Hong’s study, the prevalence of HBV patients with the diagnosis of fatty liver was much lower than the reported prevalence in the general population of Korea (15.4% vs. 32.9%). The impact of MAFLD on MACE may be underestimated; hence, the effect of TDF/TAF on MACE is supposed to be further minimized in CHB patients. In this aspect, risk factors for MACE may play a more dominant role. Moreover, Hong’s study conducted a retrospective design with chart review in one medical hospital, which may introduce selection bias and confounding factors. The cohort effect due to the late introduction of TAF to the market might also contribute to the imbalance in the proportion of patients taking lipid-lowering agents and the misinterpretation of the effect on MACE between the TAF- and TDF-treated groups.

There are several issues that need to be further clarified. First, long-term use of TDF/TAF is always required for the majority of CHB patients. Safety concerns should be taken into account. Unclear mechanisms of TDF/TAF make the impact of lipid changes on MACE uncertain. At present, whether the lipid profiles related to TDF/TAF are truly “benefit” or “bad” for MACE remains to be resolved. Second, the risk of MAFLD in CHB patients warrants stratification of MACE risk according to the grade of cardiometabolic risk factors. In the current era, metabolic derangements contribute to diseases of different organs and have become an important determinant of outcome measurements. Evaluation of MACE risk in the presence of cardiometabolic risk factors is warranted in the long-term care of CHB patients under antiviral therapy.

Authors’ contribution
PN Cheng drafted the manuscript. ML Yu reviewed and finalized the manuscript.

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Abbreviations:
HBV, hepatitis B virus; MACE, major adverse cardiovascular events; TDF, tenofovir disoproxil fumarate; TAF, tenofovir alafenamide; CHB, chronic hepatitis B; MAFLD, metabolic dysfunction-associated fatty disease
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Conflicts of Interest
The authors have no conflicts to disclose.

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Toward hepatitis C virus elimination using artificial intelligence

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Keywords: Hepatitis C virus; Antiviral; Sustained virologic response; Machine learning

Since the introduction of direct-acting antivirals (DAAs), the treatment paradigm for hepatitis C virus (HCV) infection has changed, leading to the World Health Organization’s agenda to reduce new HCV infections by 90% and deaths by 65% between 2016 and 2030. However, despite the high potency of DAAs regardless of HCV genotypes, about 1–3% of patients with chronic hepatitis C still fail to achieve a sustained virologic response (SVR). Decompensated liver cirrhosis, hepatocellular carcinoma (HCC), and high HCV RNA levels are associated with a greater risk of SVR failure. As patients who do not attain SVR should be considered for rescue therapy, predicting and responding to SVR failure in advance can play a critical role in achieving HCV elimination goals.

Under these circumstances, in the article accompanying this editorial, Lu et al. developed and validated an artificial intelligence (AI) model to predict DAA treatment failure using a nationwide cohort in Taiwan. The Taiwan HCV Registry database is a multi-center, prospective cohort that has enrolled over 30,000 patients with chronic hepatitis C receiving DAA treatment with available SVR data. This database includes various baseline demographic information and virologic factors before and after DAA treatment, and a total of 55 host and virologic factors were incorporated in the model development. The authors constructed several models using different machine learning algorithms and showed that a model employing Extreme Gradient Boosting (XGBoost) was more effective in predicting DAA treatment failure compared to other algorithms and a model based on traditional statistics (i.e., logistic regression). The XGBoost algorithm demonstrated accuracy, specificity, positive predictive value, and negative predictive value greater than 97%. The AI model detected 69.7% of the subjects who failed to achieve SVR among the top five decile subgroups. In a multivariable regression analysis, liver cirrhosis, HCC, poor compliance with DAA, and high hemoglobin A1c levels were identified as independent risk factors for SVR failure. The AI model showed that patients with higher fibrosis-4 index, bilirubin, aspartate aminotransferase, and alpha-fetoprotein levels, as well as lower albumin and platelet levels, were less likely to attain SVR. It is consistent with the result of multivariable regression analysis, as these variables are known risk factors for the development of...
liver cirrhosis or HCC.

This model may contribute to an individualized decision-making process for antiviral treatment in patients with chronic hepatitis C. Patients with decompensated cirrhosis are less likely to achieve SVR compared to those with compensated cirrhosis. International guidelines vary in their recommendations for the initiation of DAA therapy in patients with decompensated liver cirrhosis, based on liver function assessed by the Child-Pugh score or Model for End-Stage Liver Disease (MELD) score. In general, patients with a significantly higher MELD score should receive liver transplantation followed by DAA treatment, while those with a lower MELD score and no planned liver transplantation are recommended to be treated with DAs first. However, patients in the gray zone should be managed on a case-by-case basis. In this case, the risk of DAA treatment failure predicted by the AI model can help patients and physicians make appropriate decisions. If the risk of SVR failure calculated by the AI model is significantly high, it may be in the patient’s best interest to defer treatment, given the side effects and costs of DAA therapy. Chronic hepatitis C patients without decompensated liver cirrhosis who are at high risk of DAA treatment failure as predicted by the AI model may also benefit from a more potent combination of antivirals than is currently recommended by clinical guidelines to increase SVR rates and prevent the development of DAA resistance. In addition, high-risk patients who are likely to fail on existing treatment recommendations can be considered for early enrollment in clinical trials.

The risk of DAA treatment failure is likely to be determined by a combination of several variables, such as disease-related factors (e.g., serum HCV RNA level and presence of fibrosis) and host factors (e.g., age, sex, and comorbidities). It is possible to train machine learning models to identify complex non-linear associations between variables, which are challenging to capture through traditional statistical methods. Considering the heterogeneity of risk variables and patient populations, a more comprehensive model using AI may be better at accurately stratifying patients with different levels of risk, thereby providing individualized treatment strategies.

The AI model in this study utilized multiple variables from a large cohort and demonstrated impressive performance in predicting DAA treatment failure. The model showed higher predictive power than a model using conventional statistics, as well as another machine learning model that had been developed under similar conditions. A machine learning model developed to predict DAA treatment failure in the HCV-TARGET registry of patients in North America and Europe showed a predictive power of c-index of 0.69 in its validation cohort. Despite the fact that these are two distinct cohorts, which complicates a direct comparison and necessitates cross-validation between them, the model in the current study achieved a good accuracy of AUROC 0.803 in the validation cohort. This impressive performance might be attributed to the larger sample size. A large number of events are required to appropriately train an AI model. Since the rate of DAA failure is relatively low, the authors utilized a nationwide cohort to ensure that the number of DAA failure events was large enough to train the model. In fact, SVR failure was confirmed in 538 individuals, accounting for 1.6% of the entire study population.

While the AI models in this study demonstrated impressive predictive accuracy, it is important to note that there was a significant decrease in model performance in the validation cohort compared to the training cohort. The superior performance of AI models over logistic regression models was maintained in both the training and validation cohorts. However, while the performance of the logistic regression models did not differ significantly between the two cohorts (rather, it slightly increased in the validation cohort), the decrease in the performance of the AI models in the validation cohort suggests the presence of overfitting. In other words, the AI models may be overtrained in the training cohort and less accurate than expected in other populations. Although this model has been developed in a large cohort, it is limited by the fact that the study was conducted in a single ethnically homogeneous country. Therefore, external validation of this AI model in another independent international cohort is required.

In conclusion, this AI model can be used to identify patients with chronic hepatitis C who are susceptible to SVR failure.
and to recommend more intensive antiviral therapy than is recommended by current guidelines. In patients with decompensated liver cirrhosis, the AI model may also help determine the optimal timing for DAA treatment. However, given the generalizability issue of this model, it needs to be validated in another international cohort.

**Authors’ contribution**

Conceptualization, J-H Lee; Original draft, MH Hur and J-H Lee; Review and editing, MH Hur and J-H Lee.

**Conflicts of Interest**

MH Hur has no conflict of interest to disclose. J-H Lee receives research grants from Yuhan Pharmaceuticals and GreenCross Cell, and lecture fees from GreenCross Cell, Daewoong Pharmaceuticals, and Gilead Korea.

**REFERENCES**

Starting the journey: Understanding the roles of complement proteins in liver diseases through mendelian randomization

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Keywords: Liver diseases; Hepatocellular carcinoma; Complement system proteins; Mendelian randomization; Drug repositioning

With the increasing prevalence and burden of metabolic dysfunction-associated steatotic liver disease (MASLD) and alcohol-associated liver disease (ALD), the burden of chronic liver diseases (CLDs) and their associated complications, including cirrhosis and liver cancer, is increasing.¹ Global MASLD prevalence is estimated to be about 30%, with more than 50% increase from 1990–2006 (25.26%) to 2016–2019 (38%).² ALDs are also widely prevalent types of CLDs, and about half of the deaths related to CLDs are thought to be caused by heavy alcohol consumption.¹ Hepatocellular carcinoma (HCC), as one of the important complications of CLDs, is considered the main type of primary liver cancer and is ranked the third leading cause of cancer death worldwide.⁴ A deeper understanding of CLDs in various aspects, including their risk factors and new pharmacological targets for their treatment, can help to decrease their burden.

Previous studies have highlighted the critical roles of complement proteins in hepatic regeneration, liver injuries, and diseases such as ALD, MASLD, and autoimmune hepatitis (AIH).⁵,⁶ These proteins, essential in the innate immune system, function through tightly regulated enzymatic cascades and are primarily produced by the liver.⁶,⁷ The liver is susceptible to complement-mediated injury, as alterations in complement levels from liver disease itself can contribute to the exacerbation of liver disease pathogenesis. While complement proteins can be protective, promoting hepatocyte regeneration and liver cell proliferation and survival,⁸ their activation can also lead to increased inflammation and liver injury, amplifying local inflammation and potentially leading to fibrosis and cirrhosis.⁷ In HCC, complement proteins have been implicated in tumorigenesis, metastasis, and immune suppression, suggesting that targeting these pathways could probably offer new therapeutic avenues.⁹ The presence of
complement proteins in various cell types, such as platelets and neutrophils, underscores their importance in the immune response to injury and pathogens, but also highlights how their rapid mobilization can exacerbate liver disease progression.\textsuperscript{11,12} Given their important role in liver diseases, research into complement-targeted therapies holds promise for developing more effective treatments targeting CLD and HCC.

Although randomized controlled trials are considered the gold standard for establishing causal relationships between outcomes and exposures, performing such studies is not always possible due to various reasons. Therefore, Mendelian randomization (MR) serves as a method for causal inference using observational data and single nucleotide polymorphisms as instrumental variables to infer causal relationships. Although MR cannot replace RCTs, it can provide complementary information.\textsuperscript{13,14} There are several MR studies that have reported the causal association between different complement components and certain diseases such as deep vein thrombosis,\textsuperscript{15} ischemic stroke,\textsuperscript{16} periodontitis,\textsuperscript{17} multiple myeloma,\textsuperscript{18} chronic prostatitis,\textsuperscript{19} and Alzheimer’s disease.\textsuperscript{20} As establishing a causal relationship for the effect of complement components in causing liver diseases can be challenging due to the aforementioned reasons (e.g., inverse causality), MR studies can be useful in this regard. So far, no MR studies have assessed the relationship between complement components and CLDs.

Shi et al.\textsuperscript{21} in a recently published paper assessed the causal association between 28 circulating complement components and CLDs, including alcohol-related cirrhosis (ALC), MASLD, and three major autoimmune liver diseases, namely AIH, primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC), and HCC, through two-sample MR. In this study, the authors found a significant association between genetically predicted complement component levels of C1q C chain (C1QC) with AIH, C8 gamma chain (C8G) and complement factor H-related protein 5 (CFHR5) with PSC, CFHR1, CFHR2, and C1QC with ALC, C8G with MASLD, and finally CFHR2, C7, and C15 with HCC using the inverse-variance weighted (IVW) method. No significant association was reported between any of the 28 evaluated complement components and PBC. Additionally, when evaluating causal association with the weighted median (WM) method, only a significant association for ALC with CFHR1 and CFHR2, and for HCC with C7 and C15, was reported. Next, using identified complement proteins associated with each liver disease, the authors investigated to identify liver disease-associated proteins from the complement protein-protein interaction network in biological processes. They utilized network-based drug repositioning to assess whether specific complement proteins could serve as pharmacological targets for the treatment of liver diseases. They identified effective drugs for different liver diseases, supported by experimental or clinical evidence.

Taken together, the results via MR and evaluation of common biological processes between both proteins associated with liver disease and from the complement regulatory network demonstrate that certain complement components may play important roles in some non-viral liver diseases and HCC. It should be noted that genome-wide association study (GWAS) data for six liver diseases and the selection of complement components as instrument variables were from Icelanders and European ancestries retrospectively. Therefore, future studies should confirm the association using other available GWAS data from other ancestries to confirm the generalizability of the results in a wider population with different ethnic backgrounds. Furthermore, some of the associations identified between complement proteins and liver diseases through the IVW method were not confirmed via WM. This implies that the associations lack consistency across all studies, displaying significant heterogeneity. It underscores the necessity of conducting additional studies to validate these findings. There may be mediators, such as diseases or conditions like oxidative stress or cytokines and chemokines\textsuperscript{20} affecting the association between complement proteins and liver diseases, and they need to be considered when adjusting the demonstrated associations.

With a drug repositioning approach, the study has attempted to propose that the identified association between...

**Abbreviations:**
- CLD, chronic liver diseases
- AIH, autoimmune hepatitis
- ALC, alcohol-related cirrhosis
- ALD, alcohol-associated liver disease
- PSC, primary sclerosing cholangitis
- PBC, primary biliary cholangitis
- HCC, hepatocellular carcinoma
- MASLD, metabolic dysfunction-associated steatotic liver disease
- CFHR, complement factor H-related protein
- C8G, complement C8 gamma chain
- GWAS, genome-wide association study
- WM, weighted median
- IVW, inverse-variance weighted
- MR, mendelian randomization
- OR, odds ratio
- CI, confidence interval
complement proteins and some non-viral diseases can have clinical utility. Although the authors provided some experimental and clinical evidence for each identified drug, no hospital cohort data or clinical trials specifically validated the findings of this study or the utility of these identified drugs. On the other hand, although the authors provided some common biological processes that both liver disease-associated proteins and proteins from the complement regulatory network are involved in, information specifically related to complement deregulation from immunological mechanisms is still limited, and more studies are needed to shed light on the mechanisms involved for possible different effects of the complement system on non-viral diseases. While this study hypothetically provides an opportunity for a better understanding of the direction in identifying novel therapeutics, it could be considered an exploratory analysis, and there is certainly a need for extensive preclinical and clinical studies to assess the potential of a drug for an earlier phase clinical trial.

Authors’ contribution

Concept and Design: JDY, MSR-Z. Data Acquisition or Interpretation: MSR-Z, NK, JDY. Drafting the Manuscript: MSR-Z. Critical Revision: NK, JDY. Final Approval and Agreement: All Authors.

Conflicts of Interest

J.D.Y. provides a consulting service for AstraZeneca, Eisai, Exact Sciences, Exelixis, Fujifilm Medical Sciences, and Gilead Sciences. M.S.R-Z. and N.K. declare no conflict of interest.

REFERENCES


Enhancing off-nucleos(t)ide analogue outcome predictions in chronic hepatitis B with time-varying hepatitis B core-related antigen

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Keywords: HBcrAg; HBsAg; Clinical relapse; HBV DNA

Editorial

Nucleos(t)ide analogue (NA) therapy stands as the cornerstone in the treatment of chronic hepatitis B (CHB), and its cessation is considered safe only upon achieving seroclearance of hepatitis B surface antigen (HBsAg). Yet, this milestone is rarely reached during long-term NA therapy. Premature discontinuation of NA treatment is associated with virological relapse, with approximately half of patients experiencing hepatitis flare or hepatic decompensation. The current challenge lies in identifying patients who are suitable for cessation of NA treatment before HBsAg seroclearance through the utilization of various viral and host markers. Previous studies have indicated that lower serum levels of viral protein at the end of treatment (EOT), as indicated by HBsAg and hepatitis B core-related antigen (HBcrAg), are associated with a reduced risk of clinical relapse.

In untreated CHB patients, the kinetics of serum HBcrAg have been shown to correlate with various clinical outcomes, including HBsAg seroclearance and the development of adverse events such as hepatitis B e antigen (HBeAg)-negative hepatitis and liver cancer. However, it remains unclear how the time-varying HBcrAg levels post-NA cessation could be utilized to predict clinical relapse. In this study, Tsai et al. enrolled 203 HBeAg-negative CHB patients who discontinued tenofovir or entecavir therapy. Clinical relapse was defined as serum alanine aminotransferase levels exceeding 2 times the upper limit of normal, along with serum hepatitis B virus (HBV) DNA levels surpassing 2,000 IU/mL. Viral markers, including HBsAg, HBcrAg, and HBV DNA, were assessed at the EOT, one year post-NA cessation, and two years post-NA cessation, respectively. They adopted time-varying levels based on the most recent measurements to predict the subsequent development of clinical relapse.

In the univariable analysis, HBcrAg and HBsAg levels at EOT, DNA transcriptional activity. In untreated CHB patients, the kinetics of serum HBcrAg have been shown to correlate with various clinical outcomes, including HBsAg seroclearance and the development of adverse events such as hepatitis B e antigen (HBeAg)-negative hepatitis and liver cancer.

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as well as the time-varying levels of HBcrAg, HBsAg, and HBV DNA, were all associated with the development of clinical relapse. In the multivariable analysis, only the time-varying HBcrAg levels, but not time-varying HBsAg and HBV DNA levels, remained an independent risk factor for clinical relapse (adjusted hazard ratio [HR] 1.53 per log U/mL; 95% confidence interval [CI], 1.12–2.08). Low post-treatment HBcrAg levels below 1,000 U/mL were associated with a decreased risk of clinical relapse (adjusted HR of 0.41; 95% CI, 0.21–0.81). Interestingly, they found that dynamic levels of HBcrAg, rather than the pattern of post-treatment changes in HBcrAg, were useful in predicting subsequent clinical relapse. In summary, this study is the first to show that not only the levels at EOT but also the kinetic levels of HBcrAg predict the risk of clinical relapse, which may help optimize clinical management.

While this study is the first to show that the kinetic levels of HBcrAg predict clinical relapse post-NA treatment cessation, there are still some unaddressed issues. For instance, the area under the receiver operating characteristic (ROC) curve for HBcrAg levels at EOT in predicting clinical relapse is only 0.61 (95% CI, 0.53–0.69). Moreover, approximately 50% of patients had HBcrAg levels below the quantification range (<1,000 U/mL). The introduction of a novel high-sensitivity assay for HBcrAg (iTACT-HBcrAg), which is approximately 10 times more sensitive, holds promise for enhancing risk prediction of clinical relapse compared to conventional assays.12

Another crucial consideration impacting clinical practice is whether routine HBV DNA measurement should be replaced by HBcrAg measurement post-NA cessation. Previous studies have highlighted the predictive value of dynamic HBV DNA monitoring for subsequent clinical relapse.13,14 However, further research is needed to determine if dynamic HBcrAg monitoring offers additional clinical benefits beyond HBV DNA quantification.

In summary, this study underscores the importance of time-varying HBcrAg levels post-NA cessation in predicting the risk of clinical relapse. Nevertheless, more extensive data are required to elucidate how to integrate various viral markers at different time points to identify patients suitable for safe cessation of antiviral treatment and to design a comprehensive monitoring schedule aimed at initiating early antiviral treatment to prevent severe hepatic flares.

**Authors’ contribution**

Guarantor of the article: Tai-Chung Tseng; Concept: Chen-Te Huang, Tai-Chung Tseng; Manuscript drafting: Chen-Te Huang; Manuscript edition and final approval: Chen-Te Huang, Tai-Chung Tseng.

**Conflicts of Interest**

T-C. T. has served on speaker’s bureaus for Fujirebio, Bristol-Myers Squibb, and Gilead Sciences and received grant support from Gilead Sciences.

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**Abbreviations:**

NA, nucleos(t)ide analogue; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; EOT, end of treatment; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio; CI, confidence interval; and ROC, receiver operating characteristic

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Evaluation of the histological scoring systems of autoimmune hepatitis: A significant step towards the optimization of clinical diagnosis

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Keywords: Autoimmune hepatitis; Histological scoring systems; Interface hepatitis; Lobular hepatitis

Autoimmune hepatitis (AIH) is an immune-inflammatory chronic liver disease in nature; however, clinicians are well aware of heterogeneous disease phenotypes, not to mention chronological dynamics: from acute-onset and acute-on-chronic to chronic insidious manifestation. For many years, there were two commonly implemented sets of AIH diagnostic criteria: the revised International AIH study group criteria reported in 1999 (hereafter referred to as “1999 IAIHG”) and the simplified criteria proposed in 2008 (or “2008 IAIHG”). The 2008 IAIHG system has superior specificity and accuracy compared to the 1999 IAIHG system, while the 1999 IAIHG system is regarded as suitable for the assessment of atypical cases, including acute-onset AIH. Their differences may stem from the fact that the histological component of the 2008 IAIHG system emphasizes emperipolesis and hepatic rosette formation as typical features supporting the diagnosis of AIH. Recently, however, emperipolesis and rosettes have instead come to be considered etiology-independent reflections of lymphocyte-driven unicellular injury on the one hand and multicellular regeneration of hepatocytes on the other. In other words, pathologists have questioned the diagnostic specificity of these two features and more recently proposed modifications to the histological component of the 2008 IAIHG criteria, discarding those two features and adding lobular hepatitis instead. The outcome of these efforts was the 2017 UCSF criteria by Balitzer et al., followed by the consensus statement for the histological diagnosis of AIH by the International Autoimmune Hepatitis Pathology Group (2022 IAHPG).

In this issue, Ahn et al. retrospectively evaluate the utility of the two modified histological scorings among patients originally diagnosed with at least probable AIH according to 1999 IAIHG by performing a comparison among four sets of diagnostic criteria, namely 1999 IAIHG, 2008 IAIHG, and 2008 IAIHGs, with the updated histological scorings of 2017 UCSF...
and 2022 IAHPG, respectively (2008 IAIHG+2017 UCSF and 2008 IAIHG+2022 IAHPG).

First, as expected, the percentage among the cohort who received the maximum histology score was lower for 2008 IAIHG than for 1999 IAIHG (58.8% vs. 73.5%), while implementation of either 2017 UCSF or 2022 IAHPG criteria with 2008 IAIHG increased the percentage to 88.2% or 94.1%, respectively. Accordingly, the percentage of patients who met probable and definite (≥probable) AIH was 89.7% and 91.2% by 2008 IAIHG+2017 UCSF and 2008 IAIHG+2022 IAHPG, respectively, compared with 82.4% by 2008 IAIHG.

Second, and relevantly for clinicians, their sub-analysis focused on the re-evaluation of cases with acute onset or aggravation (AIH with acute presentation). AIH with acute presentation is a challenging disease phenotype because a delayed or insensitive diagnosis may lead to a worse prognosis, especially in cases with atypical serological findings. As mentioned above, 2017 UCSF and 2022 IAHPG, in particular, adopted lobular hepatitis as a histological component in order to strengthen the diagnostic power, even for AIH with acute presentation. Again, as anticipated, the percentage receiving the maximum histology score among AIH with acute presentation increased from 65.6% in 2008 IAIHG to 90.6% in 2008 IAIHG+2017 UCSF, and further to 96.9% in 2008 IAIHG+2022 IAHPG. Consequently, the percentage of patients who met probable or definite (i.e., ≥probable) AIH criteria was 81.3, 90.6, and 93.8% by 2008 IAIHG, 2008 IAIHG+2017 UCSF, and 2008 IAIHG+2022 IAHPG, respectively.

The fact that AIH criteria considering lobular hepatitis (in addition to lymphoplasmacytic interface hepatitis) outperform those considering emperipolesis and hepatic rosette formation reinforces the position that the context of demarcated hepatic inflammation or liver zonation likely matters in the multifaceted pathology of AIH (Fig. 1). Practical technical issues around pathological diagnosis may also support their findings. Recognition of emperipolesis, the presence of lymphocytes within the cytoplasm of hepatocytes, is often a difficult, time-consuming task, in particular by general pathologists, with a tendency for wide interobserver variability; the

**Figure 1.** The context of demarcated hepatic inflammation in the multifaceted pathology of AIH.

**Abbreviations:**
AIH, autoimmune hepatitis; Ig, immunoglobulins
frequency of emperipolesis varies and has been reported as low as ~15% in even a recent national survey in Japan.9

AIH is a disease without signature diagnostic features. A specific pathological signature is still being sought, the latest candidate being Kupffer cell hyaline globules.10 Kupffer cells in the inflamed liver have been speculated to catabolize and recycle excess immunoglobulins (Ig) secreted by plasma cells, giving rise to hyaline globules within the cytoplasm. Kupffer cell hyaline globules have been reported to be relatively specific in AIH compared with other liver diseases,5 but it is still controversial for their disease specificity and association with serum Ig levels.11 The need to do additional staining to identify Kupffer cells is a drawback of its routine implementation in clinical practice. So far, again, understanding the specific context of AIH inflammation *in situ* is the gold standard in AIH diagnosis, as in the findings by Ahn et al.8; there is likely room for improvement in the future with assistance from artificial intelligence.

The study by Ahn et al.8 was a retrospective study with only a relatively small number of Korean AIH patients in the absence of a control group with other etiologies. In the future, multinational evaluation to validate the sensitivity and assess the specificity of the four diagnostic systems will be important, especially one that considers acute drug-induced liver injury, a most prominent disease entity that requires careful differentiation from AIH with acute presentation.12

There is still a mountain to climb in the optimization of the diagnosis of AIH,13,14 when its pathognomonic signatures will finally be revealed to us. The findings of Ahn et al. in the present issue mark a timely advancement along that path.

**Conflicts of Interest**

The authors have no conflicts to disclose.

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Exploring the prognostic value of ultra-low-pass whole-genome sequencing of circulating tumor DNA in hepatocellular carcinoma

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Keywords: Hepatocellular carcinoma; Circulating tumor DNA; Ultra-low-pass whole-genome sequencing

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and a leading cause of cancer-related mortality worldwide. Many cases of HCC are diagnosed at advanced stages and are accompanied by underlying liver cirrhosis. Despite considerable progress in the management of HCC with both immune checkpoint inhibitors and tyrosine kinase inhibitors over the course of recent decades, the prognosis of patients with HCC remains poor. The identification of reliable biomarkers that can predict the therapeutic response to systemic treatment is expected to improve the prognosis of patients with advanced HCC. Because conventional liver biopsy, which is invasive, collects limited tissue samples, the results cannot reflect intra-tumor genetic heterogeneity, and tracking the evolution of the tumor according to treatment is difficult. Therefore, liquid biopsy, which could obtain tumor genetic information via a less invasive method, has gained substantial attention recently.

In this issue of Clinical and Molecular Hepatology, Sogbe et al. demonstrated the prognostic implications of ultra-low-pass whole-genome sequencing (ULP-WGS) of ctDNA in patients with HCC receiving systemic treatment. The ctDNA detected using ULP-WGS was associated with a worse prognosis for patients with HCC receiving systemic treatment. Among the ctDNA of patients with HCC receiving systemic therapy, losses of 5q and 16q were identified as independent prognostic factors for poor overall survival. Prior studies have focused on detecting targeted DNA aberrations, such as single-nucleotide mutations, copy number variations, and epigenetic modifications, such as methylation changes and somatic mutations, in ctDNA using high-depth genetic analysis technology. This study demonstrated that broad-coverage and low-depth genetic analyses can elucidate genetic differences between HCC and cirrhosis without HCC in terms of HCC prognosis. This finding may lead to a relevant, affordable, and cost-effective approach that can enhance the ability to predict the prognosis of HCC, thus significantly impacting...
Clinical research and clinical practice.

Recently, ctDNA in HCC has emerged as a significant research topic. CtDNA fragments are derived from cancer cells and can be found in the bloodstream of patients with cancer. With cancers such as HCC, quantitative and qualitative analyses of ctDNA may reflect the genetic and molecular characteristics of the tumor without invasiveness, thereby providing valuable information regarding the diagnosis, prognosis, and treatment response. Previous studies have suggested that cancer-specific mutations of TP53, ITH, HCK, CTNNB1, and TERT in ctDNA are commonly observed in peripheral blood samples of patients with HCC. A study performed in Korea found that MLH1 single-nucleotide variants were associated with poor survival in patients with advanced HCC, thus highlighting the importance of quantitative analyses of ctDNA.

CtDNA can be measured using various methods, such as targeted polymerase chain reaction-based technology, next-generation sequencing, whole-exome sequencing, and WGS. In the evolving landscape of genomic research, ULP-WGS has emerged as a notable alternative to traditional WGS. Although traditional WGS offers a detailed and comprehensive overview of the genome with high sequencing coverage (typically more than 30x), it is associated with substantial costs and data processing demands. In contrast, ULP-WGS provides significantly lower coverage of approximately 0.1x to 1x, thus providing a broader, albeit less detailed, view of the genomic landscape. This approach is particularly advantageous for large-scale screening and the identification of major genomic alterations, such as copy number variations, with much lower costs and computational requirements. Although ULP-WGS provides more granular information compared to that provided by WGS, its cost-effectiveness and efficiency for detecting large-scale genomic changes make it a compelling choice for specific research and clinical applications that do not require a detailed genetic map.

Recently, the prognostic implications of ctDNA evaluated by ULP-WGS for various cancers, including metastatic squamous non-small cell lung cancer, Ewing sarcoma, osteosarcoma, metastatic castration-resistant prostate cancer, cervix cancer, and metastatic triple-negative breast cancer, have been reported. Alongside these studies, the study by Sogbe et al. is noteworthy for its meaningful investigation into the potential use of ctDNA evaluated with ULP-WGS as a prognostic biomarker in patients with advanced HCC.

The detection of ctDNA and exploration of copy number alterations using ULP-WGS have significant implications. Copy number alterations, which refer to gains or losses of parts of chromosomes, play a crucial role in tumor development and progression. By analyzing copy number alterations of ctDNA using ULP-WGS, researchers and clinicians can non-invasively gain insights into the genetic landscape of tumors at a relatively low cost. In this study, the authors investigated the copy number alterations of ctDNA across various chromosomal loci using ULP-WGS and highlighted the loci where losses (8p, 4p, 13q, 16q, and 5q) and gains (1q, 8q, 7q, and 5p) were most prevalent. Notably, they identified the loss or deletion of 5q and 16q as independent biomarkers that predict poor survival in patients undergoing systemic therapy. These findings significantly contribute to the improvement of treatment strategies and can enable personalized approaches for patients with HCC.

Although research on ctDNA using ULP-WGS has presented an economically viable option for clinical applications, thus enabling the identification of genomic features at a cost-effective rate, it is important to acknowledge that this method has certain limitations. First, as Sogbe et al. underscored, its low sensitivity poses a challenge when detecting ctDNA in patients with low tumor burden. Moreover, the low resolution of ULP-WGS limits the detection of detailed genetic variants, and difficulties encountered while interpreting noise-related data further complicate its application. Additionally, the majority of studies that have utilized ULP-WGS, including the current study, were retrospective and included limited sample sizes. Hence, further prospective studies with larger sample sizes are required to validate its clinical effectiveness.

Furthermore, the predominance of sorafenib treatment among patients in this study introduced bias. In the context of the shift toward immunotherapy-based first-line treatments for HCC, such as atezolizumab plus bevacizumab or durvalumab plus tremelimumab, further research of the role of ctDNA analyzed using ULP-WGS in the prediction of progression-free survival or the tumor response to immunother-

Abbreviations:
CtDNA, circulating tumor DNA; HCC, hepatocellular carcinoma; ULP-WGS, ultra-low-pass whole-genome sequencing

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apy in patients with advanced HCC is expected to have significant implications.

In conclusion, the study by Sogbe et al.\(^6\) represents a significant contribution to the understanding of the role of ctDNA in the prognosis of HCC using ULP-WGS, thus highlighting the need for further research on this topic. Although ULP-WGS is a non-invasive and cost-effective approach to tumor genetic profiling, it is associated with challenges, including low sensitivity and resolution, that need to be addressed. It is anticipated that further investigations of ctDNA using ULP-WGS will more clearly define its potential as a prognostic biomarker for patients with advanced HCC.

Authors’ contribution
All authors were responsible for the conceptualization, interpretation of data, drafting, and critical revision of the manuscript.

Conflicts of Interest
The authors have no conflicts to disclose.

REFERENCES


Linvencovir: Paving the way for functional cure in hepatitis B

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Keywords: Hepatitis B

Functional cure, defined as unquantifiable hepatitis B virus (HBV) DNA and sustained hepatitis B surface antigen (HBsAg) loss, is the ultimate goal of antiviral treatment for chronic hepatitis B (CHB).\(^1,2\) With currently available antiviral treatments, such as nucleos(t)ide analogues (NUCs), functional cure is rarely achievable despite long-term treatment, necessitating innovative, finite new antiviral treatments.\(^3-5\) Numerous compounds have been under development to achieve functional cure. Fortunately, some have been successful in proving their efficacy, although the efficacy remains unsatisfactory.\(^6,7\) Hou et al. reported the efficacy, safety, and pharmacokinetics of Linvencovir, a novel small-molecule capsid assembly modulator (CAM), in Part 3 of the phase 1/2 study.\(^8\) Previous reports demonstrated Linvencovir’s favorable safety and pharmacokinetic profiles in healthy volunteers and viremic patients with CHB.\(^9,10\) This study assessed the efficacy in three different cohorts; 32 Linvencovir-treated patients receiving NUCs, 10 Linvencovir-treated treatment-naïve patients in combination with NUCs, and 30 Linvencovir-treated treatment-naïve patients in combination with NUCs and peg-interferon for 48 weeks, followed by an additional 24 weeks of observation without Linvencovir but with NUCs.

Unfortunately, no patient achieved functional cure at week 24 post-study treatment, which was the primary endpoint of this study. HBV DNA was effectively suppressed in all three cohorts, as expected using NUCs. Notably, serum HBV RNA was also successfully suppressed in the majority of patients by Linvencovir, despite a rebound increase after withdrawal. However, HBsAg titer, another important marker to predict functional cure, was not sufficiently decreased despite the combination of NUC and Linvencovir.

In terms of safety, Linvencovir was tolerable without significant concern in its use alongside NUCs, which is satisfactory. Collectively, Linvencovir in this Part 3 phase 1/2 study showed limited efficacy, especially in achieving functional cure, unlike previous studies using newly developed compounds for functional cure.\(^9,10\) Considering the mechanism of action in HBV natural history, treatment with CAM might not be sufficient to achieve functional cure effectively. Combination therapy with another class of drugs, such as siRNA or immunomodulators, could be an option to further increase the
rate of functional cure, which has been partly evaluated in other studies. However, this small step is important in paving the way for functional cure.

Authors’ contribution
J Yang and J Choi were responsible for drafting and finalization of the manuscript.

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Abbreviations:
HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; CHB, chronic hepatitis B; NUCs, nucleos(t)ide analogues; CAM, capsid assembly modulator
Primary biliary cholangitis (PBC) is a chronic cholestatic immune liver disease characterized by persistent cholestasis due to damage to small bile ducts within the liver. Inflammation and destruction of the interlobular bile ducts and inflammation of the portal area of the liver are common pathological features. This process can cause persistent liver damage, leading to liver fibrosis and cirrhosis. For the development of PBC, there is a complex interaction of various molecular and pathological mechanisms involved in the progressive destruction of intrahepatic bile ducts and the development of liver fibrosis. The main mechanism is an autoimmune attack on the small bile ducts in the liver. Autoantibodies, such as anti-mitochondrial antibodies, target mitochondrial antigens on bile duct epithelial cells, triggering an immune response and promoting bile duct damage. Immune-mediated inflammatory responses also play an important role, leading to an influx of lymphocytes and other immune cells into the bile ducts. Damage to the bile duct epithelial cell (BEC) caused by an immune-mediated attack disrupts bile flow, impairs bile acid metabolism, and accumulates toxic bile acids in the liver, worsening liver damage. In addition, dysregulation of bile acid metabolism, epigenetic changes, and genetic susceptibility also contribute to the pathogenesis of PBC. Chronic inflammation and BEC damage due to various mechanisms stimulate the activation of hepatic stellate cells (HSCs) and the deposition of extracellular matrix proteins, leading to hepatic fibrosis.

Junctional Protein Associated With Coronary Artery Disease (JCAD), also known as KIAA1462, junctional cadherin 5 associated or junctional protein associated with coronary artery disease is a gene identified as a risk locus for cardiovascular diseases (CVD) through genome-wide association studies. JCAD plays a crucial role in the development of CVD by regulating various molecular mechanisms involved in endothelial dysfunction, atherosclerosis, inflammation, and thrombosis. It interacts with large tumor suppressor kinase 2 (LATS2) and negatively regulates the Hippo signaling pathway. This interaction leads to increased activity of yes-associated protein (YAP), the transcriptional effector of the Hippo pathway.
Dysregulation of Hippo signaling is associated with various cardiovascular diseases, including atherosclerosis and thrombosis. JCAD has also been reported to be associated with the development of hepatocellular carcinoma in nonalcoholic steatohepatitis by inhibiting LATS2 kinase activity. These results suggest that JCAD may affect metabolic and inflammatory pathways, contributing to both hepatic and CVD.8

In the current Clinical and Molecular Hepatology issue, Xie et al.9 conducted a study on the effects of JCAD on HSCs and hepatic fibrosis in PBC, a cholestatic liver disease. This study confirmed that JCAD is an important regulator of HSC activation in cholestatic liver disease. JCAD deficiency has been shown to promote HSC activation through the Hippo-YAP signaling axis. JCAD binds to LATS, inhibits its kinase activity, and reduces the phosphorylation level of YAP. Subsequently, unphosphorylated YAP translocates to the nucleus and trans-activates downstream target genes such as connective tissue growth factor (CTGF) and cyclin D1, resulting in HSC proliferation and activation. These findings demonstrate several potential clinical applications for treating cholestatic liver diseases. Approximately 40% of patients with PBC do not achieve adequate biochemical response or disease control with standard treatment with ursodeoxycholic acid.1,2,10 While obeticholic acid, a new therapeutic agent, provides additional biochemical improvement in some patients, it may not be tolerated in individuals with advanced disease or severe pruritus symptoms.1,3 These limitations suggest that JCAD may be a potential therapeutic target for cholestatic liver diseases, including PBC. Inhibiting JCAD expression or activity may attenuate HSC activation and reduce liver fibrosis progression. These findings suggest that the Hippo-YAP signaling pathway may be a promising target for treating cholestatic liver disease. Reducing HSC activation and fibrosis progression may be possible by inhibiting YAP nuclear translocation or downstream target genes such as CTGF and cyclin D1. However, this study showed the role of JCAD only in HSCs, which play the most critical role in fibrosis. However, it is known that BECs, hepatocytes, and inflammatory cells are closely related to the occurrence and progression of PBC, but, unfortunately, there are no results on the effect of JCAD expression on these cells.

In conclusion, this study provided a new understanding of the molecular pathways of cholestatic fibrosis and also identified potential therapeutic targets to treat this condition. Additional research is needed to verify the results of this study and develop effective treatments for cholestatic liver disease in the future.

Conflicts of Interest
The authors have no conflicts to disclose.

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Abbreviations:
BEC, bile duct epithelial cell; CTGF, connective tissue growth factor; CVD, cardiovascular diseases; JCAD, junctional protein associated with coronary artery disease; LATS2, large tumor suppressor kinase 2; PBC, primary biliary cholangitis; YAP, yes-associated protein

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Clinical impact of five cardiometabolic risk factors in metabolic dysfunction-associated steatotic liver disease (MASLD): Insights into regional and ethnic differences

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Keywords: Metabolic dysfunction-associated steatotic liver disease (MASLD); Cardiometabolic risk factors; Mortality

Staetotic liver disease (SLD) serves as an overarching term encompassing various chronic liver conditions characterized by hepatic steatosis, highlighting the pivotal role of cardiometabolic risk factors in previous non-alcoholic fatty liver disease (NAFLD).¹ Unlike NAFLD, which primarily relies on exclusionary diagnostic criteria, SLD acknowledges liver diseases stemming from alcohol consumption and viral infections as concurrent pathologies. SLD terminology allows liver disease caused by alcohol and viruses to be considered a concomitant disease and is presented to enable the more holistic management of patients with SLD.²

Recently, Iwaki et al. employed the Clinical Outcome Non-alcoholic Fatty Liver Disease (CLIONE) cohort, an Asian biopsy-proven metabolic dysfunction-associated steatotic liver disease (MASLD) cohort, and reported that 99% of existing patients with NAFLD could satisfy the new MASLD definition criteria.³ The CLIONE cohort was established by the Japan Study Group of NAFLD (JSG-NAFLD) at 11 centers across Japan for the following purposes: 1) to clarify the prevalence, natural history, and prognosis of Japanese patients with MASLD; 2) for noninvasive diagnosis of MASLD or severe fibrosis; and 3) to develop pharmacological treatments for MASLD. All CLIONE data were compiled and analyzed using the REDCap database.⁴ Iwaki et al.¹ underscore the clinical significance of assessing metabolic risk factors in individuals with MASLD and emphasize the importance of resuming and reinforcing the management of these factors. Although existing research consistently demonstrates that cardiometabolic risk factors elevate liver-related mortality and overall mortality in individuals with SLD, the emerging era of MASLD warrants further inquiry into additional considerations.
Importantly, there remains a paucity of data on whether MASLD can exacerbate the incidence of fatal cardiovascular events or mortality in response to cardiometabolic risk factors. This gap in knowledge underscores the need for targeted investigations to elucidate the potential impact of MASLD on cardiovascular outcomes in the context of metabolic risk factors. Addressing this gap is crucial for enhancing our understanding of the comprehensive health implications of MASLD and informing more effective management strategies for affected individuals. In a recent nation-wide large-scale study using 9.77 million Korean health check-up records, individuals with MASLD showed higher rates of cardiovascular events and cardiovascular mortality compared to their non-MASLD counterparts and control subjects. Similarly, another study using data from 28,000 individuals who underwent magnetic resonance imaging-estimated proton density fat fraction within the UK Biobank revealed that patients with MASLD experienced a higher number of cardiovascular events than those experienced by non-MASLD subjects. However, metabolic dysfunction independently increased the incidence of non-fatal cardiovascular events, regardless of the presence of fatty liver. Interestingly, within the population with metabolic dysfunction, no significant difference in stroke incidence was observed between patients who have SLD (MASLD) and those who do not have SLD, with rates remaining at 1.3 and 1.4%, respectively. Similarly, the incidence of ischemic heart disease did not show a significant difference and was 7.0 and 6.1% for MASLD and non-SLD with metabolic dysfunction, respectively. A recent analysis of US National Health and Nutrition Examination Survey data also revealed that patients with MASLD exhibited higher overall mortality and cardiovascular mortality than normal controls. However, after adjusting for cardiometabolic risk factors, MASLD itself did not emerge as an independent risk factor for cardiovascular or overall mortality.

MASLD is closely associated with cardiovascular events and demonstrates a bidirectional relationship with components of the metabolic syndrome. However, additional evidence is required to establish whether MASLD increases cardiovascular mortality or fatal events even independently after adjusting cardiometabolic risk factors.

Another unresolved issue regarding cardiometabolic risk factors in the MASLD era is the size of the effect of cardiometabolic risk factors and whether the cutoff value for each risk factor should vary depending on region and race. As mentioned earlier, the impact and magnitude of cardiovascular mortality in patients with MASLD differed slightly in the data presented in the West and the East. Additionally, the magnitude of the impact on liver cancer occurrence differed between the CLIONE and Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) cohorts. The all-cause mortality of patients with F3 metabolic dysfunction-associated steatohepatitis in the NASH CRN and CLIONE cohorts was similar (0.89 vs. 0.82, respectively). However, in the NASH CRN cohort, the annual hepatocellular carcinoma incidence rate in patients with F3 MASLD was 0.34%, whereas in the CLIONE cohort, this rate was 1.42%. Liver-related mortality was relatively higher in the CLIONE cohort than in the NASH CRN cohort, implying a high level of extrahepatic mortality (cardiovascular or extrahepatic malignancy) in the CRN cohort.

Finally, Iwaki et al. offered valuable insights into the significance of cardiometabolic risk factors in patients with MASLD. In patients with MASLD, individuals with cardiometabolic risk factors exhibited more severe histological severity and higher mortality rates compared to individuals with cryptogenic SLD. However, in this cohort, no clear dose-dependent pattern was observed based on specific combinations of cardiometabolic risk factors. Using data from the UK biobank, Fan et al. showed variations in the impact of glucose and lipid metabolic parameters on cardiovascular events. Moreover, the scientific rationale underlying the selection of five cardiometabolic parameters and their respective cutoffs warrants further clarification. For instance, evidence highlights the importance of C-reactive protein as a prognostic factor in MASLD subjects.

In the future, additional scientific evidence will be essential for determining the selection and optimal cutoffs of the five cardiometabolic risk factors included in MASLD diagnosis. Moreover, we are also waiting on the impact size of each cardiometabolic risk factor, along with individualized cutoffs by region and race depending on regional racial differences. Fu-

Abbreviations:
SLD, steatoctic liver disease; NAFLD, non-alcoholic fatty liver disease; CLIONE, Clinical Outcome Nonalcoholic Fatty Liver Disease; MASLD, metabolic dysfunction-associated steatoctic liver disease; NASH CRN, Nonalcoholic Steatohepatitis Clinical Research Network.
ture investigations should explore the long-term impact of hard outcomes based on the number and type of cardiometabolic risk factors, facilitating risk classification and tailored interventions.

**Authors’ contribution**

JHO drafted the manuscript. DWJ reviewed and finalized the manuscript.

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**Conflicts of Interest**

The authors have no conflicts to disclose.

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Non-alcoholic fatty liver disease (NAFLD) is a condition characterized by excessive hepatic fat accumulation leading to steatosis in >5% of hepatocytes, in the absence of secondary causes and excessive alcohol consumption. Recent changes in nomenclature adopt ‘positive diagnostic criteria’ to acknowledge metabolic dysfunction underpinning the central pathogenesis of the disease, and the substitution of ‘fatty’ by ‘steatotic’. Since these entities have >90% overlap with minimal reclassification due to the new nomenclature, the rest of the article will use NAFLD for simplicity of presentation. The term NAFLD encompasses a broad spectrum of clinical conditions ranging from non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), NASH cirrhosis, and hepatocellular carcinoma (HCC). NAFL refers to pure hepatic steatosis without significant lobular inflammation or hepatocyte ballooning. Compared to NAFL, people with NASH are at risk of accelerated disease progression to significant liver fibrosis, cirrhosis or HCC. Apart from liver-related morbidities, people with NAFLD are also at a high risk for adverse cardiovascular outcomes and extra-hepatic malignancies. The world observes an alarming increase in the global prevalence of NAFLD, affecting 25–40% with regional variations. In addition, patients with NAFLD in the West and the East differ due to a multitude of factors, including genetic background, socio-economic status, healthcare coverage, diet, and physical activity. Therefore, it is important to understand the natural course of NAFLD in the context of changing epidemiology. In this issue, Le et al. conducted a large-scale systematic review and meta-analysis comprising 79 studies, including 1,377,466 subjects with NAFLD, to delineate the outcomes of NAFLD in terms of the incidence of adverse clinical events. The huge sample size allowed the inclusion of cohorts representative of various regions of the world, as well as subgroups with well-characterized clinical phenotypes. The authors defined clinical outcomes as follows: mortality (all-cause, cardiovascular-related, liver-related, and non-liver cancer-related), liver-related outcomes (fibrosis progression, cirrhosis, liver transplant, HCC, decompensation [ascites, varices, hepatic encephalopathy]), metabolic-related events (metabolic syndrome, hypertension, hyperlipidemia, diabetes)}
tes mellitus), cardiovascular events (coronary artery disease, congestive heart failure, myocardial infarction, ischemic or hemorrhagic stroke, renal impairment, and depression/anxiety), and non-liver cancer. Multiple subgroup analyses were performed to address the impact of biopsy-proven NASH, diagnostic method, geographical location, and era of study to gain insights in each subpopulation.

Consistent with the existing literature, the pooled incidence rate of mortality was highest for cardiovascular-related deaths, followed by non-liver cancer-related deaths, and liver-related mortality being the second runner-up (4.45, 3.27, and 3.10 per 1,000 person-years, respectively). The most striking finding was the regional variations in the incidence of mortality and the cause of mortality. North America and Europe surpassed Western Pacific and Southeast Asia in all-cause mortality, cardiovascular-related mortality, and non-liver cancer-related mortality. On the other hand, Western Pacific and Southeast Asia had a higher incidence of HCC compared to North America and Europe. Apart from diet and genetic polymorphisms, such differences could also be attributed to competing risks for all-cause mortality, cardiovascular-related, and non-liver cancer-related death in North America and Europe, allowing patients in the Western Pacific/Southeast Asia region to survive long enough to develop HCC. The incidence of liver-related mortality was similar across the involved regions.

The authors confirmed the implication of biopsy-proven NASH and the presence of cirrhosis, more HCC, liver-related mortality, and liver events with these histological features. The study also confirmed worse clinical outcomes for cohorts that used liver biopsy instead of non-invasive methods such as imaging or diagnostic codes to identify NAFLD—those with clinical indications for liver biopsy, and thus identified as having NAFLD by liver biopsy—demonstrated a higher risk of mortality, fibrosis progression, liver transplant, and HCC. This acknowledges the heterogeneity of clinical outcomes and highlights the fact that NAFLD diagnostic method should be taken into account when interpreting the observed differences in prognosis among NAFLD patients (Fig. 1). Over the past few decades, despite an increasing prevalence of NAFLD,

**Figure 1.** Accountability of NAFLD diagnostic method in heterogeneous clinical presentation and prognosis among NAFLD patients. NAFLD, non-alcoholic fatty liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NITs, non-invasive tests; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma.

**Abbreviations:**
NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma
the rates of non-liver events (cardiovascular and non-liver cancer events) have decreased, while those of liver events have increased. Intriguingly, with an increase in study year, the baseline median age decreased (regression coefficient of 0.19 per year). This trend is concerning, whether the underlying mechanism is increased awareness of NAFLD or the obesity pandemic, especially among the younger generation. Patients will start to live with NAFLD at an earlier age, and they also live longer due to improvement in care for cardiovascular health. Overall, the duration of illness (NAFLD) will eventually increase, contributing to ever-growing pool of patients at risk of adverse clinical outcomes.

Intrinsic to the study design, the current meta-analysis could not provide details on how disease modifiers such as genetic polymorphisms and medications influence the disease course of NAFLD. Nevertheless, the study has clearly described the clinical outcomes in a large cohort of NAFLD patients with granular details. Le et al. call for urgent attention to recognize and appreciate the multi-faceted needs of patients living with NAFLD, which is an ever-expanding pool. Clinical care for NAFLD should prioritize to minimizing morbidity and mortality from cardiovascular diseases, cancer, and liver events.

Conflicts of Interest

The authors have no conflicts to disclose.

REFERENCES


The gene expression signature of metabolic dysfunction-associated steatotic liver disease from a multiomics perspective

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Keywords: MASLD; MASH; OMICS; HCC; Gene expression

Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) and its severe clinical form, metabolic dysfunction-associated steatohepatitis (MASH), are chronic liver diseases that are becoming increasingly prevalent. Accurate identification of the stages of MASH is crucial for patient treatment, as it is associated with an increased risk of cirrhosis, liver failure, liver cancer, and mortality as it progresses.

The understanding of disease pathogenesis has advanced significantly in the past decade, largely due to the use of OMICS and high-throughput technologies. Genomics has enabled the identification of genetic variants that confer either risk or protection against steatotic liver diseases and fibrosis. Epigenetics/epigenomics has helped to clarify the relationship between MASLD and comorbidities, disease severity, and even the interaction with the microbiome, as recently described. Besides, liver transcriptomics have revealed the key mechanisms regulating gene expression in the context of MASLD and MASH.

Despite significant progress in comprehending the molecular structure of MASLD and MASH, a substantial gap remains between the insights gained from OMICs research and their practical application in clinical settings.

In this issue of Clinical and Molecular Hepatology, Oh et al. conducted holistic omics analyses on biopsy tissue and blood samples from 134 patients with MASLD, which includes both
steatosis and MASH. Whole-genome sequencing, whole-exome sequencing, whole-genome bisulfite sequencing, and total RNA sequencing were performed, revealing 1,955 MASLD-associated features. Using a Support Vector Machine learning algorithm, the researchers identified the most predictive features. Through linear regression, a signature gene set (CAPG, HYAL3, WIP11, TREM2, SPP1, and RNASE6) capable of differentiating MASLD stages was established and validated in independent MASLD cohorts and a liver cancer cohort. These findings suggest the potential of the identified gene set as a diagnostic panel for MASLD-associated diseases.

The panel genes identified belong to biological processes such as cellular response to cytokine stimuli, response to cytokine, and positive regulation of secretion and immune response, as already anticipated.\(^7\) CAPG, RNASE6, TREM2, and SPP1 are highly co-expressed in myeloid/macrophage cells and interestingly, some genes, such as TREM2, may be important in the bacterial product’s action on liver health—a relevant finding in light of the liver metataxonomic profile found in MASLD.\(^8\)

The gene set enriched in MASH may have implications for disease treatment. For instance, a simple gene list enrichment analysis can yield interesting pharmacome annotations (toppgene.cchmc.org). In this case, the gene products may serve as targets for homochlorcyclizine or terfenadine analogs.

The elevated expression of CAPG, HYAL3, WIP11, TREM2, SPP1, and RNASE6 in MASH was mechanistically explained by the gene location in open chromatin regions.

Furthermore, the analysis of differentially hypermethylated loci revealed promising candidates, including PACS2 (Phosphofurin Acidic Cluster Sorting Protein 2), a coding gene located in the endoplasmic reticulum and mitochondrion that is involved in endoplasmic reticulum calcium ion homeostasis. The authors also identified ZNF331 (Zinc Finger Protein 331) as one of the differentially hypomethylated loci. This gene encodes a zinc finger protein that contains a KRAB (Kruppel-associated box) domain, which is found in transcriptional repressors that may be methylated and silenced in cancer cells.

Epigenetic modifications might explain the enhancement of gene expression in MASH compared to steatosis, although differentially methylated regions do not contain the panel genes. The main finding is robust, as it was reproduced in an in vivo rodent model and simulated in an in vitro organoid model.

It remains to be explained how the differential expression of the 1,393 genes between steatosis and MASH seems not to be related to the somatic mutations the authors found. In this scenario, the contributions of these mutations and gene variants should be further investigated. Perhaps they are associated with many germline gene variants, a topic solely explored for the pathognomonic gene variants associated with the disease, such as PNPLA3, TM6SF2, and so on.\(^9\) Besides, the chosen approach missed noncoding variants. Genetic variation in non-coding regions through regulatory non-coding RNAs, either small or long, may be associated with MASLD.\(^10\)

Finally, it is worth adding two notes of caution. First, the panel composed by CAPG, HYAL3, WIP11, TREM2, SPP1, and RNASE6 seems to equally discriminate between normal liver histology and liver steatosis, the severity of histological characteristics of MASH, and hepatocellular carcinoma, which may indicate a lack of specificity regarding disease progression. Second, the novel biomarker is based on gene expression solely available after a liver biopsy. It would be important to demonstrate that the biomarker is available, at least as a liquid biopsy, to be clinically useful.

**Authors’ contributions**

All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

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**Abbreviations:**

MASLD, Metabolic Dysfunction-Associated Steatotic Liver Disease; MASH, metabolic dysfunction-associated steatohepatitis
Conflicts of Interest
The authors have no conflicts to disclose.

REFERENCES


Prognostic value of ultra-low-pass whole-genome sequencing of circulating tumor DNA in hepatocellular carcinoma under systemic treatment

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Study Highlights

- The detection of ctDNA using ultra-low-pass whole-genome sequencing carried worse prognosis in HCC patients under systemic treatment. Furthermore, the loss of the long arms of chromosomes 5 and 16 was associated with worse survival among ctDNA-positive patients receiving systemic treatment. Ultra-low-pass whole-genome sequencing may provide a relevant affordable tool to improve the prediction of prognosis in HCC, which is important for clinical research and practice.
INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide with an unfavorable prognosis, particularly for patients with advanced disease. HCC most often develops on a cirrhotic liver, primarily due to viral hepatitis, alcohol-related liver disease, or metabolic liver disease.\(^1,2\) According to the Barcelona Clinic Liver Cancer (BCLC) classification, patients with very early and early stages (BCLC 0-A) are the best candidates for ablative therapies, such as radiofrequency ablation, liver resection, or liver transplantation.\(^3\) Intermediate stage HCC (multinodular liver-only disease, BCLC-B) is usually addressed with locoregional treatments when tumor burden is low, whereas advanced stages, such as BCLC-C with high tumor burden, or those tumors with vascular invasion or extrahepatic spread (BCLC-C) deserve systemic treatment.\(^3\) However, within a specific tumor stage, patients may exhibit different prognosis or be treated with different therapies.\(^4,5\)

In this scenario of multiple therapeutic options, clinical, laboratory and pathological features may help inform clinical decision-making by providing important prognostic information. Microvascular invasion or satellite nodules, for instance, may establish an indication for \textit{ab initio} liver transplantation\(^6\) or adjuvant immunotherapy.\(^7\) Serum bilirubin and albumin, or composite scores that incorporate them, such as Child-

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**Background/Aims:** New prognostic markers are needed to identify patients with hepatocellular carcinoma (HCC) who carry a worse prognosis. Ultra-low-pass whole-genome sequencing (ULP-WGS) (≤0.5× coverage) of cell-free DNA (cfDNA) has emerged as a low-cost promising tool to assess both circulating tumor DNA (ctDNA) fraction and large structural genomic alterations. Here, we studied the performance of ULP-WGS of plasma cfDNA to infer prognosis in patients with HCC.

**Methods:** Plasma samples were obtained from patients with HCC prior to surgery, locoregional or systemic therapy, and were analyzed by ULP-WGS of cfDNA to an average genome-wide fold coverage of 0.3x. ctDNA and copy number alterations (CNA) were estimated using the software package ichorCNA.

**Results:** Samples were obtained from 73 HCC patients at different BCLC stages (BCLC 0/A: n=37, 50.7%; BCLC B/C: n=36, 49.3%). ctDNA was detected in 18 out of 31 patients who received systemic treatment. Patients with detectable ctDNA showed significantly worse overall survival (median, 13.96 months vs not reached). ctDNA remained an independent predictor of prognosis after adjustment by clinical-pathologic features and type of systemic treatment (hazard ratio 7.69; 95%, CI 2.09–28.27). Among ctDNA-positive patients under systemic treatments, the loss of large genomic regions in 5q and 16q arms was associated with worse prognosis after multivariate analysis.

**Conclusions:** ULP-WGS of cfDNA provides clinically relevant information about the tumor biology. The presence of ctDNA and the loss of 5q and 16q arms in ctDNA-positive patients are independent predictors of worse prognosis in patients with advanced HCC receiving systemic therapy. \(\text{Clin Mol Hepatol 2024;30:177-190}\)

**Keywords:** Liver cancer; Liquid biopsy; Copy number alterations; Prognosis; Biomarkers

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**Abbreviations:**  
HCC, hepatocellular carcinoma; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; WGS, whole-genome sequencing; LP-WGS, low-pass whole-genome sequencing; ULP-WGS, ultra-low-pass whole-genome sequencing; CNAs, copy number alterations; OS, overall survival; PFS, progression-free survival; IQR, interquartile range; HR, hazard ratio; CI, confidence interval
Pugh or albumin-bilirubin grade, may define the indication of systemic or locoregional therapies. Among non-invasive tumor-derived biomarkers, only serum alpha-fetoprotein (AFP) has enough high-level supporting evidence to be used in clinical practice. High AFP levels may contraindicate liver transplantation or define the indication of Ramucirumab, a VEGFR-2 inhibitor. However, AFP has limited value, as only a minority of patients have increased levels (10% of patients at early stages have AFP >400 ng/dL scaling up to 40% in advanced stages). On the other hand, early changes in AFP may predict the benefit of systemic agents like Ramucirumab or the combination of Atezolizumab plus Bevacizumab.

Thus, there is a clear need for novel biomarkers that may help in predicting the prognosis and monitoring treatment response, thereby guiding a more personalized therapy. Liquid biopsy using peripheral blood content can provide information about the primary tumor in a non-invasive manner. This is because tumors shed different elements into the blood, including tumor nucleic acids (DNA and RNAs), circulating tumor cell, and exosomes, which carry with them the molecular and genetic fingerprint of each patient’s disease and could constitute a valid alternative to traditional biopsy for diagnosis, stratification, and treatment response.

The analysis of cell-free DNA (cfDNA) in plasma provides an opportunity for minimally invasive tumor profiling since a fraction of plasma cfDNA in cancer patients is tumor-derived (circulating tumor DNA [ctDNA]). When looking for single nucleotide variations (SNV) or small indel in tumors or ctDNA, the use of next-generation sequencing (NGS) panel assay with very high sequencing depth (5,000–12,000X) is needed, especially to confidently detect SNV belonging to low abundance clones. Instead, for large structural variations, ctDNA fraction calculation, and CNA inference, performing whole-genome sequencing (WGS) is more suitable than targeted panels, due to the higher breadth of coverage (percentage of target bases that are sequenced). Since HCC has no known targetable mutations, the utility of NGS panels in clinical practice is limited. On the other hand, the majority of HCCs exhibit high chromosomal instability. This characteristic may have prognostic implications and has been studied using WGS in tumor tissue. CNAs are significant subclasses of somatic mutations. They involve amplifications or deletions of large chromosomal regions, resulting in the overexpression of oncogenes or the loss of tumor suppressor genes, thereby promoting carcinogenesis. Recently, WGS has been applied to study large structural variations and copy number alterations (CNA) in ctDNA. As an illustration, cfDNA WGS (with an approximate sequencing depth of five times the whole genome, or 5x) has shown promise in identifying clinically significant tumor genomic alterations. However, conducting WGS at this depth entails substantial sequencing costs, which may render this approach less viable for routine clinical practice. Several studies have successfully employed low-pass whole-genome sequencing (LP-WGS) instead, which uses a lower depth coverage and cost. However, even with 1.5x depth coverage, the cost of this approach remains prohibitive for routine clinical practice. To overcome this limitation, ultra-low-pass whole-genome sequencing (ULP-WGS) (<0.5x) has emerged as a low-cost promising alternative to estimate ctDNA and tumor CNAs.

It has recently been reported that CNAs and ctDNA fraction correlate with tumor burden, progression-free survival (PFS), and overall survival (OS) in early HCC patients receiving radical treatments (surgery and radiofrequency ablation). Importantly, when WGS (5x depth) was used, similar patterns of CNAs were observed between plasma ctDNA and tumor tissue. Similar results were reported in patients with advanced HCC who underwent transarterial chemoembolization (TACE) with an average deep coverage of 3x. Still, the validity of LP-WGS or ULP-WGS, which could be affordable and reliable prognostic tools in patients with HCC, remains unexplored.

In the present study, we aimed to test the clinical impact of using ctDNA and CNA detection by ULP-WGS of plasma cfDNA as a blood-based biomarker to identify patients with HCC who carry a worse prognosis, including patients with advanced HCC undergoing systemic treatment.

**MATERIALS AND METHODS**

**Study design**

Blood samples were prospectively and retrospectively collected from patients with a diagnosis of HCC and patients with cirrhosis without HCC at the Liver Unit of Clinica Universidad de Navarra between 2017 and 2022. Samples of HCC patients were prospectively collected between November 2021 and November 2022. Samples from cirrhotic controls were obtained retrospectively between 2017 and 2022.
Among these samples, two belonged to patients with HCC who underwent liver transplantation, and three were from patients with cirrhosis without HCC. Informed consent from the HCC patient group was obtained before treatment, which included surgery (liver transplantation and resection), locoregional therapies (transarterial radioembolization and ablative therapies), and systemic treatments (sorafenib and immunotherapy). Patients with cirrhosis without HCC provided informed consent during a regular follow-up visit. This study was approved by the Research Ethics Committee of the Universidad de Navarra. Samples and data from patients included in the study were provided by the Biobank of the University of Navarra and were processed following standard operating procedures approved by the Ethical and Scientific Committees. All patients underwent clinical management and follow-up in the HPB Oncology Area of Clínica Universidad de Navarra.

**Blood sample processing and cfDNA extraction**

Whole blood samples (10 mL) were collected in EDTA (BD Biosciences, San Jose, CA, USA) and centrifuged at room temperature (2,000×g for 10 minutes). Isolated plasma was centrifuged a second time at room temperature (2,500×g for 10 minutes) in LoBind Eppendorf tubes to remove residual cells. Purified plasma was frozen at –80°C until cfDNA isolation. Purified plasma was thawed on ice, followed by a short centrifugation at 4°C (11,000×g for 15 minutes). cfDNA was extracted using the QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany). Extracted cfDNA concentration was measured using the Qubit dsDNA High-Sensitivity assay (Thermo-Fisher, Waltham, MA, USA). Extracted cfDNA was stored in LoBind Eppendorf tubes at –80°C until further analysis.

**Library preparation and ultra-low pass whole-genome sequencing**

Library construction of cfDNA was performed using the NEBNext Ultra II DNA Library Prep Kit (NEB) according to the manufacturer’s instructions. A total of 2.5 ng of cfDNA input was used for ULP-WGS. Sequencing libraries were pooled and sequenced with a NextSeq2000 (Illumina) using 100 bp paired-end runs with an average coverage of 0.3×.

**Data analysis**

Fastq files from the sequencing platform were quality filtered with TrimGalore, and sequences shorter than 50 bp were removed. Then, sequences were aligned with Bowtie2 using the hg19 database. Bam files were sorted and indexed with Samtools. Finally, duplicates were tagged using MarkDuplicates feature from Picard tools. Following the preprocessing, data were analyzed using ichorCNA package according to the workflow proposed by their developers. To identify large-scale CNAs and aneuploidies, we used the software package ichorCNA. IchorCNA uses a Hidden Markov Model to predict the segments of CNAs and to estimate the ctDNA fraction from ULP-WGS of cfDNA. The workflow consists of three steps: (1) computing read coverage, (2) data normalization, and (3) CNA prediction and estimation of ctDNA fraction. The analysis proceeded with a series of steps aimed at enhancing the accuracy of the results. Initially, guanine-cytosine content and mappability bias correction, depth-based local copy number estimations, and the estimation of tumor fraction based on copy number were carried out using the ichorCNA tool. Local read depth was corrected, considering guanine-cytosine bias and identifying regions with low mappability. Additionally, artifacts were eliminated by comparing the data to ichorCNA’s integrated healthy control reference. The CNAs were predicted with specific parameters tailored to the sample type, including the recommended low tumor fraction parameters for cfDNA samples and the default parameters for tumor and germline samples. Subsequently, ichorCNA utilized these binned, bias-corrected copy number values to create a two-component model, distinguishing between tumor-derived and non-tumor-derived fragments. From this model, the fraction of reads originating from the tumor, referred to as the tumor fraction, was derived.

**Statistical analysis**

The patients were categorized into two groups (positive or negative) based on the presence or absence of detectable ctDNA. This binary predictor variable was tested for association with clinical and demographic features using the Fisher’s exact or chi-square tests and t-test as appropriate. Categorical variables were reported as frequencies and percentages, and continuous variables were reported as medians, ranges, or interquartile range. OS and PFS were estimated by the Ka-
plan–Meier method. The association between OS with ctDNA positivity was tested using the log-rank test. Cox proportional hazards models were used to assess the association of ctDNA with other prognostic factors in the group of patients receiving systemic treatment. All P-values were two-sided; a P-value <0.05 was considered statistically significant. All analyses were performed using SPSS 25.0 (IBM Co., Armonk, NY, USA).

RESULTS

Patient characteristics

Blood samples were obtained from 73 patients with HCC. Patients most frequently had non-viral etiology (65.8%) and were in Child-Pugh class A (76.8%) (Table 1). Half of the patients were in very early or early BCLC stage, and 42% received systemic therapy that was mostly sorafenib. The median follow-up period was 37.38 months (range 0.5–65.6 months), and 25 patients (34.2%) had died at the time of analysis. As control group, blood samples were also obtained from 41 patients with cirrhosis without HCC or another malignancy. Most patients had non-viral etiology (75.6%) and were in Child-Pugh class B (70.7%). None of these patients developed malignancy during a median follow-up period of 22.47 months (range 1 to 58.4 months) (Table 2).

Circulating tumor DNA is detected by ULP-WGS in HCC patients but not in cirrhotic patients

The median cfDNA concentration was 33.7 ng/mL (range 6.05–495) in the HCC cohort and 31.95 ng/mL (range 17–345) in the cohort of patients with cirrhosis. ctDNA was detected in 22 of 73 patients with HCC (30.1%), and the median percent of ctDNA fraction was 27% (range 14–70%). Among patients receiving systemic treatment, ctDNA was detected in 18 of 31 patients (58.1%). Using ULP-WGS, we did not detect ctDNA in any of the patients in the cirrhotic cohort.

Detection of ctDNA is associated with clinical features and outcomes in HCC patients

We, therefore, analyzed the association between the presence of ctDNA with baseline clinical and laboratory features

<table>
<thead>
<tr>
<th>Table 1. Characteristics of HCC patients</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>73</td>
</tr>
<tr>
<td>Male sex</td>
<td>66 (90.4)</td>
</tr>
<tr>
<td>Age, years</td>
<td>65.10 (59.81–70.38)</td>
</tr>
<tr>
<td>Etiology</td>
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<tr>
<td>Alcoholic</td>
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<tr>
<td>HCV</td>
<td>19 (26.0)</td>
</tr>
<tr>
<td>HBV</td>
<td>6 (8.2)</td>
</tr>
<tr>
<td>MAFLD</td>
<td>15 (20.5)</td>
</tr>
<tr>
<td>Cryptogenic</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Hemochromatosis</td>
<td>2 (2.7)</td>
</tr>
<tr>
<td>Child-Pugh class</td>
<td></td>
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<tr>
<td>A/5</td>
<td>18 (24.7)</td>
</tr>
<tr>
<td>A/6</td>
<td>38 (52.1)</td>
</tr>
<tr>
<td>B/7</td>
<td>9 (12.3)</td>
</tr>
<tr>
<td>Other</td>
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<tr>
<td>ALBI grade</td>
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<tr>
<td>1</td>
<td>30 (41.1)</td>
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<td>2</td>
<td>42 (57.5)</td>
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<tr>
<td>3</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>BCLC stage</td>
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<tr>
<td>0/A</td>
<td>37 (50.7)</td>
</tr>
<tr>
<td>B/C</td>
<td>36 (49.3)</td>
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<tr>
<td>Macrovascular invasion</td>
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<tr>
<td>Extrahepatic spread</td>
<td>14 (19.2)</td>
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<tr>
<td>Bilobar involvement</td>
<td>25 (34.2)</td>
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<tr>
<td>Locoregional treatment</td>
<td>5 (6.8)</td>
</tr>
<tr>
<td>Systemic treatment</td>
<td>31 (42.5)</td>
</tr>
<tr>
<td>· Sorafenib</td>
<td>20 (27.4)</td>
</tr>
<tr>
<td>· Anti-PD1</td>
<td>5 (6.8)</td>
</tr>
<tr>
<td>· Anti-PD1+Anti-CTLA4</td>
<td>2 (2.74)</td>
</tr>
<tr>
<td>· Anti-PD-L1+Anti-VEGF</td>
<td>2 (2.74)</td>
</tr>
<tr>
<td>· Anti-PD-L1</td>
<td>1 (1.37)</td>
</tr>
<tr>
<td>· Anti-CTLA4</td>
<td>1 (1.37)</td>
</tr>
<tr>
<td>AFP</td>
<td></td>
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<tr>
<td>&lt;20 ng/mL</td>
<td>44 (60.3)</td>
</tr>
<tr>
<td>≥20 ng/mL</td>
<td>29 (39.7)</td>
</tr>
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</table>

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

HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; MAFLD, metabolic dysfunction-associated fatty liver disease; ALBI, albumin-bilirubin; BCLC, Barcelona Clinic Liver Cancer; Anti-PD1, anti-programmed cell death protein 1; Anti-CTLA4, cytotoxic T lymphocyte-associated antigen 4; Anti-PD-L1, anti-programmed death-ligand 1; Anti-VEGF, anti-vascular endothelial growth factor.
(Table 3). The patients who tested positive for ctDNA more frequently had advanced HCC, including BCLC B/C stage, macrovascular invasion, extrahepatic spread, tumor size ≥5 cm, and high levels of AFP (≥20 ng/mL), compared to those who tested negative (P < 0.05). There were no significant differences in age, sex, etiology (viral vs. non-viral), tumor number, and degree of involvement (bilobar vs. unilobar) between patients with and without detectable ctDNA.

Patients were followed for a median of 37.4 months (range 0.53–65.64 months). Among patients receiving systemic treatment, the detection of ctDNA was associated with inferior OS (Fig. 1). Median OS was not reached in the ctDNA-negative group, while it was 13.9 months (95% confidence interval [CI] 6.46–21.46) in the ctDNA-positive group (P = 0.01). PFS was also numerically lower for patients with detectable ctDNA, but the difference was not statistically significant (P = 0.119). Median PFS was 8.7 months (95% CI 2.21–15.26) in the ctDNA-negative group and 4.2 months (95%CI 2.67–5.80) in the ctDNA-positive group.

The four patients in the ctDNA-positive group, who did not receive systemic treatment due to being candidates to surgery or loco-regional therapy, were alive and free from recurrence at the end of follow-up. Two of them were treated with hepatectomy (BCLC-A stage and BCLC-0 stage), and the other two were treated with radioembolization (BCLC-A stage and BCLC-B stage).

After adjusting for factors such as macrovascular invasion, extrahepatic spread, tumor size ≥5 cm, AFP ≥20 ng/mL, and type of treatment (sorafenib vs. immunotherapy), a multivariable Cox proportional hazard regression analysis revealed a significant association between the presence of ctDNA and OS in patients with HCC receiving systemic treatments. The hazard ratio (HR) was 7.69 (95% CI, 2.09–28.27). HRs for macrovascular invasion, extrahepatic spread, tumor size ≥5 cm, AFP ≥20 ng/mL, and treatment with sorafenib were 1.84 (95% CI, 0.67–5.02), 0.35 (95% CI, 0.09–1.24), 1.12 (95% CI, 0.33–3.83), 1.61 (95% CI, 0.38–6.77), and 4.92 (95% CI, 1.54–15.67), respectively. Treatment with immunotherapy was protective (HR 0.20; 95% CI 0.06–0.64) (Supplementary Table 1).

The percentage of ctDNA among the ctDNA-positive patients was not associated to OS, but the sample size was low (P = 0.142), with a median survival of 13.37 months (95% CI, 9.45–17.28) for those with high ctDNA fraction compared to 21.61 months (95% CI, 7.41–35.82) for those with low ctDNA fraction.

In a subgroup analysis based on the BCLC stage, a non-significant trend towards worse OS in the presence of ctDNA was observed in the more advanced stages. No BCLC-0 patient and only two BCLC-A patients had died at the end of follow-up. Among 14 BCLC-B patients (six ctDNA-positive), the median overall survival was not reached in the ctDNA-negative group and was 13.9 months in the ctDNA-positive group (P = 0.184). Among 22 BCLC-C patients (13 ctDNA-positive), the median overall survival was 40.1 months in the ctDNA-negative group and 16.3 months in the ctDNA-positive group (P = 0.148).

### Identification of genetic features of HCC using ULP-WGS

ULP-WGS data analysis showed CNAs at different chromosomal loci. According to the previously described structural genomic variations in HCC sequencing, certain chromosomal alteration patterns were commonly found in patients with HCC. The most frequent chromosomal arm gains were 1q (63.6%), 8q (59.1%), 7q (27.2%), and 5p (22.7%), and the
The most frequent chromosomal arm losses were 8p (54.5%), 4q (45.4%), 13q (45.4%), 16q (40.9%), and 5q (36.3%) (Fig. 2A). The frequency of CNA per patient was highly variable, and a minority of patients’ ctDNA had only one chromosomal arm affected. While 22.7% of patients had only one gain, another 22.7% had more than seven gains (Fig. 2B). In the case of losses, 9.1% of patients had one loss, while 50% had more than seven losses (Fig. 2C). Considering either gains and loss-

| Table 3. Association between ctDNA detection and clinical features in patients with HCC |
|----------------------------------------|----------------------------------|-----------------|-------------------|
| **Clinical features**                  | **Positive ctDNA**               | **Negative ctDNA** | **P-value**       |
| Number of patients                     | 22                              | 51               |                   |
| Age, years                             | 63.68 (59.45-71.11)             | 65.6 (59.9-69.77) | 0.491             |
| Male                                   | 20 (90.90)                      | 46 (90.19)       | 1                 |
| Etiology                               |                                  |                  |                   |
| Viral etiology                         | 8 (36.36)                       | 17 (33.33)       | 0.802             |
| Non-viral etiology                     | 14 (63.63)                      | 34 (66.67)       |                   |
| BCLC stage                             |                                  |                  | 0.000*            |
| 0/A                                    | 3 (13.63)                       | 34 (66.67)       |                   |
| B/C                                    | 19 (86.36)                      | 17 (33.33)       |                   |
| Macrovacular invasion                  |                                  |                  | 0.023*            |
| Yes                                    | 8 (36.36)                       | 6 (11.76)        |                   |
| No                                     | 14 (63.63)                      | 45 (88.23)       |                   |
| Extrahepatic spread                    |                                  |                  | 0.000*            |
| Yes                                    | 10 (45.45)                      | 4 (7.84)         |                   |
| No                                     | 12 (54.54)                      | 47 (92.15)       |                   |
| Bilobar involvement                    |                                  |                  | 0.062             |
| Yes                                    | 11 (50)                         | 14 (27.45)       |                   |
| No                                     | 11 (50)                         | 37 (72.54)       |                   |
| Tumor number                           |                                  |                  | 0.179             |
| <3                                     | 11 (50)                         | 34 (66.66)       |                   |
| ≥3                                     | 11 (50)                         | 17 (33.33)       |                   |
| Tumor size                             |                                  |                  | 0.000*            |
| <5 cm                                  | 10 (45.45)                      | 46 (90.19)       |                   |
| ≥5 cm                                  | 12 (54.54)                      | 5 (9.80)         |                   |
| AFP                                    |                                  |                  | 0.000*            |
| ≥20 ng/mL                              | 16 (72.72)                      | 9 (17.64)        |                   |
| <20 ng/mL                              | 6 (27.27)                       | 42 (82.35)       |                   |
| Treatments                             |                                  |                  | 0.000*            |
| Surgical                               | 2 (9.09)                        | 35 (68.62)       |                   |
| Locoregional                           | 2 (9.09)                        | 3 (5.88)         |                   |
| Systemic                               | 18 (81.81)                      | 13 (25.49)       |                   |
| Systemic treatment                     |                                  |                  |                   |
| Sorafenib                              | 11 (61.11)                      | 9 (69.23)        | 0.718             |
| Immunotherapy                          | 7 (38.88)                       | 4 (30.76)        |                   |

Values are presented as number only, median (interquartile range), or number (%).
HCC, hepatocellular carcinoma; ctDNA, circulating tumor DNA; BCLC, Barcelona Clinic Liver Cancer; AF, alpha-fetoprotein.
*Statistical significance, P<0.05.
es, only 18.2% of patients had only one gain or loss, while 59.1% had more than seven chromosomal arm gains or losses (Fig. 2D).

In ctDNA-positive patients, more than seven CNA (either gains or losses) was associated with inferior OS. Median OS was 54.6 months (95% CI 21.06–88.21) in the less than seven CNA group, and 10.5 months (95% CI 3.28–17.80) in the more than seven CNA group ($P=0.006$).

There was no difference in the OS of patients with gain of 1q, 8q, 7q, or 5p compared to those without a corresponding chromosomal-arm gain. Among the patients under systemic treatment with positive ctDNA, those with loss of 5q and 16q exhibited a significantly worse OS compared to those without a corresponding chromosomal-arm loss (both $P<0.05$).

The median OS was 10.38 months (95% CI, 0–22.91) with 5q loss and 21.61 months (95% CI, 10.09–33.13) without the loss (Fig. 3A). The median OS was 5.97 months (95% CI, 0.98–10.97) with 16q loss and 21.61 months (95% CI, 7.41–35.82) without it (Fig. 3B).

A significant association was found between 5q loss and OS after adjusting for AFP ≥20 ng/ml, macrovascular invasion, tumor size ≥5 cm, and extrahepatic spread. HR for 5q loss was 8.92 (95% CI, 1.79–44.38). HRs for AFP ≥20 ng/mL, macrovascular invasion, tumor size ≥5 cm, and extrahepatic spread were 2.52 (95% CI, 0.55–11.40), and 0.30 (95% CI, 0.06–1.43), respectively (Supplementary Table 2).

Moreover, an independent association was observed between 16q loss and the OS, after adjusting for variables such as AFP ≥20 ng/ml, macrovascular invasion, tumor size ≥5 cm, and extrahepatic spread. HR for 16q loss was 5.29 (95% CI, 1.24–22.50). HRs for AFP ≥20 ng/mL, macrovascular invasion, tumor size ≥5 cm, and extrahepatic spread were 0.39 (95% CI, 0.06–2.47), 1.31 (95% CI, 0.32–5.33), 2.30 (95% CI, 0.49–10.84), and 0.43 (95% CI, 0.07–2.49), respectively (Supplementary Table 3).

**DISCUSSION**

Based on our results, detectable ctDNA serves as a minimally invasive biomarker indicating a worse prognosis in patients with HCC undergoing systemic therapy, independent of clinicopathologic characteristics and type of systemic treatment. Patients with detectable ctDNA were more likely to exhibit unfavorable biological tumor features, including AFP ≥20 ng/mL, macrovascular invasion, tumor size ≥5 cm, type of systemic treatment, and extrahepatic spread.

Our results are supported by recent studies that demonstrated the association of ctDNA detected by ULP-WGS with
poor OS in various cancer types, including metastatic squa-
mo us non–small cell lung cancer,\textsuperscript{23} Ewing sarcoma, and osteosarcoma,\textsuperscript{24} metastatic castration-resistant prostate cancer,\textsuperscript{25} cervix cancer,\textsuperscript{26} and metastatic triple-negative breast cancer.\textsuperscript{27} Our study is the first to show the same phenome-
non in advanced HCC patients. The identification of ctDNA is a biomarker for tumor aggressiveness and may allow more accurate risk stratification, treatment planning, and surveil-
lance.

ULP-WGS presents several advantages. Among them, easy processing, low cost, and rapid readout standout as the most relevant advantages for routine clinical practice. If confirmed in larger series, ULP-WGS could help in prognostic assess-
ment. Tumor staging with clinical and imaging features allow allocating patients in groups with different prognosis.\textsuperscript{3} AFP and AFP-L3\% can identify a group of patients with worse prognosis across stages.\textsuperscript{28} However, accurate individual prog-
nostication is still an unmet need in HCC. Contrary to AFP measurement, ULP-WGS is not focused on a specific type of genetic alteration. It can identify and group various genetic alterations, contributing to a reduction in result variability. Notably, detecting ctDNA and CNAs with this approach possesses a distinctive capacity to encapsulate comprehensive somatic information about HCC. This unique attribute may

Figure 2. Distribution of large copy number alterations along the whole genome and the cohort. (A) Diagram showing the relative length of each chromosome arm in the human genome (gray rounded shaped vertical symbols) with, besides, the number of patients with either gains (blue) and losses (green) in each chromosome arm. (B) Percentage of patients with a specific number of concomitant CNAs gains in different arms. (C) Percentage of patients with a specific number of concomitant CNAs losses in different arms. (D) Percentage of patients with a specific number of concomitant CNAs losses in different arms. CNA, copy number alterations.
overcome those challenges related to tumor heterogeneity. Tissue biomarkers, such as Heat Shock Protein 70, can offer diagnostic and prognostic utility.\(^{29,30}\) However, accessing this information requires biopsies or surgical specimens, which come with inherent risks. ULP-WGS could also potentially help in monitoring the response to treatment and provide a dynamic picture of the disease course.

Limitations of ULP-WGS include its lower sensitivity and the need of a relatively high tumor burden for effective detection of ctDNA and CNAs, as shown by the poor performance in BCLC 0/A patients. In such earlier stages or for the detection of minimal residual disease, more sensitive methods would be needed, such as the detection of tumor-derived SNV using Droplet Digital PCR or deep-targeted sequencing.\(^{25,31}\) Studies in prostate cancer have also shown that ctDNA was not detected in patients with local vs. metastatic disease.\(^{25,31}\) The potential mechanisms include reduced necrosis and vascularization of localized small tumors with diminished proliferative rate.\(^{31,32}\) Understanding the strengths and limitations of ULP-WGS underscores the importance of tailoring the approach to the specific clinical context and disease stage for optimal utility.

**Figure 3.** Overall survival of patients with advanced HCC according to prognostically-relevant CNAs. (A) Survival curve of patients with detectable ctDNA according to the presence or absence of 5q loss. (B) Survival curve of patients with detectable ctDNA according to the presence or absence of 16q loss. All patients were treated with systemic treatments. LogRank test was used for analyzing the median survival differences. Tick marks indicate censored data. 5q, long arm of chromosome 5; 16q, long arm of chromosome 16. ctDNA, circulating tumor DNA; HCC, hepatocellular carcinoma; CNA, copy number alterations.
The utility of detecting ctDNA using cfDNA WGS in patients with HCC has been reported in a cohort of 117 subjects with early tumors receiving surgery or radiofrequency ablation. In this cohort, the sequencing depth coverage of the WGS was relatively high (5x). Higher levels of ctDNA were associated with poor recurrence-free and OS. The most frequent CNAs included gains in 20p, 8q, 1q, and 20q, as well as losses in 17p, 4q, 19p, and 16q. In another cohort of 34 HCC patients undergoing surgery, ctDNA detected by WGS (with a deep coverage of 5x) had prognostic value. Similar findings were observed in a longitudinal cohort of 64 subjects with advanced HCC receiving TACE. In this cohort, the average depth of sequencing coverage was 3x. Notably, they found that the changes in ctDNA during TACE treatment correlated with tumor burden and had predictive value for treatment response and prognosis. The most common CNAs were gains in the regions of chromosomes 1q, 6p, 8q, 20q, and 20q along with losses in chromosomes 4q, 13q, 8p, 16q, and 17p.

In the present study, we discovered various genomic features in ctDNA that were prognostically relevant, including the detection of CNAs at different chromosomal loci in HCC. Among patients with detectable ctDNA undergoing systemic therapy, the loss or deletion of 5q and 16q emerged as independent biomarkers predicting worse survival, regardless of other clinicopathologic features usually associated with bad prognosis, such as high AFP, macrovascular invasion, or extrahepatic spread. Furthermore, most patients exhibited more than seven concurrent CNAs, indicating a high chromosomal instability and molecular heterogeneity, which ultimately lead to disease progression.

In our study, the most frequent losses were 8p, 4p, 13q, 16q, and 5q, all reported as frequent in HCC, in large cohort studies involving the WGS of tumor samples. The loss of 5q and 16q chromosome arms was observed in a group of patients with poor survival. The loss at 5q was reported in HCC. Notably, the genomic loci of 5q13.2 encompass cancer related genes, including GTF2H2, NAIP, and OCLN. In a previous study involving 29 HCC patients, the loss of 5q was observed in the tissue of nine patients (31%). Multivariate analysis revealed that allelic loss on chromosome 5q34 band served as an independent prognostic factor for poor survival. On the order hand, the long arm of chromosome 16 carries the epithelial cadherin (E-cadherin) gene, a finding supported by previous studies in HCC. E-cadherin is a cell adhesion protein implicated as an invasion and metastasis suppressor. A meta-analysis involving 2,439 patients demonstrated that reduced expression of E-cadherin correlated with a poor prognosis in HCC. It is also associated with metastasis, vascular invasion, advanced differentiation grade, and advanced disease stage.

The three most frequent losses at chromosomes 8p, 4p, and 13q were not associated with survival. Chromosome 8p harbors a cluster of six genes, including DLC1, CCDC25, ELP3, PROSC, SH2D4A, and SORBS3, all of which are tumor suppressor genes. Also, the inhibitor of growth family member 2 (ING2) is a tumor suppressor gene located on chromosome 4q. Frequent allelic losses at chromosome 13q have been observed in HCC. The retinoblastoma gene (RB1), located in this chromosome, is believed to play a role in HCC.

In our cohort, the most frequent gains were 1q, 8q, 7q, and 5p. These amplifications encompass well-known driver oncogenes, including MCL1 (1q21.3), MET (7q31.2), MYC (8q24.21), and TERT (5p15.33). CNAs are important subclasses of somatic mutations, with aberrant chromosomal regions of amplifications or deletions commonly associated with overexpressed oncogenes or the loss of tumor suppressor genes. CNAs are a hallmark of human cancer and are believed to contribute to carcinogenesis, tumor progression, and the development of therapy resistance. In a previous study involving patients with metastatic prostate and breast cancer, tumor-derived CNAs were detected in ctDNA using ULP-WGS, and these were found to be concordant with those observed in the corresponding tumor tissue.

Our study has some limitations. First, the technique used to assess ctDNA and CNAs exhibited low sensitivity, particularly in patients at early stages. Moreover, the retrospective nature of the study raises concerns about potential bias. Another limitation include the heterogeneity of treatments: the majority of patients received sorafenib and the patients treated with immunotherapy received different regimes. Consequently, a validation cohort with a larger sample size and consecutive blood samples, ideally involving patients undergoing first-line systemic treatments such as combination therapies of Atezolizumab-Bevacizumab or Durvalumab-Tremelimumab, would be highly valuable.

In summary, our study explores the utility of using ULP-WGS of cfDNA to detect the presence of ctDNA and CNAs in HCC. Our findings demonstrate that the detection of ctDNA...
and CNAs can provide clinically relevant information regarding HCC prognosis. The assessment of ctDNA and CNAs has the potential to serve as a prognostic biomarker in advanced HCC, to help provide better information about unique genomic features, tumor progression, drug resistance, and novel therapeutic targets.

**Authors’ contribution**


**Acknowledgements**

We particularly acknowledge the contributions of the patients for their participation and the Biobank of the University of Navarra for its collaboration, as well as Amaya Redin from the Liver Unit of Clínica Universidad de Navarra.

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**Conflicts of Interest**

B5 reports consultancy fees from Adaptimmune, Astra Zeneca, Bayer, BMS, Boston Scientific, Eisai, Eli Lilly, Incyte, Ipsen, Novartis, MSD, Roche, Sanofi, Sirtex Medical, Terumo; speaker fees from Astra Zeneca, Bayer, BMS, Eisai, Eli Lilly, Incyte, Ipsen, Novartis, Roche, Sirtex Medical, Terumo; research grants (to the Institution) from BMS and Sirtex Medical. J.A. is member of a scientific steering committee of Roche Spain and has received fees for talks by Pfizer and Roche. Rest of the authors have nothing to declare.

**SUPPLEMENTARY MATERIAL**

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

**REFERENCES**


Efficacy, safety, and pharmacokinetics of capsid assembly modulator linvencorvir plus standard of care in chronic hepatitis B patients

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Graphical Abstract

Efficacy, safety, and pharmacokinetics of capsid assembly modulator linvencorvir plus standard of care in chronic hepatitis B patients

Mean HBsAg change from baseline in patients with baseline HBsAg<1.0 mIU/mL.

Mean HBV DNA change from baseline in patients with baseline HBV DNA<1.00 log10 IU/mL.

Mean HBV RNA change from baseline in patients with baseline HBV RNA<1.00 log10 copies/mL.

* Only include compliant and followed-up with NUC patients.
Background/Aims: Four-week treatment of linvencorvir (RO7049389) was generally safe and well tolerated, and showed anti-viral activity in chronic hepatitis B (CHB) patients. This study evaluated the efficacy, safety, and pharmacokinetics of 48-week treatment with linvencorvir plus standard of care (SoC) in CHB patients.

Methods: This was a multicentre, non-randomized, non-controlled, open-label phase 2 study enrolling three cohorts: nucleos(t)ide analogue (NUC)-suppressed patients received linvencorvir plus NUC (Cohort A, n=32); treatment-naïve patients received linvencorvir plus NUC without (Cohort B, n=10) or with (Cohort C, n=30) pegylated interferon-α (Peg-IFN-α). Treatment duration was 48 weeks, followed by NUC alone for 24 weeks.

Results: 68 patients completed the study. No patient achieved functional cure (sustained HBsAg loss and unquantifiable HBV DNA). By Week 48, 89% of treatment-naïve patients (10/10 Cohort B; 24/28 Cohort C) reached unquantifiable HBV DNA. Unquantifiable HBV RNA was achieved in 92% of patients with quantifiable baseline HBV RNA (14/15 Cohort A, 8/8 Cohort B, 22/25 Cohort C) at Week 48 along with partially sustained HBV RNA responses in treatment-naïve patients during follow-up period. Pronounced reductions in HBeAg and HBcrAg were observed in treatment-naïve patients, while HBsAg decline was only observed in Cohort C. Most adverse events were grade 1–2, and no linvencorvir-related serious adverse events were reported.

Conclusions: 48-week linvencorvir plus SoC was generally safe and well tolerated, and resulted in potent HBV DNA and RNA suppression. However, 48-week linvencorvir plus NUC with or without Peg-IFN did not result in the achievement of functional cure in any patient. (Clin Mol Hepatol 2024;30:191-205)

Keywords: Linvencorvir; RO7049389; Capsid assembly modulator; Chronic hepatitis B; Phase 2
INTRODUCTION

Hepatitis B virus (HBV) infection remains a major global health challenge, and is associated with life-threatening consequences.1,2 Functional cure, defined as unquantifiable HBV DNA and sustained hepatitis B surface antigen (HBsAg) loss,3 improves long-term prognosis and is a major therapeutic goal for chronic hepatitis B (CHB) therapy.4-7 Currently available treatments for CHB, including nucleos(t)ide analogues (NUCs) and pegylated interferon (Peg-IFN), have limitations. NUCs, which inhibit HBV DNA synthesis, are unable to fully suppress viral replication in some patients (or do so very slowly), especially in hepatitis B e antigen (HBeAg) positive patients and those with high viral load.5,9 NUCs must be taken life-long, have no direct effect on HBV RNA or covalently closed circular DNA (cccDNA), and rarely lead to functional cure.5,9,10 Peg-IFN therapy is finite, but results in low rates of functional cure and is associated with side effects.12 There is therefore a need for novel, well tolerated treatments that can augment viral suppression and help clear HBsAg in combination with current standard of care (SoC).10

The HBV capsid is involved in multiple steps of the HBV life cycle and is an important target of antiviral therapies in development.13,14 Several capsid assembly modulators (CAMs), which inhibit viral replication by inducing the formation of aberrant non-capsid polymers (CAM-A, previously known as Class I) or morphologically normal but nucleic acid-free empty capsids (CAM-E, previously known as Class II),13 have reached phase 1 and 2 clinical development.13 Studies to date have shown that 24-week treatment with CAM and NUC leads to suppression of HBV DNA and RNA, but has limited effect on HBV antigens.14,15,16

Linvencorvir (RO7049389) is a novel small molecule CAM that induces aberrant capsid assembly, leading to the degradation of viral core protein, thereby inhibiting pregenomic RNA (pgRNA) encapsidation and HBV DNA replication. Further, linvencorvir also induces the disassembly of nucleocapsids, potentially interfering with cccDNA biosynthesis.18 A first-in-human, three-part phase 1/2 study of linvencorvir has been conducted in healthy volunteers and CHB patients. In Part 1 of the phase 1/2 study, linvencorvir showed favourable safety and pharmacokinetic profiles in healthy volunteers following single ascending doses up to 2,500 mg, and multiple ascending doses up to 1,200 mg/day for 2 weeks.19 In Part 2, 4-week monotherapy with linvencorvir up to 1,000 mg/day was generally safe and well tolerated, and had potent antiviral activity in viremic CHB patients.19 Here, we report Part 3 (phase 2 stage) of the phase 1/2 study, in which we evaluated the efficacy, safety and pharmacokinetics of linvencorvir in combination with SoC therapies (NUC with or without Peg-IFN-α) for 48 weeks in virologically-suppressed and treatment-naïve CHB patients.

MATERIALS AND METHODS

Study design and population

This multicenter, non-randomized, non-controlled, open-label phase 2 study (Part 3 of the first-in-human linvencorvir trial) was performed at 16 sites in Taiwan (n=5), Mainland China (n=3), New Zealand (n=2), Thailand (n=2), Australia (n=1), Bulgaria (n=1), Hong Kong (n=1) and Singapore (n=1). This study comprised three treatment cohorts, in which NUC-suppressed or treatment-naïve CHB patients received open-label treatment with linvencorvir plus a first-line NUC (entecavir [ETV], tenofovir alafenamide [TAF], or tenofovir disoproxil fumarate [TDF]) with or without Peg-IFN-α for 48 weeks (Fig. 1). In Cohort A, NUC-suppressed patients received linvencorvir plus NUC therapy for 48 weeks. In Cohort B, treatment-naïve patients received linvencorvir alone for the first 4 weeks of the treatment period, followed by linvencorvir plus NUC therapy for the remaining 44 weeks. Treatment-naïve patients enrolled in Cohort C received linvencorvir plus NUC and Peg-IFN-α therapy throughout the 48-week study treatment period. After the study treatment period, all patients were followed up for 24 weeks with NUC monotherapy, or without NUC if they met protocol-defined NUC stopping criteria (HBsAg below 100 IU/mL and HBV DNA below the lower limit of quantification [LLOQ; 20 IU/mL]) at the end of study treatment (Week 48). During the off-treatment follow-up period, if alanine aminotransferase (ALT) >2 times the upper limit of normal (ULN; 41 U/L for men and 33 U/L for women) was accompanied by confirmed virological relapse, NUC treatment may be restarted at the discretion of the investigator and applicable CHB guidelines.

Eligible patients were aged 18–60 years with CHB (a positive HBsAg or HBV DNA test or HBeAg-positive for more than 6 months before screening), and HBsAg concentration above 250 IU/mL at screening. NUC-suppressed patients were re-

quired to have been treated with NUC monotherapy (ETV, TAF, or TDF) for at least 12 months, and must have been on the same NUC therapy for at least 3 months before screening. These patients should have HBV DNA below LLOQ at screening, and ALT ≤2×ULN at screening and Day -1. Treatment-naïve patients were required to have previously received anti-HBV treatments for less than 30 days in total, and to have not received any anti-HBV treatment within 3 months prior to the first study dose. Treatment-naïve patients also required HBV DNA of at least 2×10⁴ IU/mL (HBeAg-positive patients) or 2×10³ IU/mL (HBeAg-negative patients) at screening, and ALT levels between 1–5×ULN at screening and below 5×ULN at Day -1. Full details of the eligibility criteria are provided in the Supplementary Material.

The study was conducted in accordance with Good Clinical Practice standards and the Declaration of Helsinki. The study protocol was approved by the institutional review boards or ethics committees from all participating study centres, and written informed consent was obtained from each participant included in the study.

**Procedures**

In all three treatment cohorts, linvencorvir 600 mg was administered orally once a day in the fasted state (≥2 hours after a meal and ≥2 hours before the next meal). NUC (ETV, TAF, or TDF) and Peg-IFN-α therapy were administered according to the local label or guidelines. Investigators could refer to Peg-IFN stopping rules recommended in major guidelines.⁵,²¹ If Peg-IFN was stopped before the end of the 48-week treatment period, linvencorvir and NUC were to be continued until the end of the treatment period. At the end of the study treatment period, NUC therapy was continued for 24 weeks unless patients met the NUC stopping criteria.

Safety-related clinical and laboratory evaluations, and blood sample collections for the determination of HBV viral dynamic responses were conducted on day-1, during the study treatment period (every 2 weeks for the first 4 weeks and every 4 weeks thereafter), and during the follow-up (every 8 weeks for patients not meeting the NUC stopping criteria; every 2 weeks for the first 12 weeks and every 4 weeks thereafter for patients meeting the NUC stopping criteria). Details of methodologies for determining HBV genotype, and measuring viral dynamic markers are provided in the Supplementary Material. In particular, plasma HBV RNA was quantitatively assessed at Roche Diagnostic International Ltd (for non-Chinese sites) or Q2 Solutions for Chinese sites using a COBAS® HBV RNA test on the Roche COBAS® 6800 System.²²,²³ Safety assessments included monitoring and recording the occurrence and severity of adverse events (AEs), physical examinations, safety laboratory assessments, vital signs, and 12-lead electrocardiograms (ECGs). AEs and ALT and aspartate aminotransferase (AST) elevations were graded according to the Division of AIDS criteria (Supplementary Table 1).

Plasma samples for pharmacokinetics (PK) analysis were...
collected at the following time points: (1) pre-dose and 1–8 hours post-dose on day 1 and Weeks 4 (Cohort B only) and 24, (2) pre-dose and 1–4 hours post-dose at all other scheduled visits during study treatment; and (3) before and 1–24 hours after the last dose of study treatment.

Endpoints

The primary endpoint in this study was HBV DNA below the LLOQ (20 IU/mL) with HBsAg loss (<0.05 IU/mL) at 24 weeks post-treatment (defined as functional cure in the protocol). Secondary efficacy endpoints included serum HBV DNA and RNA below the LLOQ, HBsAg and HBeAg loss and anti-HBs and anti-HBe seroconversion, quantitative change from baseline for the HBV markers including serum HBV DNA, HBV RNA and HBV antigens. Secondary efficacy endpoints were assessed in each cohort overall and the following patient subgroups: HBeAg-positive and HBeAg-negative, and low and high baseline HBsAg (cutoff: 4 log10 IU/mL). Relationships between secondary efficacy endpoints and HBV genotype and high baseline HBV DNA (>7 log10 IU/mL) were also explored. Other secondary endpoints were the incidence of AEs and most common AEs, and the PK profile of linvencorvir and its metabolites when used in combination with SoC therapies.

Statistical analysis

The sample size for this study was intended to support the assessment of the functional cure rate. Individual cohort sample sizes of at least 10–30 were planned to ensure that the lower 95% confidence interval was above 5–14% if there was an observed functional cure rate of 30%, assuming binomial distribution.

All patients who received at least one dose of linvencorvir were included in the safety and efficacy analysis populations. Efficacy analyses were based on the actual number of patients with valid results at each study visit. For the PK analysis, patients who significantly violated inclusion or exclusion criteria, who deviated significantly from the protocol, or for whom data were unavailable or incomplete which may have influenced the PK analysis were excluded.

For continuous variables, descriptive statistics were calculated. Values below the LLOQ were imputed to numeric values below the LLOQ value to make a conservative calculation of change from baseline values (Supplementary Table 2). For categorical data, the number and proportion of study participants in each category were summarized. Spearman’s rank correlation was calculated to determine the relationship between graded treatment-emergent ALT elevations and categorized maximal declines in HBsAg. PK parameters were calculated from a non-compartment analysis using Phoenix software (WinNonlin models version 6.4).

RESULTS

Patient characteristics

Between June 14, 2019 and October 19, 2020, 72 (44%) of 163 screened patients were enrolled in the study: 32 NUC-suppressed patients in Cohort A, 10 and 30 treatment-naïve patients in Cohorts B and C, respectively (Fig. 2). All 72 patients received linvencorvir, and 68 (94.4%) patients completed the 72-week study. Linvencorvir treatment was discontinued early for non-safety reasons in four patients (on days 15 and 62 for two patients in Cohort A, and on days 83 and 237 for two patients in Cohort C).

Baseline demographics and clinical characteristics are shown in Table 1. In Cohorts A and C, patients were predominantly Asian and male, whereas 5 (50%) patients were Asian and 5 (50%) patients were male in Cohort B. HBV DNA levels were below the LLOQ in all Cohort A patients, but 15 (46.9%) patients had quantifiable HBV RNA. Mean baseline HBV DNA levels were 5.73 log10 IU/mL in Cohort B and 6.91 log10 IU/mL in Cohort C. Two (20%) and 18 (60%) patients in Cohorts B and C, respectively, had a high viral load (HBV DNA >7 log10 IU/mL). Eight (80%) and 27 (90%) patients in Cohorts B and C, respectively, had baseline quantifiable HBV RNA. In Cohort A, six (19%) patients had HBV genotype C, as did five patients (50%) in Cohort B, and 11 patients (37%) in Cohort C. NUC-suppressed patients were mainly HBeAg-negative (66% [21/32]), but treatment-naïve patients were mainly HBeAg-positive (63% [25/40]). Mean baseline HBsAg levels across the three cohorts ranged from 3.2 log10 IU/mL in Cohort A to 3.96 log10 IU/mL in Cohort C. More than half of the Cohort C patients had high baseline HBsAg levels (≥ 4 log10 IU/mL).
Primary endpoint

In this study, no patient achieved HBV DNA < LLOQ with HBsAg loss at Week 24 post-study treatment (functional cure).

HBV DNA responses

In NUC-suppressed patients (Cohort A), mean HBV DNA levels remained below the LLOQ throughout the study. In treatment-naïve patients, HBV DNA levels declined by a mean (standard deviation [SD]) of 4.45 (1.86) and 5.80 (1.81) log10 IU/mL at Week 48 in Cohorts B and C, respectively (Fig. 3A). With higher baseline HBV DNA levels, HBeAg-positive patients achieved larger reductions in HBV DNA than HBeAg-negative patients (mean [SD] HBV DNA declines of 5.48 [1.19] vs. 2.90 [1.62] log10 IU/mL, respectively, in Cohort B; 6.97 [0.74] vs. 3.80 [1.20] log10 IU/mL, respectively, in Cohort C) (Fig. 3B). All ten (100%) Cohort B patients achieved HBV DNA below the LLOQ at Week 48, including two HBeAg-positive patients with high viral load. By Week 48, HBV DNA levels reached below the LLOQ in 86% (24/28) of Cohort C patients who completed 48-weeks of study treatment, including in 76% (13/17) of HBeAg-positive patients with high viral load. All the remaining four patients who had not achieved unquantifiable HBV DNA during the study treatment had significantly reduced viral DNA levels (<150 IU/mL) at Week 48. At the end of study treatment, all Cohort B patients entered into the 24-week follow-up with NUC treatment. Five patients in Cohort C met the NUC stopping criteria at Week 48, so they were followed without NUC. During the NUC-alone follow-up period, 96% (26/27) of NUC-compliant Cohorts B and C patients with unquantifiable HBV DNA by Week 48 sustained HBV DNA below the LLOQ; the four patients who had not achieved unquantifiable HBV DNA by Week 48 attained HBV DNA below the LLOQ or maintained low HBV DNA levels. Among the five patients who entered off-treatment follow-up, four patients experienced HBV DNA rebound to quantifiable levels at around Week 56. Three out of them were not retreated at the investi-
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NUC-suppressed Cohort A (n=32)</th>
<th>Treatment-naive Cohort B (n=10)</th>
<th>Cohort C (n=30)</th>
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<td>47.2 (8.3)</td>
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<td>32.8 (7.7)</td>
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<td>13 (41%)</td>
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<td>5 (50%)</td>
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<td>1 (3%)</td>
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<tr>
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</tr>
<tr>
<td>B</td>
<td>8 (25%)</td>
<td>0</td>
<td>13 (43%)</td>
</tr>
<tr>
<td>C</td>
<td>6 (19%)</td>
<td>5 (50%)</td>
<td>11 (37%)</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>5 (50%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>17 (53%)</td>
<td>0</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>20.66 (7.30)</td>
<td>59.40 (36.57)</td>
<td>94.10 (42.96)</td>
</tr>
<tr>
<td>Normal</td>
<td>32 (100%)</td>
<td>1 (10%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>(&gt;1)–(2)×ULN</td>
<td>0</td>
<td>7 (70%)</td>
<td>11 (37%)</td>
</tr>
<tr>
<td>(&gt;2)–(5)×ULN</td>
<td>0</td>
<td>2 (20%)</td>
<td>17 (57%)</td>
</tr>
</tbody>
</table>

Data are presented as mean (standard deviation) or number (%).

ALT, alanine aminotransferase; HBV, hepatitis B virus; HBeAg, hepatitis core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LLOQ, lower limit of quantification; NUC, nucleos(t)ide analogue; ULN, upper limit of normal.

*Only one patient received Lamivudine from Aug to Sep in 2011 (the exact start and end dates were unknown) before screening in 2020.

†Calculated from patients who were HBeAg-positive.
gators’ discretion and the remaining patient restarted NUC treatment from Week 60 with HBV DNA subsequently declining to below the LLOQ.

**HBV RNA responses**

Among the patients with quantifiable baseline HBV RNA, HBV RNA levels were suppressed to below the LLOQ at Week 48 in 93% (14/15), 100% (8/8) and 88% (22/25) of patients in Cohorts A, B and C, respectively. The mean (SD) 48-week declines in HBV RNA for patients with quantifiable baseline HBV RNA were 1.82 (1.05) log_{10} copies/mL in Cohort A, 3.45 (1.41) log_{10} copies/mL in Cohort B, and 4.20 (1.78) log_{10} copies/mL in Cohort C (Fig. 4). During the follow-up without linvencorvir, HBV RNA levels rebounded to approximately the baseline levels in Cohort A patients, but mean reductions from baseline of 2.16 (1.66) and 3.27 (1.71) log_{10} copies/mL were retained at Week 72 in Cohorts B and C patients, respectively. Patients in all three cohorts with unquantifiable baseline HBV RNA maintained HBV RNA levels below the LLOQ during the study treatment and NUC-alone follow-up periods.

**Figure 3.** Mean HBV DNA levels over 72 weeks. (A) Three cohorts overall and (B) HBeAg-positive and HBeAg-negative subgroups of treatment-naive patients in Cohorts B and C. *Excluded one non-compliant patient during the FU period. **One patient was retreated with NUC from Week 60. Error bars represent standard deviation. HBV, hepatitis B virus; EOT, end of treatment; FU, follow-up; NUC, nucleos(t)ide analogue; Peg-IFN, pegylated interferon.
HBsAg responses

No HBsAg loss or anti-HBs seroconversion occurred among patients completing the study. No apparent mean declines for Cohort A and B in HBsAg were observed during the study (Fig. 5A), but two HBeAg-positive patients in Cohort B had maximal HBsAg declines of 0.40–0.45 log10 IU/mL. In Cohort C, at Week 48, mean (SD) HBsAg decline was 1.39 (0.98) log10 IU/mL and numerically larger mean (SD) HBsAg declines occurred in HBeAg-positive and patients with baseline HBsAg ≥4 log10 IU/mL (1.64 (0.90) log10 IU/mL and 1.72 (0.88) log10 IU/mL, respectively). HBV genotype B and C patients achieved mean (SD) HBsAg declines of 1.35 (0.62) and 1.74 (1.13) log10 IU/mL from baseline levels of 3.80 (0.76) and 4.41 (0.91) log10 IU/mL, respectively (Table 2, Fig. 5A). At Week 48, 21% (6/28) and 68% (19/28) of patients achieved HBsAg levels <2 and 3 log10 IU/mL, respectively (Table 2). HBsAg declines were concurrent with treatment-emergent grade 2–4 ALT elevations which mostly occurred in treatment-naïve patients, with statistically significant positive correlations between graded ALT elevations and categorical maximal HBsAg declines (Spearman’s rho 0.432, \( P = 0.017 \) for Cohort C and 0.697, \( P = 0.025 \) for Cohort B) (Supplementary Fig. 1).

HBeAg and HBcrAg responses

At Week 48, NUC-suppressed HBeAg-positive Cohort A patients had mean (SD) HBeAg decline of 0.23 (0.23) log10 IU/mL from 0.41 (0.75) log10 IU/mL at baseline. Treatment-naïve, HBeAg-positive Cohorts B and C patients had mean (SD) HBeAg declines of 1.48 (0.84) and 2.10 (0.90) log10 IU/mL, respectively (Fig. 5B). Among the HBeAg-positive treatment-naïve patients, 50% (3/6) and 39% (7/18) achieved HBeAg loss and anti-HBe seroconversion occurred in 17% (1/6) and 33% (6/18) in Cohorts B and C, respectively. At Week 48, HBcrAg levels declined from baseline by mean (SD) of 0.13 (0.24), 1.23 (0.76), and 1.76 (1.1) log10 U/mL in Cohorts A, B, and C, respectively (Fig. 5C). During the follow-up period, levels of HBeAg and HBcrAg were generally sustained in treatment-naïve patients.

Adverse events

AEs occurred in 69% (22/32) of NUC-suppressed patients in Cohort A, 90% (9/10) of treatment-naïve patients in Cohort B, and all 30 treatment-naïve patients in Cohort C (Table 3). Headache, pyrexia, and increased ALT levels were among the most commonly reported AEs. Increased ALT levels occurred mainly at Weeks 2–8, and resolved within 14 weeks with no accompanying bilirubin/indirect bilirubin increase, although a mild increase in bilirubin occurred in a NUC-suppressed patient who had pre-existing liver disease (cholestasis and Gilbert syndrome). Moreover, in all five patients with grade 4 ALT elevations, linvencorvir was interrupted per protocol, but no further ALT elevations occurred after re-administering. Most AEs were grades 1–2. Grade 3–4 AEs were reported in
Figure 5. HBsAg (A), HBeAg (B) and HBcrAg (C) mean changes from baseline over 72 weeks. Patients with baseline value below the LLOQ were excluded from change from baseline analysis. HBsAg ≥4 log means baseline HBsAg level ≥4 log_{10} IU/mL; HBsAg <4 log means baseline HBsAg level <4 log_{10} IU/mL. Error bars represent standard deviation. EOT, end of treatment; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; LLOQ, lower limit of quantification; NUC, nucleos(t)ide analogue.
four Cohort A patients (13%), two Cohort B patients (20%), and 11 Cohort C patients (37%). There were eight serious AEs and one death (due to malignant melanoma), none of which were related to linvencorvir. Most treatment-related AEs occurred in Cohort C: 74 related to linvencorvir, 25 related to NUC and 266 related to Peg-IFN-α. Four AEs were assessed as being related to linvencorvir in each of Cohorts A and B (Table 3). There were no trends of clinically significant changes in vital signs or ECG data.

**Pharmacokinetics**

Linvencorvir was rapidly absorbed and eliminated, with low accumulation of linvencorvir and its major metabolites in plasma after 48 weeks of dosing. The PK profiles of linvencorvir, with or without SoC (NUC with or without Peg-IFN-α), were considered similar. The plasma concentration of NUCs and Peg-IFNs remained stable during the study treatment period.

**DISCUSSION**

In this study, no patient achieved functional cure at 24 weeks post-48-week treatment with linvencorvir 600 mg/day plus NUC with or without Peg-IFN-α. Linvencorvir plus NUC with or without Peg-IFN-α did demonstrate potent suppression of HBV DNA and RNA. Linvencorvir plus NUC and Peg-IFN-α in treatment-naïve patients led to the greatest overall declines in HBV antigens. Linvencorvir was generally safe and well tolerated in combination with SoC.

HBV DNA was maintained below the LLOQ throughout the study in NUC-suppressed patients and was suppressed below the LLOQ in the majority of treatment-naïve patients, including HBeAg-positive patients with high viral load. Moreover, after linvencorvir cessation, HBV DNA generally remained suppressed by NUC monotherapy. While complete suppression of HBV DNA is an essential part of functional cure, 30% to 50% HBeAg positive and/or patients with high viral load cannot achieve HBV DNA<LLOQ within 1–3 years NUC monotherapy. Furthermore, some CHB patients may develop low-level viremia even with long-term NUC treatment. For these NUC difficult-to-treat patients, addition of linvencorvir to NUC may be a potential therapeutic strategy. Larger and longer trials would be necessary to test this hypothesis.

Serum HBV RNA was suppressed to below the LLOQ in the majority of NUC-suppressed and treatment-naïve patients during study treatment, which reflected target engagement by linvencorvir. During the off-linvencorvir follow-up period, retained HBV RNA declines were only observed in treatment-

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**Table 2. HBsAg levels in treatment-naïve patients in Cohort C**

<table>
<thead>
<tr>
<th>HBsAg</th>
<th>Linvencorvir+NUC+Peg-IFN-α (Cohort C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
</tr>
<tr>
<td>Baseline</td>
<td>n=30</td>
</tr>
<tr>
<td>log₁₀ IU/mL</td>
<td>3.96 (0.90)</td>
</tr>
<tr>
<td>&lt;3 log₁₀ IU/mL</td>
<td>6 (20%)</td>
</tr>
<tr>
<td>&lt;2 log₁₀ IU/mL</td>
<td>0</td>
</tr>
<tr>
<td>At Week 48</td>
<td>n=28</td>
</tr>
<tr>
<td>CFB, log₁₀ IU/mL</td>
<td>–1.39 (0.98)</td>
</tr>
<tr>
<td>Genotype B</td>
<td>–1.35 (0.62)</td>
</tr>
<tr>
<td>Genotype C</td>
<td>–1.74 (1.13)</td>
</tr>
<tr>
<td>≥0.5 log₁₀ IU/mL CFB</td>
<td>21 (75.0%)</td>
</tr>
<tr>
<td>&gt;1.0 log₁₀ IU/mL CFB</td>
<td>20 (71.4%)</td>
</tr>
<tr>
<td>&gt;2.0 log₁₀ IU/mL CFB</td>
<td>7 (25.0%)</td>
</tr>
<tr>
<td>&lt;3 log₁₀ IU/mL</td>
<td>19 (68%)</td>
</tr>
<tr>
<td>&lt;2 log₁₀ IU/mL</td>
<td>6 (21%)</td>
</tr>
</tbody>
</table>

Data are presented as mean (standard deviation) or number (%).

CFB, change from baseline; HBsAg, hepatitis B surface antigen; NUC, nucleos(t)ide analogue; Peg-IFN, pegylated interferon.

Overall, n=12; HBeAg+, n=9; HBeAg-, n=3. Overall, n=11; HBeAg+, n=8; HBeAg-, n=3.
naïve patients, suggesting that initial HBV RNA declines in treatment-naïve patients may be more readily retained than secondary declines in NUC-suppressed patients. This partially sustained HBV RNA suppression, together with durable declines in HBCrAg and HBeAg, may indicate suppression of cccDNA transcriptional activity or a reduction in cccDNA levels,\(^{24,25}\) which is rarely observed in NUC therapy alone.\(^{26}\) Consistent with this hypothesis, CAMs have been shown in vitro to induce disassembly of nucleocapsids, thereby interfering with cccDNA reservoir establishment and replenishment.\(^{26-28}\) Linvencorvir showed little benefit in HBsAg reduction on top of NUC, however, when combined with Peg-IFN-α, HB-

---

Table 3. Overview of AEs in NUC-suppressed (Cohort A) and treatment-naïve (Cohorts B and C) patients

<table>
<thead>
<tr>
<th></th>
<th>Linvencorvir+NUC</th>
<th>Linvencorvir+NUC+Peg-IFN-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cohort A (n=32)</td>
<td>Cohort B (n=10)</td>
</tr>
<tr>
<td>Patients with at least one AE, n (%)</td>
<td>22 (69%)</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>Total number of AEs</td>
<td>110</td>
<td>48</td>
</tr>
<tr>
<td>Total number of treatment-related AEs</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>NUC</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Linvencorvir</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Peg-IFN</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Most common AEs*, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>3 (9%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Pyrexia</td>
<td>0</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>ALT increased</td>
<td>1 (3%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Alopecia</td>
<td>0</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Platelet count decreased</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>AST elevation</td>
<td>1 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>Decreased appetite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patients with at least one AE with fatal outcome</td>
<td>0</td>
<td>1 (10%)*</td>
</tr>
<tr>
<td>SAE</td>
<td>1 (3%)*</td>
<td>1 (10%)*</td>
</tr>
<tr>
<td>AE leading to withdrawal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AE leading to Linvencorvir/Peg-IFN interruption or modification</td>
<td>1 (3%)/NA</td>
<td>1 (10%)/NA</td>
</tr>
<tr>
<td>Related AE</td>
<td>3 (9%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Related to Linvencorvir</td>
<td>3 (9%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Related to NUC</td>
<td>0</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Related to Peg-IFN</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Grade 3–4 AE</td>
<td>4 (13%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Non-ALT elevation-associated Grade 3–4 AE</td>
<td>3 (9%)</td>
<td>1 (10%)</td>
</tr>
</tbody>
</table>

AEs, adverse events; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NA, not applicable; NUC, nucleos(t)ide analogue; Peg-IFN, pegylated interferon; SAE, serious adverse event; ULN, upper limit of normal; URTI, upper respiratory tract infection.

*Occurring with ≥30% incidence in at least one cohort. \(^{1}\)Gastroesophageal reflux disease onset on Day 364. \(^{2}\)The patient died on Day 535 due to malignant melanoma (SAE) onset on Day 425, with unresolved cellulitis and lymphadenitis (SAE diagnosed on Day 446). \(^{3}\)One patient had SAEs of URTI (Day 185) and cellulitis (Day 251); one patient had SAEs of hypersensitivity (Day 286) and dizziness (Day 472). \(^{4}\)Peg-IFN-α. AEs: thyroid disorder; allergic dermatitis. \(^{5}\)Per protocol, patients with ALT >10×ULN should interrupt linvencorvir and Peg-IFN treatment (Cohort C only).
sAg declines were observed in treatment-naïve patients. Notably, the HBsAg mean decline observed in Cohort C was larger than it was in a previous study of TDF plus Peg-IFN combination therapy. Moreover, HBsAg levels declined comparably in Cohort C patients with HBV genotypes C and B. It has been reported that HBsAg decline was significantly lower in patients with either HBV genotypes C or D than in patients with HBV genotypes A and B with one-year Peg-IFN plus NUC treatment. However, due to the limited sample size, the baseline differences, and the lack of a control group of Peg-IFN plus NUC, any additional benefit to HBsAg reduction from linvencorvir on top of Peg-IFN and NUC needs to be confirmed.

There were no unexpected safety concerns when linvencorvir was administered in combination with NUC or NUC plus Peg-IFN-α. AEs occurring in patients receiving linvencorvir plus NUC and Peg-IFN-α were consistent with the safety profile of Peg-IFN-α. As the observations seen in the previous study with 4-week linvencorvir monotherapy, transient treatment-emergent ALT elevations were observed almost exclusively in treatment-naïve patients but not in NUC-suppressed patients, and were accompanied by declining levels of viral antigens, including HBsAg. These ALT elevations are consistent with the natural history of CHB patients with active viral replication and are considered indicators of the host immune response against HBV rather than drug-induced liver injury.

Limitations of this study include its non-randomized, non-controlled design with no stratification, as well as the small sample size. The small sample size and unbalanced baseline characteristics detract from the validity of subgroup analyses.

In conclusion, linvencorvir is generally safe and well tolerated when added to SoC therapy for CHB. Linvencorvir on top of SoC potently suppresses HBV replication, including in HBeAg-positive patients and those with high viral load, however limited benefit is shown towards HBsAg loss. Next-generation CAMs with higher potency and greater inhibitory activity towards cccDNA reservoir maintenance may result in different outcomes towards the achievement of functional cure in CHB patients.

Authors’ contribution
All authors approved the final manuscript for submission. Jinlin Hou, Man-Fung Yuen contributed to the study design and conduct, data acquisition, data interpretation, and manuscript drafting and revision. Wen Zhang contributed to the data analyses, data interpretation, and manuscript drafting and revision. Qingyan Bo contributed to the study design, data analyses, data interpretation, and manuscript revision. Edward Gane and Wenhong Zhang contributed to the study design and conduct, data acquisition, data interpretation, and manuscript revision. Rozalina Balabanska, Jiming Zhang, Tien Huey Lim, Qing Xie, Chau-Ting Yeh, Sheng-Shun Yang, Xiex Liang, Piyawat Komolmit, Apinya Leerapun, Ting-Tsong Chang, Tsung-Hui Hu, Seng Gee Lim, Wan-Long Chuang and Barbara Leggett contributed to the study conduct, data acquisition, data interpretation, and manuscript revision. Zenghui Xue, Ethan Chan and Yuchen Zhang contributed to the data analyses, data interpretation, and manuscript revision. Qiaoqiao Xie, Xue Zhou and Miriam Triyatni contributed to the data interpretation, and manuscript revision. All authors reviewed and approved the final manuscript for submission.

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Conflicts of Interest
Jinlin Hou received grants from Bristol Myers Squibb and Johnson & Johnson; and declared other financial or non-financial interests with AbbVie, Arbutus, Bristol Myers Squibb, Gilead Sciences, Johnson & Johnson, and Hoffmann-La Roche.

Edward Gane is an advisory committee or review panel member for AbbVie, Arbutus, Arrowhead, Assembly Biosciences, Availa, Clear B Therapeutics, Dicerna, Finch Therapeutics, Gilead Sciences, Janssen, Novartis, Hoffmann-La Roche, and Vir Bio; and has received speaking and teaching fees from AbbVie, Aligos, DrugFarm, Enanta, Gilead, GlaxoSmithKline, Janssen, Merck, and Novartis.

Sheng-Shun Yang has received speaking fees from AbbVie, Bristol-Myers Squibb, Gilead Sciences, Ipsen, and Merck, Sharp & Dohme, and served as an advisory board member for AbbVie, Gilead Sciences, Hoffmann-La Roche, Sysmex, and Ipsen.

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Seng Gee Lim received payment or honoraria for lectures from Gilead, Janssen, Hoffman-La Roche, Sysmex and GSK; served as advisory board member for Gilead, Abbott, Hoffmann-La Roche, GSK, Janssen, Sysmex, Abrutus, Assembly, and Grifols; served in leadership role in ICE-HBV, HBV Forum, AASLD Asia Pacific Advisory Board, and ANRS-MIE Advisory Board; and receipt of research support from Gilead, Abbott, Hoffmann-LaRoche, Sysmex, Fibronostics, and Merck.

Man-Fung Yuen serves as advisory board member/ consultant for and/or received research funding from AbbVie, Aligos Therapeutics, AlCuris, Antios Therapeutics, Arrowhead Pharmaceuticals, Arbutus Biopharma, Assembly Biosciences, Bristol Myer Squibb, Bluejay Therapeutics, Clear B Therapeutics, Dicerna Pharmaceuticals, Finch Therapeutics, Fujirebio Incorporation, GlaxoSmithKline, Gilead Sciences, Immunocore, Janssen, Merck Sharp and Dohme, and Hoffmann-La Roche, Vir Biotechnology.

Rozalina Balabanska, Wenhong Zhang, Jiming Zhang, Tien Huey Lim, Qing Xie, Chau-Ting Yeh, Xieer Liang, Pyawat Komolmit, Apinya Leerapun, Ting-Tsung Chang, Tsung-Hui Hu, Wan-Long Chuang, and Barbara Leggett declare no competing interests.

Zenghui Xue, Ethan Chen, Yuchen Zhang, Qiaoqiao Xie and Wen Zhang are employees of Hoffmann-La Roche. Qingyan Bo and Xue Zhou were former employees of Hoffmann-La Roche.

**SUPPLEMENTARY MATERIAL**

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

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infection. J Hepatol 2022;77:1265-1275.
JCAD deficiency attenuates activation of hepatic stellate cells and cholestatic fibrosis

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Study Highlights
• JCAD was highly expressed in fibroblast-like cells in PBC specimens and in mice with bile duct ligation.
• Global JCAD knock-out alleviated bile ductular reaction and fibrosis after BDL.
• JCAD knock-down abrogated HSC activation via the Hippo-YAP pathway.
• HSC-specific JCAD deletion further augured its promoting effect in HSC activation.
• JCAD is a potential therapeutic target in cholestatic liver disease.
INTRODUCTION

Primary biliary cholangitis (PBC), a classic representative of chronic cholestatic hepatobiliary cell injury, is an autoimmune disease primarily affecting the intrahepatic small bile ducts. The 5-year accumulative incidence of decompensation, HCC and death/liver transplantation in PBC patients was 6.95%, 1.54% and 4.02%, respectively. Cholestatic liver diseases including primary biliary cholangitis (PBC) are associated with active hepatic fibrogenesis, which ultimately progresses to cirrhosis. Activated hepatic stellate cells (HSCs) are the main fibrogenic effectors in response to cholangiocyte damage. JCAD regulates cell proliferation and malignant transformation in nonalcoholic steatohepatitis-associated hepatocellular carcinoma (NASH-HCC). However, its participation in cholestatic fibrosis has not been explored yet.

Background/Aims: Cholestatic liver diseases including primary biliary cholangitis (PBC) are associated with active hepatic fibrogenesis, which ultimately progresses to cirrhosis. Activated hepatic stellate cells (HSCs) are the main fibrogenic effectors in response to cholangiocyte damage. JCAD regulates cell proliferation and malignant transformation in nonalcoholic steatohepatitis-associated hepatocellular carcinoma (NASH-HCC). However, its participation in cholestatic fibrosis has not been explored yet.

Methods: Serial sections of liver tissue of PBC patients were stained with immunofluorescence. Hepatic fibrosis was induced by bile duct ligation (BDL) in wild-type (WT), global JCAD knockout mice (JCAD-KO) and HSC-specific JCAD knockout mice (HSC-JCAD-KO), and evaluated by histopathology and biochemical tests. In situ-activated HSCs isolated from BDL mice were used to determine effects of JCAD on HSC activation.

Results: In consistence with staining of liver sections from PBC patients, immunofluorescent staining revealed that JCAD expression was identified in smooth muscle α-actin (α-SMA)-positive fibroblast-like cells and was significantly up-regulated in WT mice with BDL. JCAD deficiency remarkably ameliorated BDL-induced hepatic injury and fibrosis, as documented by liver hydroxyproline content, when compared to WT mice with BDL. Histopathologically, collagen deposition was dramatically reduced in both JCAD-KO and HSC-JCAD-KO mice compared to WT mice, as visualized by Trichrome staining and semi-quantitative scores. Moreover, JCAD deprivation significantly attenuated in situ HSC activation and reduced expression of fibrotic genes after BDL.

Conclusions: JCAD deficiency effectively suppressed hepatic fibrosis induced by BDL in mice, and the underlying mechanisms are largely through suppressed Hippo-YAP signaling activity in HSCs. (Clin Mol Hepatol 2024;30:206-224)

Keywords: Primary biliary cholangitis; Cholestasis; Hepatic stellate cells; JCAD; Hippo-YAP signaling

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Abbreviations:
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BC, bile canaliculi; BDL, bile duct ligation; BECs, bile epithelial cells; CCL2, C-C motif chemokine ligand 2; CTGF, connective tissue growth factor; DMEM, Dulbecco’s modified Eagle’s medium; ECs, endothelial cells; ECM, extracellular matrix; EdU, 5-ethynyl-2’-deoxyuridine; HQ, hydroxyproline quantitation; HSCs, hepatic stellate cells; H&E, hematoxylin and eosin; IL6, interleukin-6; IBDM, intrahepatic bile duct mass; IHC, immunohistochemical; JCAD, junctional protein associated with coronary artery disease; LSEC, liver sinusoidal endothelial cells; LD, lipid droplets; PBC, primary biliary cholangitis; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; α-SMA, smooth muscle α-actin; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; YAP, Yes-associated protein

ease/cirrhosis cases represented 1,758 of 9,473 liver transplantsations done in the United States until 2023, according to the Organ Procurement and Transplantation Network (OPTN) (www.hrsa.gov). Despite not being a leading indication for liver transplantation, the true incidence of PBC is rising as more screening tests are performed in otherwise healthy persons. PBC is more common than men among women of middle age worldwide. The disease ratio among females to males is 10:1. The incidence and prevalence are widely variable across different countries. The prevalence of PBC in the United States per million persons is 654 for women and 121 for men. The overall prevalence of PBC was 118.75 cases per million in the Asia-Pacific region.

Hepatic fibrosis is a wound-healing process responding to various modes of chronic liver injury, including cholestasis. A variety of etiologies, such as toxicity of medications, viral infection, even pregnancy or contraceptives, may lead to cholangiocyte injury and cholestasis as a result of the accumulation of hydrophobic bile acids, which are toxic to bile epithelial cells (BECs) and hepatocytes. In response to injury, BECs proliferate to compensate for their respective loss and maintenance of bile duct integrity. Repeated cycles of BEC injury-proliferation evokes inflammation and collagen deposition, manifested as bile duct reaction and cholestatic fibrosis. Hepatic stellate cells (HSCs) and portal fibroblasts (PFs) are activated and proliferate more rapidly when cytokines, such as transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), C-C motif chemokine ligand 2 (CCL2), and interleukin-6 (IL6), etc. are released by proliferative BECs. Activated HSCs are the main cellular effectors in liver fibrosis in response to cholangiocyte damage. This ultimately results in an excess of extracellular matrix (ECM) production and accelerates the development of fibrosis. Hence, activated HSCs are considered as a therapeutic target for the treatment of cholestatic fibrosis.

Junctional protein associated with coronary artery disease (JCAD), a cell-cell junctional protein localized between vascular endothelial cells (ECs), promotes endothelial dysfunction and atherosclerosis. It has been demonstrated that JCAD promotes YAP activation in human EC, while JCAD depletion decreased the percentage of nuclear-localized YAP and reduced expression of atherogenic genes (such as CTGF and CYR61) by enhancing the phosphorylation of YAP. Moreover, our previous studies demonstrated that JCAD promoted the transformation of NASH into HCC by interacting with the kinase domain of LATS2 and inhibiting its phosphokinase activity, which further resulted in a decreased level of YAP phosphorylation and an increase in YAP nuclear translocation. However, to our knowledge, its participation in cholestatic fibrosis has not been explored yet, nor has the role of JCAD in HSC activation through the Hippo-YAP signaling axis been investigated.

In the present study, enhanced expression of JCAD was found in activated fibroblast-like cells in the portal triads in patients with PBC. Bile duct ligation (BDL) was performed to induce cholangitis and fibrosis in global and HSC-specific JCAD knock-out (KO) mice. The findings demonstrated that JCAD depletion alleviated BDL-induced BEC proliferation and liver fibrosis, two major repairing responses to cholestatic injury. Moreover, JCAD acted as a novel upstream regulator of the Hippo-YAP signaling pathway to positively affect HSC activation.

METHODS AND MATERIALS

Human subjects and ethic issues

The procedures regarding human subjects were approved by the Ethic Committee of Fudan University School of Basic Medical Sciences (2022-C008). Formalin-fixed, paraffin-embedded liver slices from five PBC patients and five control livers from non-PBC patients were obtained from the Biorepository of Pathologic Specimens in the Department of Pathology, School of Basic Medical Sciences, Fudan University. All patient identification was removed, and the sections were re-numbered for annotation. Patient information is briefly summarized in Table 1.

Animal experiments and ethic approval

Mice were housed in specific pathogen-free (SPF) facilities at Fudan University and Shanghai Model Organisms. All experimental protocols (2021-0302-051, 2023-0301-088) were approved by the Ethic Committee for Experimental Animal Use and Care, Fudan University School of Basic Medical Sciences, and performed in line with the NIH Guidelines for Experimental Animal Handling and Use, as well as the national, municipal, and university regulations. Breeding pairs of global homozygous JCAD knockout C57BL/6J (JCAD-KO) mice...
were obtained from RIKEN Center for Life Science Technologies, Kobe, Hyogo, Japan, and utilized to generate offspring, and offspring chimeric mice were identified by PCR using primer pairs as reported.

Only male JCAD-KO mice were used in the present study in order to avoid gender differences in response to cholestatic insults. The wild-type (WT) C57BL6/J mice were used as controls. WT and JCAD-KO mice (at least 10 weeks of age) were subjected to sham or BDL without cholecystectomy for up to 14 days as we described previously.

Mice were sacrificed 2 weeks after BDL procedures, and liver tissue was collected and stored at –80°C for histopathologic, biochemical, and molecular analyses as reported in our previous study.

Hepatic stellate cell-specific conditional JCAD knockout mice (JCAD\textsuperscript{flox/flox}, GFAP-cre\textsuperscript{+/+}, named as HSC-JCAD-KO) were generated by crossing floxed JCAD mice containing the exon 3 open-reading frame of JCAD (generated in Shanghai Model Organisms, Shanghai, China) with the GFAP-cre/ERT2 strain mice (a generous gift from Prof. Yong Xu in Nanjing Medical University, Nanjing, China), as reported.

To activate cre-enzyme for JCAD deletion, 5- to 6-week-old male mice were injected daily with tamoxifen (TAX, 100 mg/kg) for 5 consecutive days, followed by a 7-day interval for the clearance of tamoxifen. After verification with genome typing and tamoxifen treatment (Supplementary Fig. 8A–D), mice with the correct genomic type and phenotype were subjected to BDL. Wild-type littermates (JCAD\textsuperscript{flox/flox}, GFAP-cre\textsuperscript{-/-}, defined as WT in short) were used as controls to determine whether JCAD deficiency in HSCs results in minimal or no fibrosis in homozygous mice. All experiments were performed in male mice.

Statistical analysis
All data were expressed as mean±standard deviation (SD). SPSS software was used for statistical analysis. When two groups were compared, Student’s t-test was used after normal distribution test. When the experimental design involves more than 2 groups, one-way ANOVA was used to compare the least significant difference (LSD) test. If normality or homogeneity of variance is not met, Kruskal-Wallis test and Mann-Whitney U test were used for multiple comparisons between groups. All PRIs for identification of mouse genome types were listed in Supplementary Table 1.

Table 1. Clinical characteristics of subjects

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age</th>
<th>Clinical diagnosis</th>
<th>Clinical signs and laboratory tests</th>
<th>Pathological diagnosis</th>
<th>Grade (Scheuer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>35</td>
<td>Biliary liver injury</td>
<td>ANA (+), AMA (+), AMA-M2 (+), IgG (+)</td>
<td>PBC stage 1</td>
<td>CH-G1 S0</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>52</td>
<td>AIH-PBC</td>
<td>ANA1:640, AMA (+), AMA-M2 (+)</td>
<td>PBC stage 1</td>
<td>CH-G2 S1</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>57</td>
<td>PBC</td>
<td>ANA (+), AMA-M2 (+), GP210 (+)</td>
<td>PBC stage 2</td>
<td>CH-G2 S1</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>49</td>
<td>Liver insufficiency</td>
<td>ANA (+), AMA (+), AMA-M2 (+), GP210 (+), IgG (+)</td>
<td>PBC stage 1</td>
<td>CH-G1-2 S0</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>19</td>
<td>Liver insufficiency</td>
<td>ANA (+), AMA-M2 (+)</td>
<td>PBC stage 2</td>
<td>CH-G2 S1</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>50</td>
<td>AIH</td>
<td>AMA (-), AMA-M2 (-), IgG (-)</td>
<td>Nearly normal liver tissue</td>
<td>CH-G0 S0</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>47</td>
<td>Liver insufficiency</td>
<td>AMA (+), AMA-M2 (+), IgG (+)</td>
<td>Non-specific inflammatory reaction</td>
<td>CH-G0 S0</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>42</td>
<td>Liver insufficiency</td>
<td>AMA (-), AMA-M2 (-), IgG (-)</td>
<td>Non-specific inflammatory reaction</td>
<td>CH-G0 S0</td>
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<tr>
<td>9</td>
<td>F</td>
<td>55</td>
<td>Liver insufficiency</td>
<td>AMA (-), AMA-M2 (-), IgG (-)</td>
<td>Non-specific inflammatory reaction</td>
<td>CH-G0 S0</td>
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<tr>
<td>10</td>
<td>F</td>
<td>59</td>
<td>AIH</td>
<td>AMA (-), AMA-M2 (-), IgG (-)</td>
<td>Non-specific inflammatory reaction</td>
<td>CH-G0 S0</td>
</tr>
</tbody>
</table>

The exclusion indicators: HBsAg (-), HBeAg (-), Copper (-), and Pers blue (-) are used to detect all subjects.

F, female; M, male; PBC, primary biliary cholangitis; AIH, autoimmune hepatitis; AMA, anti-mitochondrial antibody; ANA, antinuclear antibodies; GP210, antinuclear antibodies against glycoprotein 210; CH-G, S represent inflammation and fibrosis respectively.
two given groups. *, **, *** represent as P<0.05, 0.01 and 0.001; P-value<0.05 was considered as statistically significant.

**RESULTS**

**JCAD is highly expressed in the livers of both patients with PBC and mice with biliary obstruction**

Liver sections from control subjects without chronic cholestatic liver disease and patients with PBC were examined for counter-staining of JCAD with α-SMA protein. In control livers, positive α-SMA staining was localized to fibroblast-like cells in the portal triads as evidenced in H&E staining showing the bile ductule (Fig. 1, top panel). JCAD staining was largely negative in this area in control livers. However, immunofluorescent staining of serial sections revealed that JCAD was strongly positive in the biopsied tissue of PBC patients, and was overlapped in α-SMA-positive fibroblast-like cells (Fig. 1 white arrow), which illustrated that JCAD, a membrane conjunction protein, was expressed most in activated HSCs or possibly in PFs too in the portal triads when it was in an early phase of bile duct reaction. Moreover, positive JCAD staining was visualized in BECs with the ductular reactions in sections of biopsied tissue from PBC patients (Fig. 1 yellow arrow). These findings indicate that enhanced JCAD activity in BECs and HSCs may participate in the biliary ductular reaction and fibrosis of cholestatic disorders in human.

To study the potential role of JCAD in cholestatic insult, BDL was utilized as an animal model of cholestasis. Liver tissue was harvested 14 days after BDL, which resulted in acute obstructive cholestasis and portal fibrosis at this time point. The gross examination and histopathology showed a huge gallbladder, massive liver necrosis and profound fibrosis (Supplementary Fig. 1A). JCAD expression was examined in the liver after BDL. A significant increase in JCAD protein levels was detected by Western blotting analysis (Fig. 1B, C) in consistency with elevated mRNA levels compared to sham-operated mice (Supplementary Fig. 1B). Immunostaining confirmed that JCAD expression was increased in fibrotic liver after BDL compared to sham-operated mice (Fig. 1D). In agreement with the immunofluorescent staining of liver sections from PBC patients, JCAD expression in cholestatic livers was enhanced along with upregulated α-SMA, CK19 and YAP expression (Fig. 1B, C). Thus, it is tempting to speculate that JCAD is an important player in the regulation of biliary ductular reaction and fibrosis following BDL in mice or in cholestatic disorders in humans.

**BDL-induced hepatic injury was ameliorated in JCAD-KO mice**

To test whether increased JCAD levels are critical for hepatic response, BDL was performed in WT and JCAD-KO mice. Liver histopathology revealed that the WT-BDL mice displayed increased focal necrosis, portal inflammation and bile leakage (Supplementary Fig. 1A). In JCAD-KO-BDL mice, the degree of focal necrosis was attenuated histopathologically compared to WT-BDL (Fig. 2A). The ratio of liver over body weight did not differ between WT and JCAD-KO mice after BDL. In contrast, JCAD-KO-BDL mice restored the reduction in liver weight and body weight compared to WT-BDL mice (Supplementary Fig. 2A). Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated in WT-BDL mice; however, they were reduced remarkably in JCAD-KO-BDL mice (Supplementary Fig. 2B). Similarly, serum levels of bilirubin and bile acids were strikingly decreased in JCAD-KO-BDL mice compared to WT-BDL mice.

**JCAD deficiency abrogated bile ductular reaction and fibrosis after BDL**

Effects of JCAD deficiency on intrahepatic bile duct mass were assessed with immunohistochemical staining for CK-19 (a specific marker of bile duct epithelial cells), and it was found that the positivity of CK-19 staining in JCAD-KO-sham mice was similar to WT-sham mice (Fig. 2B, Supplementary Fig. 3B). Comparing WT-BDL with WT-sham or JCAD-KO-sham mice revealed a large increase in ductal mass in WT-BDL mice; whereas JCAD-KO-BDL mice exhibited a significant decrease in intrahepatic ductal mass (Fig. 2B–D). In addition, JCAD deficiency was manifested with decreased intrahepatic bile duct mass, as evidenced by decreased Ki67-positive cell counts and 5-ethynyl-2’-deoxyuridine (EdU) incorporation (Fig. 2C, Supplementary Fig. 3A, B).

To further confirm whether JCAD modulates in vivo cholestatic fibrogenesis, fibrotic progression and collagen deposition were assessed. As shown in Figure 2E, 2F, BDL caused re-
Figure 1. JCAD was highly expressed in PBC specimens and bile duct ligation mice. (A) Representative micrographs of H&E and immunofluorescent staining of liver-biopsied specimens. The H&E staining image was taken at original magnification (100×). Scale bars=400 μm. The immunofluorescent co-staining of JCAD with α-SMA was positive in fibroblast-like cells in the liver lobules, but much more positive in the portal triads from PBC patients compared to the control. Red: JCAD; Green: α-SMA; Blue: DAPI. Image amplification: 400×. Scale bars=50 μm. PV: Branch of the hepatic portal vein; B: Bile ductule; BECs: Biliary epithelial cells; HSCs: Hepatic stellate cells. (B) Western blot analysis of JCAD protein level in BDL mouse livers (n=3). β-Actin was used as a loading control. P-YAP/YAP ratio indicates phosphorylation levels of Yap. (C) Semi-quantitative analysis of Western blot of JCAD, α-SMA, and CK19 proteins (n=3). (D) Representative confocal micrographs of α-SMA and JCAD in WT mice post BDL. Green: α-SMA; Red: JCAD; Blue: DAPI. Images were taken at original magnification (400×). JCAD, junctional protein associated with coronary artery disease; PBC, primary biliary cholangitis; YAP, yes-associated protein; WT, wild-type. Scale bars=50 μm. All data were expressed as mean±standard deviation. *, **, ***P<0.05, 0.01 and 0.001 vs. WT-Sham mice.
Figure 2. Liver histopathologic features of mice subjected to sham or bile duct ligation surgery and effects of JCAD depletion. (A) Liver damage was assessed by H&E staining in liver sections from all groups. Images were taken at original magnification (100×), scale bars=200 μm. (B) Representative image of immunohistochemistry for CK-19. Images were taken at original magnification (100×), scale bars=200 μm. (C, D) Representative confocal micrographs of double-staining. For (C), CK19 was stained in red and EdU was stained in green. Images were taken at original magnification (400×), scale bars=40 μm or 50 μm. For (D), CK19 was stained in red and α-SMA was stained in green. Images were taken at original magnification (630×), scale bars=50 μm. (E, F) Fibrosis was shown by Masson and Sirius red staining. Images were taken at original magnification (100×), scale bars=200 μm. (G) Semiquantitative score of hepatic fibrosis by a pathologist (n=6). (H) Liver hydroxyproline content (μg/mg tissue) (n=6). JCAD, junctional protein associated with coronary artery disease; WT, wild-type; BDL, bile duct ligation; EdU, 5-ethynyl-2’-deoxyuridine. All data were expressed as mean±standard deviation. *, **, *** P<0.05, 0.01, and 0.001 vs. WT-Sham mice; #, ##, ### P<0.05, 0.01, and 0.001 vs. WT-BDL mice.
Figure 3. JCAD deficiency protects mice from cholestatic liver fibrosis and biliary duct reaction following BDL. (A) Total protein levels of α-SMA, CK19, and JCAD-Hippo signaling axis in mouse liver were assayed by Western blot (n=3). β-Actin was used as a loading control. P-YAP/YAP ratio indicates phosphorylation levels of Yap. (B) Relative mRNA levels of JCAD, α-SMA, TGF-β and CK19 in mouse liver were determined by quantitative RT-PCR analysis (n=6). (D) Levels of TGF-β1 were examined by ELISA (ABclonal, RK00057) in hepatic homogenates and serum respectively from these mice (n=6). JCAD, junctional protein associated with coronary artery disease; YAP, yes-associated protein; WT, wild-type; BDL, bile duct ligation. All data were expressed as mean±standard deviation. ***, **** P<0.05, 0.01, and 0.001 vs. WT-Sham mice; #, ##, ### P<0.05, 0.01, and 0.001 vs. WT-BDL mice.
markable collagen accumulation in the liver compared to the sham-operated mice in Sirius red and Masson’s trichrome staining. In contrast, collagen deposition was dramatically reduced in JCAD-KO-BDL mice (Fig. 2E, F). Consistently, semi-quantitative score of hepatic fibrosis and photometric analysis of Masson/Sirius red staining verified that the collagen fiber-deposited area was significantly increased in the BDL-mice; however, it was remarkably decreased in JCAD-KO mice after BDL (Fig. 2G, Supplementary Fig. 3C). This finding was further confirmed by immunofluorescent staining for α-SMA, CK-19, and in situ EdU labeling (Fig. 2C, D). Liver hydroxyproline content was significantly decreased in JCAD-KO-BDL mice compared to WT-BDL mice (Fig. 2H). Moreover, Western blot images showed that α-SMA and CK-19 protein levels were elevated in BDL-induced fibrotic liver, and JCAD-KO dramatically reduced expression of these two markers at protein levels (Fig. 3A, B). Similarly, gene expression of α-SMA, TGF-β, CTGF, and procollagen type-1 was significantly up-regulated in WT-BDL mice compared to sham-operated WT; whereas, expression of these genes was much suppressed in JCAD-KO-BDL mice (Fig. 3C, Supplementary Fig. 3D). Given that TGF-β1 is a well-known fibrogenic cytokine, expression of TGF-β1 in the liver and secretion of TGF-β1 in serum from these mice were determined. As expected, the levels of TGF-β1 in hepatic homogenates and serum were remarkably decreased in JCAD-KO-BDL compared to WT-BDL mice (Fig.

Figure 4. Liver histology by transmission electron microscopy (TEM). Ultrastructural cell morphology in livers from wild-type (I) and JCAD-KO (III) mice before performed BDL surgery versus wild-type (II) and JCAD-KO (IV) mice 14 days after BDL. Morphological analysis of bile canaliculi and small bile ducts (A, B), mitochondria and ribosomes (B), liver sinusoid (C), and hepatic Stellate cells (D). Note: loss of microvilli and canalicular dilation, damaged mitochondria and ribosomes, activated HSCs in WT-BDL (II) mice. In comparison with the wild-type, liver pathological damage of JCAD-KO-BDL (IV) mice was minimized. BC, bile canaliculi; M, mitochondria; RER, rough endoplasmic reticulum; N, nucleus; Gly, glycogen; Lys, lysosome; LSEC, liver sinusoid endothelial cells; HSC, hepatic stellate cells; LD, lipid droplet; KC, Kupffer cell; JCAD, junctional protein associated with coronary artery disease; WT, wild-type; BDL, bile duct ligation. Arrows point to the microvilli. Original magnification: 14,000× (B, D), 5,800× (A, C).
Figure 5. Inhibition of JCAD expression by an RNAi approach suppressed HSC activation and proliferation. (A) Immunofluorescent co-staining of JCAD with α-SMA in immortalized human LX2 cells transfected with siRNAs against human JCAD gene. Red: JCAD; Green: α-SMA; Blue: DAPI. Image amplification: 630×. Scale bars=40 μm. (B) Total protein levels of the JCAD in immortalized human LX2 and HSC cells transfected with siRNAs against the human JCAD gene (n=3). β-Actin was used as a loading control. (C) mRNA levels of JCAD, Col-Iα1, TIMP1 and CTGF in immortalized LX2 and HSC cells transfected with siRNAs against human JCAD gene (n=3). (D) The proliferation of LX2 and HSC cells transfected with siRNAs against the human JCAD gene, following TGF-β (10 ng/mL) treatment for 48 hours, was detected using Cell Titer-Lumi™ assay (n=3). The experiment was repeated at least 3 times. JCAD, junctional protein associated with coronary artery disease; HSC, hepatic stellate cells. All data were expressed as mean±standard deviation. * P<0.05, ** P<0.01, and *** P<0.001 compared to Ctrl-siRNA or Ctrl-DMEM. # P<0.05, ## P<0.01, and ### P<0.001 compared to Ctrl-TGF-β.
Figure 6. Primary stellate cells isolated from JCAD-KO mice tended to stay in a quiescent state and were resistant to TGF-β activation. Primary HSCs isolated from WT and JCAD-KO mice. Primary HSCs were cultured on coverslips for 5 days, and then treated with TGF-β (10 ng/mL) for 24 hours. (A) Differences in cell morphology viewed by an open field of microscope between primary HSC from WT and JCAD-KO mice respectively on day 1 and 5 after isolation. (B) Gene expression levels of α-SMA and CTGF in the primary hepatic stellate cell on day 1 and 5 after isolation (n=3). (C) Gene expression levels of α-SMA, C-myb, TGF-β1, Col-Iα1, Col-3α1, fibronectin, CTGF, and TIMP1 at different experimental set-ups (n=3). (D) Proliferation related-gene expression levels of PCNA and cyclin D1 in primary HSC (n=3). The experiment was repeated at least 3 times. JCAD, junctional protein associated with coronary artery disease; HSC, hepatic stellate cells; WT, wild-type; BDL, bile duct ligation; CTGF, connective tissue growth factor. All data were expressed as mean±standard deviation. *, **, ***P<0.05, 0.01, and 0.001 compared to WT-Day 1 or WT-Ctrl. #, ##, ###P<0.05, 0.01, and 0.001 compared to WT-Day 5 or WT-TGF-β.
Collectively, these findings documented that JCAD deficiency mitigated bile ductular reaction and fibrosis after BDL in mice. Moreover, the liver tissue was examined by transmission electron microscopy using semi-thin tissue sections, which enables the resolution and visualization of ultrastructural details. As shown in Figure 4I, hepatocytes from sham-operated WT mice exhibited a normal ultrastructure of the nucleus, mitochondria and lysosomes. The plasma membrane of these subcellular organelles is mostly smooth and flat except where it is enfolded to form the bile canaliculi (BC) in the bilatera...
Figure 7. JCAD mediates hepatic stellate cell activation via the Hippo-YAP signaling pathway. (A) YAP transcriptional activity was increased by JCAD. LX2 cells were transiently transfected with JCAD-siRNA or myc-JCAD plasmid, TEAD-responsive 8X GTIIC luciferase reporter gene. Renilla luciferase reporter plasmid pRL-SV40 was used as an internal control, and YAP-siRNA or myc-YAP as a positive control (n=3). (B) LX2 cells were transfected with siRNAs against the human JCAD gene and treated with Lats-IN-1 (1 μM or 5 μM) for 24 hours. LX2 cells were treated with YAP inhibitor verteporfin (0.5 and 1 μM) for 48 hours. Total protein levels of α-SMA and YAP were assayed by Western blot (n=3). (C) LX2 cells transfected with siRNAs against the human JCAD gene were treated with Lats-IN-1 (5 μm) for 24 hours. LX2 cells were treated with YAP inhibitor verteporfin (1 μm) for 24 hours. Fibrosis-related gene expression was quantified by qRT-PCR (n=3). (D) Immunofluorescent co-staining of YAP and α-SMA in immortalized human LX2 cells transfected with the siRNA against human YAP gene. The nucleus was visualized with DAPI staining. Red: YAP; Green: α-SMA; Blue: DAPI. Image amplification: 1,260×. Scale bars=20 μm. (E) Total protein levels of the YAP and α-SMA in immortalized human LX2 cells transfected with the siRNA against the human YAP gene were analyzed by Western blot analysis. JCAD, junctional protein associated with coronary artery disease; YAP, yes-associated protein. All data were expressed as mean±standard deviation. *, **, *** P<0.05, 0.01, and 0.001 compared to Ctrl-siRNA or Ctrl-siRNA-DMEM. $#, $$, $$$ P<0.05, 0.01, and 0.001 compared to Ctrl-siRNA-Lats-IN-1.
Figure 8. HSC-specific JCAD deletion attenuated liver fibrosis in mice. (A–C) Paraffin sections were stained with H&E, Masson’s trichrome, and Sirius. (D, E) Representative pictures of quantifying changes in protein fiber alignment in the liver as visualized by SHG/TPEF microscopy. (F) Total protein levels of α-SMA in mouse liver were assayed by Western blot (n=3). (G) Liver hydroxyproline levels were measured with a kit as described in Supplemental methods (n=6). (H) Primary HSCs were isolated from normal mice, JCAD-KO and HSC-JCAD-KO mice that underwent respectively 0, 5, and 14 days after BDL. The gene expression levels of TGF-β and CTGF were analyzed. HSCs from 3 livers were pooled to increase the yield and was seen as one ex vivo experiment, repeated 6 times. HSC, hepatic stellate cells; JCAD, junctional protein associated with coronary artery disease; SHG/TPEF, second harmonic generation/two-photon excitation fluorescence; BDL, bile duct ligation; CTGF, connective tissue growth factor; WT, wild-type. All data were expressed as mean±standard deviation. *, **, ***P<0.05, 0.01, and 0.001 compared to WT-Sham or primary HSC isolated from WT mice at day 0. #, ##, ###P<0.05, 0.01, and 0.001 compared to WT-Sham or primary HSC isolated from WT mice at day 5 or 14.
suppressed in primary HSCs isolated from JCAD-KO-BDL mice compared to those from WT-BDL mice (Fig. 6C). In summary, these findings from in vitro or ex vivo experiments argued that JCAD is essential for HSC activation in mice with BDL.

Inhibition of JCAD by RNAi knock-down abrogated HSC activation via the Hippo-YAP pathway

In order to prove whether JCAD acts on HSCs through the Hippo-YAP signaling axis, a YAP/TEAD-based dual-luciferase reporter assay was utilized. As shown in Figure 7A, JCAD depletion reduced YAP/TAZ/TEAD transcriptional activity, while JCAD over-expression caused a significant induction of 8xGTIIC luciferase reporter activity, with YAP siRNA and HA-YAP transfection as negative and positive controls. Furthermore, JCAD was knocked-down using siRNA transfection in LX2 cells, and thereafter, they were treated with Lats-IN-1 one day after transfection. Lats-IN-1 is a potent and ATP-competitive inhibitor of Lats1/2 kinases and drives Yap-dependent proliferation in post-mitotic mammalian cells.24 It was observed that with the effective JCAD inhibition, the degree of HSC activation was attenuated along with decreased YAP expression (Fig. 7B). Once Lats-IN-1 was supplemented, expression of YAP and α-SMA was increased in Ctrl (scrambled)-siRNA-transfected LX2 cells; whereas their expression was not significantly changed in JCAD-siRNA-transfected cells with blocking LATS1/2 kinase activity by Lats-IN-1 (Fig. 7B, C).

Since the findings from above-mentioned experiments indicated that JCAD is positively correlated with the expression of YAP, the function of YAP during HSC activation was evaluated with two different strategies: firstly, LX2 cells were treated with the pharmacological inhibitor of YAP: verteporfin (VP), that disrupts the YAP/TEAD complex. Treatment of LX2 cells with VP at the time of seeding abolished the induction of α-SMA and YAP expression 48 hours after seeding, and suppressed expression of TGF-β, TIMP1, CTGF, and COL-1a1 genes, indicating that they were not increased in LX2 cells with YAP RNAi knockdown (Supplementary Fig. 4B). Collectively, these results demonstrated that YAP is essential for HSC activation, and JCAD facilitated HSC activation via the Hippo-YAP signaling axis.

HSC-specific JCAD deletion attenuated liver fibrosis in mice

Finally, in order to further prove the role of JCAD in HSC activation, HSC-specific JCAD knock-out mice were subjected to sham or BDL surgical procedure. As shown in Western blotting and qRT-PCR analyses, JCAD levels were down-regulated in HSCs isolated from tamoxifen (TAX)-injected mice compared to corn oil-injected mice (Supplementary Fig. 7C). The H&E staining in liver sections indicated that conditional inactivation of JCAD in HSCs did not significantly affect the extent of liver injury (Fig. 8A) and serum ALT and AST levels were similar between WT and HSC-JCAD-KO mice (data not shown). However, liver fibrogenesis was appreciably ameliorated in HSC-JCAD-KO mice after BDL as evidenced by Masson’s trichrome (Fig. 8B) and Sirius red staining (Fig. 8C), showing less fibrogenesis in HSC-JCAD-KO mice compared to WT mice after a BDL procedure.

Second harmonic generation/two-photon excitation fluorescence (SHG/TPEF) microscopy with artificial intelligence (AI) analysis provides a standardized evaluation of liver fibrosis and collagen fiber quantitation on a continuous scale. This approach was employed to gain an in-depth understanding of fibrogenic process resulting from the BDL model. As shown in Figure 8D, 8E, HSC-JCAD-KO mice were undergone milder fibrogenesis than WT-mice. The percentage of fibrotic area was further determined in 5 separate regions: the portal tract, periportal (the area within 100 μm around the portal tract), transitional (zone 2), pericentral, and central vein areas (Supplementary Fig. 5A). The combination of periportal, transitional, and pericentral areas represents the perisinusoidal area with zone 2 being the largest component. Zonal analysis of fibrotic dynamics revealed that BDL procedure resulted in a large amount of collagen deposition in the entire liver, especially in the perisinusoidal area (Supplementary Fig. 5B, C); whereas there was less fibrotic deposition in the portal tract and central vein areas. Fibrotic extent was significantly mitigated in the HSC-specific JCAD-KO mice undergone BDL, es-
especially in the perisinusoidal area (Supplementary Fig. 5B, C).

These observations were further verified by protein expression of α-SMA in the liver, which was highly elevated in WT-BDL mice, but alleviated in HSC-JCAD-KO-BDL mice (Fig. 8F, Supplementary Fig. 6A). Moreover, measurements of hydroxyproline levels verified that liver fibrosis was significantly attenuated in HSC-JCAD-KO mice compared to WT mice after BDL (Fig. 8G, P=0.0541). In consistency with global JCAD knockout mice, expression of fibrosis-associated genes in HSC-JCAD-KO mice after BDL was strikingly reduced (Supplementary Fig. 6B). Furthermore, HSCs were isolated 5 or 14 days after BDL, representing early and late time points of in situ HSC activation. Thereafter, expression of fibrotic genes was determined to assess extent of in situ HSC activation. As shown in Fig. 8H, Supplementary Fig. 6C, expression of Tgf-β, Ctgf, α-SMA, Col-1α1, Col-3α1 and Timp1 was markedly down-regulated in HSCs from HSC-JCAD-KO mice in comparison to those from WT mice. These findings further confirmed that JCAD was considerably essential for HSC activation in the progression of cholestatic fibrosis, implying that JCAD may be an alternative target to inhibit fibrogenesis.

DISCUSSION

Cholestatic disorders, including primary biliary cholangitis, are associated with active hepatic fibrogenesis, which ultimately advances to end-stage liver disease (cirrhosis). Available treatments for cholestatic disorders, such as ursodeoxycholic acid (UDCA), may only minimize clinical manifestations, however hardly blocks its progression to cirrhosis. In the present study, it has demonstrated that JCAD, a novel upstream regulator of the Hippo signaling pathway, is co-localized with α-SMA-positive fibroblast-like cells in the portal zone, especially in bile duct epithelial cells and activated HSCs in the livers of both patients with PBC and mice with biliary obstruction. JCAD interacts with the kinase domain of LATS2 in the Hippo signaling pathway, inhibiting its phosphokinase activity, which further results in increasing nuclear translocation of un-phosphorylated YAP. In the current study, it is hypothesized that as a transcription coactivator in the Hippo-YAP signaling axis, YAP is actively involved in HSC activation, proliferation and fibrogenic process. Therefore, increased JCAD could be critical for cholestatic fibrogenesis; whereas JCAD deficiency ameliorates fibrotic deposition in a mouse model cholestasis caused by BDL, highlighting a potentially therapeutic avenue for selectively targeting an aberrant JCAD-Hippo-YAP axis in cholestatic disorders.

BDL is a well-known experimental model to induce cholestatic injury and periportal biliary fibrosis, which etiologically and pathogenically mimics cholestatic fibrosis in humans to a great extent. Although PFs may participate in bile duct re-action and early ductular injury repair, recent data from lineage-tracing experiments indicate that among populations of ECM-producing or fibrogenic cells, HSCs are considered to be the major cell type for excessive production of EMC components in the BDL model and that activation of HSCs is a key initiating event in hepatic fibrogenesis. Once activated, they transform into myofibroblast-like cells that drive excessive production of collagen content, resulting in fibrotic deposition in the liver. In the present study, JCAD was first identified as a critical regulator for HSC activation in the progression of cholestatic fibrosis. Given that JCAD was strongly positive in the portal triads of the liver tissue from PBC patients, consistent with the elevation of YAP protein in fibrotic livers after BDL, JCAD may function as a crucial signaling molecule in the regulation of biliary hyperplasia and fibrosis following BDL or in cholestatic disorders. In preliminary experiments, incubation of either human HSC lines or primary mouse HSCs with diluted bile collected from BDL mice significantly increased JCAD protein and mRNA levels, indicating bile acids, the main components in the bile, may stimulate activation of JCAD during cholestasis (Supplementary Fig. 7A–C).

In order to in-depth delineate pathophysiological role of JCAD, JCAD-KO and HSC-specific conditional JCAD knockout (JCADlox/lox, GFAP-cre+) mice were utilized to examine the role of JCAD during liver fibrosis especially in HSCs in the present study. Either global deficiency or HSC-specific JCAD knockout significantly suppressed liver fibrosis after BDL, as evidenced by remarkably decreased liver hydroxyproline content and improved histopathology. Primary HSCs were isolated from WT and JCAD-KO mice to further evaluate whether JCAD is directly involved in influencing HSC activation and proliferation. JCAD deprivation significantly diminished fibrosis-associated genes and decreased HSC proliferation regardless whether they were plated on plastic dishes for culture-induced activation or under TGF-β stimulation. Furthermore, in situ-activated HSCs derived from JCAD-KO mice undergone BDL exhibited reduced expression of fibrosis-associated genes in comparison to WT animals, which fur-
ther supports the notion that JCAD is critical for HSC activation in cholestatic conditions.

Moreover, the findings in the present study delineated that JCAD acts on HSC activation through the Hippo-YAP signaling axis. It was demonstrated that JCAD regulates cell proliferation and tumor progression by interacting with the kinase domain of LATS2 and inhibiting its phosphokinase activity, impeding the phosphorylation of YAP, and increasing nuclear translocation of un-phosphorylated YAP in NASH-HCC in our previous study. However, its participation in HSC activation and fibrogenesis has not been explored in well-established animal models. It is initially confirmed that HSCs expressed JCAD at both RNA and protein levels, and that its expression was in the same trend along with fibrogenic markers, such as α-SMA, while suppressing JCAD expression in stellate cells decreased YAP expression in the present study. Next a YAP/TEAD-based dual luciferase reporter was used to further demonstrate the critical role JCAD through the Hippo-YAP axis in HSCs. As expected, JCAD inhibition by siRNAs decreased the transcriptional activity of YAP/TEAD. This finding goes along with recent studies, which demonstrated that the Hippo pathway interacts with the TGF-β/Smad pathway through interaction of YAP/TEAD with Smad at transcription level. As the downstream target gene, CTGF, was overexpressed in activated HSCs in the present study, which is a strong cytokine eliciting the synthesis and secretion of ECM proteins, in majority of fibrillar collagens for fibrous deposition. Furthermore, when YAP was inhibited with siRNAs or with VP, a well-recognized YAP inhibitor, in HSC lines, expression of fibrotic genes was remarkably suppressed, which is in accordance with the reports by others, indicating the participation of the Hippo-YAP signaling axis in HSC activation and EMC production.

To further clarify that JCAD increases YAP nuclear translocation via LATS2, a LATS2 kinase inhibitor, Lats-IN-1, was used. Notably, Lats-IN-1 effectively increased the protein level of un-phosphorylated YAP, along with increased expression of fibrosis-associated genes and proteins. However, their expression was not significantly changed in JCAD-siRNA-transfected cells after blocking LATS2 kinase activity by Lats-IN-1. These results confirmed the significance of Hippo-YAP signaling axis in the initiation and perpetuation of HSC activation. In speculation, suppressing JCAD subsequently restrained the nuclear translocation of YAP might be a possible strategy in ameliorating liver fibrosis.

JCAD was originally recognized as a molecular component of the E-cadherin-based cell-cell junction in vascular endothelial cells. A recent study demonstrated that JCAD has a functionally conserved domain that resembles ROCK1/2 in p38 MAPK pathway, which is critical for F-actin stress fiber formation. Moreover, JCAD has been shown to interact with several actin-binding proteins (e.g., TRIOBP), to regulate F-actin. It is known that the reorganization of the F-actin cytoskeleton is associated with HSC activation and that the p38 MAPK pathway is involved in this process. Hence, it is intriguing to determine whether F-actin plays a part regarding how JCAD affects HSC activation as further investigation. In addition, it is worthy to note that global JCAD-KO presented a more profound phenotype than HSC-JCAD-KO in the present study. Two potential explanations might be attributed to this phenomenon. Firstly, although advanced genetic techniques have provided strong evidence that HSCs are the predominant source of myofibroblasts in cholestatic liver injury, it was reported that PFs contribute to biliary fibrosis caused by BDL or 3,5-diethoxycarbonyl-1,4-dihydrocollidine diet diet at early stages. Secondly, as visualized in immuno-histochemical staining in serial paraffin sections of PBC patients, JCAD was strongly positive in the portal area, which suggests that the function of JCAD in cholangiocytes in cholestasis-associated fibrosis is worthy of investigation. Therefore, further studies as described above are warranted to investigate the role of JCAD in other cell types in cholestatic diseases.

In conclusion, the findings in the present study demonstrated that JCAD is a positive regulator of hepatic fibrogenesis in cholestatic conditions. HSC-specific JCAD knockout effectively halted hepatic fibrosis induced by bile duct ligation, and the underlying mechanisms are associated with suppressed Hippo-YAP signaling activity in HSCs. The elucidation of the critical role of this junctional protein in HSCs would facilitate the development of molecular interventions for cholestasis-associated fibrosis.

Authors’ contribution

The authors declare that they have participated in the preparation of the manuscript and have seen and approved the final version. Li Xie and Jian Wu designed all experiments; Li Xie, Hui Chen, Li Zhang, Yuan Zhou, Yong-Yu Yang, Chang Liu, Yu-Li Wang, Yue Ma, and Ya-Jun Yan performed all experiments and analyzed the data. Li Xie wrote the manuscript.
with the help of Jia Ding. Xiao Ten and Qiang Yang provided SHG/TPEF microscopy and AI analyses. Xiu-Ping Liu and Jian Wu provided the conceptual framework for the study. Jian Wu is responsible for the manuscript’s finalization.

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Conflicts of Interest

All authors declare that no conflict of interest is involved in participation or contribution to the present work.

SUPPLEMENTARY MATERIAL

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

REFERENCES

Prognosis of biopsy-confirmed metabolic dysfunction-associated steatotic liver disease: A sub-analysis of the CLIONE study

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Graphical Abstract

1,398 Japanese patients with biopsy-confirmed nonalcoholic fatty liver disease (NAFLD)

Cardiometabolic criteria

<table>
<thead>
<tr>
<th>Presence</th>
<th>Absence</th>
</tr>
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<tbody>
<tr>
<td>NAFL</td>
<td>452 (32.7)</td>
</tr>
<tr>
<td>NASH</td>
<td>929 (67.3)</td>
</tr>
</tbody>
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P=0.008

Approximately 99% of NAFLD cases corresponded to MASLD
INTRODUCTION

The main shortcomings of the terms nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) are their dependence on exclusionary confounding factors and the potential use of stigmatizing language. Three large

Study Highlights

- What is already known about this topic?
  MASLD was recently proposed as an alternative disease concept to NAFLD.

- What does this study add?
  Around 99% of NAFLD cases met MASLD criteria, while the NAFLD-only group, which comprised patients with NAFLD that did not meet the MASLD criteria, exhibited less severe histopathological characteristics and a favorable prognosis.

- How may this study affect research, practice, or policy?
  A prognosis of MASLD should be considered equivalent to a prognosis of NAFLD.

Background/Aims: Metabolic dysfunction-associated steatotic liver disease (MASLD) was recently proposed as an alternative disease concept to nonalcoholic fatty liver disease (NAFLD). We aimed to investigate the prognosis of patients with biopsy-confirmed MASLD using data from a multicenter study.

Methods: This was a sub-analysis of the Clinical Outcome Nonalcoholic Fatty Liver Disease (CLIONE) study that included 1,398 patients with NAFLD. Liver biopsy specimens were pathologically diagnosed and histologically scored using the NASH Clinical Research Network system, the FLIP algorithm, and the SAF score. Patients who met at least one cardiometabolic criterion were diagnosed with MASLD.

Results: Approximately 99% of cases (n=1,381) were classified as MASLD. Patients with no cardiometabolic risk (n=17) had a significantly lower BMI than patients with MASLD (20.9 kg/m^2 vs. 28.0 kg/m^2, P<0.001), in addition to significantly lower levels of inflammation, ballooning, NAFLD activity score, and fibrosis stage based on liver histology. These 17 patients had a median follow-up of 5.9 years, equivalent to 115 person-years, with no deaths, liver-related events, cardiovascular events, or extrahepatic cancers. The results showed that the prognosis for pure MASLD was similar to that for the original CLIONE cohort, with 47 deaths and one patient who underwent orthotopic liver transplantation. The leading cause of death was extrahepatic cancer (n=10), while the leading causes of liver-related death were liver failure (n=9), hepatocellular carcinoma (n=8), and cholangiocarcinoma (n=4).

Conclusions: Approximately 99% of NAFLD cases were considered MASLD based on the 2023 liver disease nomenclature. The NAFLD-only group, which is not encompassed by MASLD, had a relatively mild histopathologic severity and a favorable prognosis. Consequently, the prognosis of MASLD is similar to that previously reported for NAFLD. (Clin Mol Hepatol 2024;30:225-234)

Keywords: Prognosis; Steatotic liver disease; Liver biopsy

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Abbreviations:
CI, confidence interval; DM, diabetes mellitus; HCC, hepatocellular carcinoma; HR, hazard ratio; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis

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pan-national liver associations (AASLD, EASL, and ALEH) announced the official development and finalization of a new liver disease nomenclature in June 2023. The term “steatotic liver disease (SLD)” was selected as a comprehensive label to encompass the different causes of steatosis, and NAFLD has been renamed metabolic dysfunction-associated steatotic liver disease (MASLD). MASLD includes patients with hepatic steatosis and at least one of five cardiometabolic risk factors. Furthermore, metabolic dysfunction-associated steatohepatitis (MASH) is an alternative term for NASH.

Song et al. recently found little difference in the prevalence of NAFLD (25.7%) and MASLD (26.7%) in a random subset of 1,016 community subjects from Hong Kong who were examined using proton-magnetic resonance spectroscopy. Hagström et al. reported that 99% of patients with biopsy-confirmed NAFLD (n=1,333) had MASLD (n=1,329) based on cardiometabolic criteria. The 10-year mortality rates for patients with NAFLD and MASLD were 10.4% and 10.3%, respectively. Nonetheless, this well-documented liver biopsy case report originates from Sweden, one of the Nordic countries. The Asian-based study Clinical Outcomes in Nonalcoholic Fatty Liver Disease (CLIONE) found that Westerners and Asians have significantly different NAFLD prognoses. In this study, we examined the prognosis of pure MASLD as a sub-analysis of the CLIONE study.

MATERIALS AND METHODS

We utilized a dataset from the CLIONE study carried out in Asia. Detailed information on how this cohort was formed and the primary findings of the CLIONE study can be found in a separate publication. This study was approved by the Institutional Review Board of Saga University Hospital (approval no. 2020-04-R-02; June 30, 2020), which waived the requirement for informed consent due to the use of pre-existing data. This study adhered to the reporting guidelines provided by Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).

Data sources

We employed a database from the Japan Study Group of NAFLD (JSG-NAFLD) to acquire information concerning patients with biopsy-confirmed NAFLD. All data for the study were collected and organized using REDCap electronic data capture tools, which are hosted by Osaka Metropolitan University.

Study cohort

We identified all patients who received a diagnosis of biopsy-confirmed NAFLD from December 1, 1994, to December 31, 2020. We tracked this cohort’s progress until March 31, 2021, to determine clinical outcomes related to hepatocellular carcinoma (HCC). Patients with excessive alcohol consumption (>30 g/day for men; >20 g/day for women) and those with liver disease caused by viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cholangitis, or biliary obstruction were excluded from the study.

Clinical assessment

We collected data on several factors including body mass index (BMI), blood pressure, daily alcohol consumption, smoking habits, past medical history, and current medication usage. Blood samples were collected after an overnight fast of at least 8 hours to measure plasma glucose, lipids, and liver-related biochemistry values. Diagnoses of type 2 diabetes mellitus (DM), hypertension, and dyslipidemia were based on established diagnostic criteria.

Cardiometabolic (CM) criteria in the MASLD definition are as follows: i) BMI ≥23 kg/m² (for Asian populations); ii) insulin resistance, defined as fasting blood glucose ≥100 mg/dL, HbA1C ≥ 5.7%, diagnosis of DM, or treatment for DM; iii) high blood pressure indicated by blood pressure ≥130/85 mmHg or the use of antihypertensive medication; iv) elevated triglycerides, with plasma triglyceride levels ≥150 mg/dL or the use of lipid-lowering medication; and v) dyslipidemia, characterized by plasma HDL-cholesterol levels ≤40 mg/dL (for males) and ≤50 mg/dL (for females) or the use of lipid-lowering treatment. Cardiometabolic criteria are considered met when at least one of the above five criteria is met.

Liver histology

Liver biopsy samples were acquired percutaneously with the guidance of ultrasound. Formalin-fixed and paraffin-embedded liver sections were stained using hematoxylin and
eosin or azan before being sent for centralized evaluation. NAFLD was defined as the presence of ≥5% hepatic steatosis, following the criteria established by Kleiner and colleagues. Grading and staging were based on the NAFLD activity score (NAS) of the NASH Clinical Research Network system (NASH-CRN) according to Brunt et al. and Kleiner et al. by an experienced pathologist (S.A.) at Saga University, who was blinded to the patients’ clinical and laboratory data. MASH was diagnosed according to steatosis activity fibrosis (SAF) and the fatty liver inhibition of progression (FLIP) algorithms. In this study, active MASH was defined as NAS of 4 or higher and a fibrosis stage of 2 or higher.

Follow-up evaluation

The follow-up period commenced on the date of biopsy and extended until the date of the last visit, patient death, or receipt of an orthotopic liver transplantation. Patients were monitored at intervals ranging from 3 to 12 months following NAFLD diagnosis, with repeated measurements of anthropometric parameters and metabolic assessments during each follow-up visit. For liver-related events, which encompassed the composite endpoint of gastroesophageal varices/bleeding, HCC, or decompensated cirrhosis, only the initial occurrence of these events following liver biopsy was considered. Recurring cases were not included in the analysis. In addition, we continued to record other cancer events, cardiovascular diseases (coronary artery disease, heart failure, arrhythmia), and stroke. The definition of cardiovascular disease was taken from our previous report. In cases of hospitalizations, we recorded the diagnosis upon admission. Additionally, we recorded the date and cause of death for all individuals. The duration of follow-up was determined as the time from the date of the biopsy to the date of the most recent follow-up.

Statistical analysis

Continuous and ordinal variables are expressed as the mean (standard deviation [SD]) and were compared using the unpaired t-test. Some data are expressed as number (%). Categorical variables were compared using Fisher’s exact test. Clinical outcomes (overall mortality and liver-related events) are presented as Kaplan–Meier curves. Presence or absence of cardiometabolic criteria, histologic features such as fibrosis stage, presence of MASH based on the FLIP algorithm, and high or low cumulative number of cardiometabolic risk factors were compared using the log-rank test. Two-sided P-values <0.05 were considered significant. All statistical tests, except those for spline curve creation, were conducted using JMP® 17.2.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Baseline characteristics

Cardiometabolic criteria were met by 1,381 of the 1,398 patients in the CLIONE cohort, which accounts for 98.8% of the total cohort (Table 1). Table 2 describes the differences in clinical characteristics according to the presence or absence of cardiometabolic criteria. Patients with cardiometabolic criteria (n=1,381) had a significantly higher BMI (28.0 vs. 20.9 kg/m², P<0.001), TG level (159 vs. 107 mg/dL, P<0.029), and FBS (114 vs. 86.6 mg/dL, P=0.002) than those without and had significantly lower HDL-C levels (49.6 vs. 59.9 mg/dL, P=0.009). Table 3 shows the differences in pathological features according to the presence or absence of cardiometabolic criteria. Inflammation, ballooning, NAFLD activity score, fibrosis stage, and the proportion of MASH and active MASH were significantly higher in patients with cardiometabolic criteria than in those without.

Clinical outcomes

Median follow-up duration of patients with pure MASLD (n=1,381) was 4.6 years (range: 0.3–21.6 years), for a total of 8,759 person-years. The number of patients lost to follow-up was 717 (51.3%) at 5 years, 284 (20.3%) at 5–10 years, and 257 (18.4%) at 10–15 years. Kaplan–Meier curve analysis of all-cause mortality and liver-related events, stratified by the presence or absence of cardiometabolic criteria in NAFLD patients, is presented in Figure 1. The Kaplan–Meier curves of overall mortality and liver-related events stratified by fibrosis stage and presence of MASH are presented in Figure 2. When patients were divided into four groups by fibrosis stage, overall survival and liver-related event-free rates were significantly stratified (log-rank P=0.009 and P<0.001, respectively). Furthermore, when patients were divided into two groups by...
Table 1. MASLD diagnosis criteria

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BMI</td>
<td>1,224/1,395</td>
<td>87.7%</td>
</tr>
<tr>
<td>2. Insulin resistance</td>
<td>1,065/1,294</td>
<td>82.3%</td>
</tr>
<tr>
<td>3. Blood pressure</td>
<td>770/1,089</td>
<td>70.7%</td>
</tr>
<tr>
<td>4. Elevated triglycerides</td>
<td>577/1,303</td>
<td>44.3%</td>
</tr>
<tr>
<td>5. Dyslipidemia</td>
<td>722/1,204</td>
<td>51.6%</td>
</tr>
<tr>
<td>6. Cardiometabolic criteria*</td>
<td>1,381/1,398</td>
<td>98.8%</td>
</tr>
</tbody>
</table>

MASLD, metabolic dysfunction-associated steatotic liver disease; BMI, body mass index. *At least one of 1–5 above criterion.

Table 2. Differences in clinical characteristics in the presence and absence of cardiometabolic criteria (n=1,398)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Numbers</th>
<th>Cardiometabolic criteria (+) (n=1,381)</th>
<th>Cardiometabolic criteria (–) (n=17)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>1,398</td>
<td>54.6 (14.2)</td>
<td>45.6 (14.8)</td>
<td>0.009</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>1,398</td>
<td>592 (42.9)</td>
<td>7 (41.2)</td>
<td>1.00</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1,395</td>
<td>28.0 (4.7)</td>
<td>20.9 (1.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>1,265</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>994 (79.5)</td>
<td></td>
<td>11 (78.6)</td>
<td>0.32</td>
</tr>
<tr>
<td>Past</td>
<td>109 (8.7)</td>
<td></td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>148 (11.8)</td>
<td></td>
<td>3 (21.4)</td>
<td></td>
</tr>
<tr>
<td>DM (yes)</td>
<td>1,397</td>
<td>587 (42.5)</td>
<td>0 (0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension (yes)</td>
<td>1,397</td>
<td>797 (57.7)</td>
<td>7 (41.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidemia (yes)</td>
<td>1,398</td>
<td>61.3 (39.6)</td>
<td>46.2 (38.5)</td>
<td>0.120</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>1,398</td>
<td>88.4 (60.9)</td>
<td>59.8 (51.1)</td>
<td>0.054</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>1,393</td>
<td>88.9 (96.4)</td>
<td>63.9 (47.3)</td>
<td>0.28</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>1,343</td>
<td>268 (103)</td>
<td>279 (74.0)</td>
<td>0.65</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>1,377</td>
<td>0.87 (0.45)</td>
<td>0.93 (0.41)</td>
<td>0.57</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>1,383</td>
<td>4.3 (0.4)</td>
<td>4.3 (0.3)</td>
<td>0.71</td>
</tr>
<tr>
<td>Hg (g/dL)</td>
<td>1,334</td>
<td>14.4 (1.5)</td>
<td>13.9 (1.6)</td>
<td>0.198</td>
</tr>
<tr>
<td>Platelet (&gt;109 /μL)</td>
<td>1,397</td>
<td>220 (70.4)</td>
<td>210 (37.4)</td>
<td>0.53</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>1,204</td>
<td>200 (38.8)</td>
<td>221 (36.1)</td>
<td>0.056</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>1,299</td>
<td>159 (89.5)</td>
<td>107 (18.6)</td>
<td>0.029</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>1,204</td>
<td>49.6 (13.7)</td>
<td>59.9 (8.1)</td>
<td>0.009</td>
</tr>
<tr>
<td>FBS (mg/dL)</td>
<td>1,296</td>
<td>114 (34.4)</td>
<td>86.6 (9.9)</td>
<td>0.002</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>1,085</td>
<td>285 (284)</td>
<td>275 (358)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Values are presented as number (percent).

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus; GGT, gamma-glutamyltransferase; HDL, high-density lipoprotein; Hg, hemoglobin; SD, standard Deviation; TC, total cholesterol; TG, triglycerides.
the presence of MASH, overall survival and liver-related event-free rates were significantly stratified (log-rank $P=0.007$ and $P<0.001$, respectively).

In Appendix Figure 1, CM risk factors had no effect on long-term prognosis, including mortality or the incidence of liver-related events, when the cumulative number of risk factors was divided into low (0–2) and high (3–5) groups.

In contrast, the 17 patients who did not meet CM criteria had a median follow-up duration of 5.9 years (range, 0.2–16.7 years), equivalent to 115 person-years, without death, liver-related events, cardiovascular events, or extrahepatic cancer. Therefore, the number of deaths and causes of death in patients with pure MASLD (n=1,381) were the same as those reported in the original CLIONE study. During the observation

### Table 3. Differences in pathological features in the presence and absence of cardiometabolic criteria (n=1,398)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cardiometabolic criteria (+) (n=1,381)</th>
<th>Cardiometabolic criteria (–) (n=17)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0*</td>
<td>8 (0.6)</td>
<td>0 (0)</td>
<td>0.20</td>
</tr>
<tr>
<td>1</td>
<td>967 (70.0)</td>
<td>16 (94.1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>270 (19.6)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>136 (9.8)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>65 (4.7)</td>
<td>4 (23.5)</td>
<td>0.007</td>
</tr>
<tr>
<td>1</td>
<td>870 (63.0)</td>
<td>12 (70.6)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>365 (26.4)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>81 (5.9)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Ballooning</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>453 (32.1)</td>
<td>11 (64.7)</td>
<td>0.019</td>
</tr>
<tr>
<td>1</td>
<td>609 (44.1)</td>
<td>5 (29.4)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>329 (23.8)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
<tr>
<td>NAFLD activity score (NAS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–2</td>
<td>272 (19.7)</td>
<td>11 (64.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3–4</td>
<td>744 (53.9)</td>
<td>5 (29.4)</td>
<td></td>
</tr>
<tr>
<td>5–8</td>
<td>365 (26.4)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
<tr>
<td>Fibrosis stage (F)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>231 (16.7)</td>
<td>10 (58.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1</td>
<td>536 (38.8)</td>
<td>3 (17.7)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>391 (28.3)</td>
<td>3 (17.7)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>197 (14.3)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
<tr>
<td>4+</td>
<td>26 (1.9)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>MASH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>452 (32.7)</td>
<td>11 (64.7)</td>
<td>0.008</td>
</tr>
<tr>
<td>Yes</td>
<td>929 (67.3)</td>
<td>6 (35.3)</td>
<td></td>
</tr>
<tr>
<td>Active MASH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAS≥4+F≥2</td>
<td>462 (33.5)</td>
<td>1 (5.9)</td>
<td>0.017</td>
</tr>
<tr>
<td>Others</td>
<td>919 (66.5)</td>
<td>16 (94.1)</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as number (percent).

NAFLD, nonalcoholic fatty liver disease; MASH, metabolic dysfunction-associated steatohepatitis.

*Includes cases of burned-out MASH (no increased fat and fibrosis stage 4).
Figure 1. Overall mortality and liver-related events stratified according to cardiometabolic criteria in patients with NAFLD (n=1,398). (A) Overall mortality and (B) liver-related events according to the presence of cardiometabolic criteria. NAFLD, nonalcoholic fatty liver disease.

Figure 2. Overall mortality and liver-related events stratified according to histological characteristics in patients with pure MASLD (n=1,381). (A) Overall mortality and (B) liver-related events according to fibrosis stage. (C) Overall mortality and (D) liver-related events according to the presence or absence of MASH. MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease.
period, 47 patients with pure MASLD died and one patient underwent orthotopic liver transplantation. The primary cause of death was extrahepatic cancer (n=10). The leading liver-related causes of death were liver failure (n=9), HCC (n=8), and cholangiocellular carcinoma (n=4) (Supplementary Table 1). Five-year and 10-year mortality rates were 1.9% and 4.9%, respectively. Finally, during the observation period, 77 new cases of liver-related events, 51 new cases of cardiovascular disease, and 21 new cases of stroke occurred (Supplementary Table 2). Finally, the types of new cancers observed during follow-up were hepatocellular carcinoma (n=37), breast cancer (n=16), and stomach cancer (n=10) (Supplementary Table 3).

**DISCUSSION**

Analysis of a subset of data from the CLIONE study revealed that approximately 99% of NAFLD cases could be classified as MASLD cases. The NAFLD-only group that did not meet the MASLD criteria exhibited milder histopathologic severity than the MASLD cohort and had a favorable prognosis. The prognosis of MASLD was similar to that of NAFLD.

Several studies have reported that MASLD is largely synonymous with NAFLD. Indeed, as shown in Table 1, about 99% of SLD patients met the CM criteria for MASLD. SLD has a bidirectional association with components of metabolic syndrome (MetS), and type 2 diabetes increases the risk of cirrhosis and related complications. Furthermore, the definition of MAFLD also includes MetS factors. Cardiometabolic criteria do not include high-sensitivity CRP or Homeostatic Model Assessment for Insulin Resistance, which is more realistic for clinical practice. In MAFLD, the group without metabolic factors showed less frequent fibrosis development than the group with metabolic factors. The prevalence of significant fibrosis also increased with the number of metabolic abnormalities. Furthermore, it has been reported that patients with steatotic liver but no metabolic risk factors have a better prognosis than those with metabolic risk factors. In this study, we demonstrated that the NAFLD-only group, which comprised NAFLD patients without CM risk factors that would have resulted in the classification of MASLD, had a milder histologic severity and better prognosis than the MASLD group. Our findings are consistent with those of previous studies. Interestingly, the cumulative number of CM risk factors did not affect long-term outcomes such as mortality or occurrence of liver-related events. Compared to CM risk factors, we believe that the presence or absence of MASH and liver fibrosis have more influence on the long-term prognosis of MASLD.

The cohort we studied had a lower mortality rate than those reported for Western cohorts. One possible reason for the lower mortality in Asian studies is that there are fewer CVD-related deaths in Asia than in the West. In a previous Western cohort of NAFLD, the CVD-related mortality rate in patients with biopsy-proven NAFLD was in the 20% range, whereas that in our cohort was only 4.2%. The prognosis of MASLD needs to be discussed in the Asian population. Previous reports on the prognosis of MASLD (formerly called NAFLD) indicated that the leading cause of death was cardiovascular disease (CVD). Leung and colleagues followed 307 patients in Hong Kong with biopsy-confirmed MASLD for an average of 49 months. Six patients died during the observation period, but only one was due to cardiovascular disease (ruptured abdominal aortic aneurysm). In Japan, the unadjusted mortality rates for heart diseases per 100,000 people in 2000, 2010, and 2019 were 117, 144, and 163 for men and 116, 155, and 172 for women, respectively. In contrast, the age-adjusted mortality rates for all heart diseases showed a consistent decline from 1995 to 2019. A well-developed universal health insurance system, high statin adherence rates among high-risk patients, and the use of superior therapeutic devices may contribute to the lower CVD mortality rate in Japan than in Western countries. It should also be noted that the impact on clinical outcomes differs between population-based big data studies and hospital-based biopsy cohort studies. In the Korean health examination big data, the cumulative CVD incidence rate for MASLD was 8.5 per 1,000 person-years.

Our study's key strengths include the large size of the patient cohort; verification of all NAFLD cases via liver biopsy; assessment of biopsy features by a single, experienced liver pathologist to eliminate inter-observer differences; and adoption of well-established scoring systems, such as the FLIP algorithm, for grading and staging liver biopsy features.

This study also had some limitations. Many of these limitations are inherent to retrospective studies, including the lack of specific treatment protocols, the lack of follow-up endoscopic evaluations, the lack of tracking of alcohol consump-
tion, the lack of imaging in non-cirrhotic patients, interval censoring, and residual confounding effects. Therefore, the number of liver-related events may have been underestimated in our cohort. Waist diameter could also not be assessed because it was not included in routine clinical observations. Second, we did not assess patatin-like phospholipase domain-containing protein 3 (PNPLA3) polymorphisms, which are more common among Asian than Western populations.\(^{27}\) PNPLA3 polymorphisms are detected in homozygous forms in approximately 20% of the general Japanese population.\(^{28}\) Recently, Seko et al.\(^{29}\) reported the impact of PNPLA3 polymorphisms on liver-related events. In that study, 1,550 Japanese patients with MASLD were followed for a mean of 7.1 years. Multivariate analysis identified the presence of PNPLA3 CG/GG (hazard ratio 16.04, \(P=0.006\)) as a predictor of liver-related events. Evaluation of single nucleotide polymorphisms, including PNPLA3, will aid in our understanding of the pathogenesis of SLD. Third, only 17 patients did not meet the CM criteria for MASLD, and although Table 3 shows significant differences in some histopathologic factors, the data should be interpreted with caution since the significance could change if the histologic data changed in even one case. Finally, selection bias may have been present because all participants in this study were diagnosed by liver biopsy.

In conclusion, approximately 99% of NAFLD cases could be classified as MASLD cases, whereas those with NAFLD only who did not meet the MASLD criteria had milder histopathological traits and a more positive outlook. Therefore, the prognosis of MASLD closely resembles that of what was previously referred to as NAFLD. Further studies with a larger number of patients will help clarify the pathogenesis of MASLD and patients with NAFLD-only who do not meet the MASLD criteria.

Authors’ contribution

Writing: HF; data collection: all authors.; statistical analyses: HF, AT, and SU; revision and editing: HF and YK; acceptance of the final version: all authors.

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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Global incidence of adverse clinical events in non-alcoholic fatty liver disease: A systematic review and meta-analysis

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Study Highlights

• Incidence rates for mortality, liver, and non-liver adverse clinical outcomes among persons with NAFLD were assessed using a meta-analytic approach. Incidence of mortality and HCC differed among NAFLD patients in North America, Europe, and Asia. Incidence of adverse clinical outcomes did not differ by sex. Those with NASH developed liver-related events at a significantly higher rate than NAFLD. The incidence of decompensated cirrhosis among those with NAFLD is increasing.
INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), a complex chronic liver disease associated with metabolic disorders in particular obesity and type 2 diabetes mellitus, is a major global health problem affecting more than 30% of the global population as of 2019. NAFLD carries a significant clinical burden including increased risk for hepatocellular carcinoma (HCC), non-liver cancer, cardiovascular disease (CVD), as well as increased risk for all-cause, liver-related, and CVD-related mortality. With progress in the diagnosis and treatment of viral hepatitis, NAFLD is poised to become the leading cause of liver-related morbidity and mortality in the world. NAFLD is already the leading etiology for cirrhosis in Mexico and is now the fastest growing indication for liver transplantation and HCC in liver transplant candidates in the United States. Similar trends for NAFLD-related HCC have been noted in Europe. NAFLD also carries a significant economic burden with annual direct medical costs estimated at $101 billion in the United States, €35 billion in Europe, and even higher in those with diabetes mellitus (DM).

Background/Aims: Nonalcoholic fatty liver disease (NAFLD) is associated with a multitude of adverse outcomes. We aimed to estimate the pooled incidence of NAFLD-related adverse events.

Methods: We performed a systematic review and meta-analysis of cohort studies of adults with NAFLD to evaluate the pooled incidence of adverse events.

Results: 19,406 articles were screened, 409 full-text articles reviewed, and 79 eligible studies (1,377,466 persons) were included. Mean age was 51.47 years and body mass index 28.90 kg/m². Baseline comorbidities included metabolic syndrome (41.73%), cardiovascular disease (CVD) (16.83%), cirrhosis (21.97%), and nonalcoholic steatohepatitis (NASH) (58.85%). Incidence rate per 1,000 person-years for mortality included: all-cause (14.6), CVD-related (4.53), non-liver cancer-related (4.53), and liver-related (3.10). Incidence for liver-related events included overall (24.3), fibrosis progression (49.0), cirrhosis (10.9), liver transplant (12.0), and hepatocellular carcinoma (HCC) (3.39). Incidence for non-liver events included metabolic syndrome (25.4), hypertension (25.8), dyslipidemia (26.4), diabetes (19.0), CVD (24.7), renal impairment (30.3), depression/anxiety (29.1), and non-liver cancer (10.5). Biopsy-proven NASH had higher incidence of HCC (P=0.043) compared to non-NASH. Higher rates of CVD and mortality were observed in North America and Europe, hypertension and non-liver cancer in North America, and HCC in Western Pacific/Southeast Asia (P<0.05). No significant differences were observed by sex. Time-period analyses showed decreasing rates of cardiovascular and non-liver cancer mortality and increasing rates of decompensated cirrhosis (P<0.05).

Conclusions: People with NAFLD have high incidence of liver and non-liver adverse clinical events, varying by NASH, geographic region, and time-period, but not sex. (Clin Mol Hepatol 2024;30:235-246)

Keywords: NAFLD; Cirrhosis; Meta-analysis; Epidemiology

Abbreviations:
CLD, chronic liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; DM, type 2 diabetes mellitus; HTN, hypertension; HLD/DLD, hyperlipidemia/dyslipidemia; HCC, hepatocellular carcinoma; MetS, metabolic syndrome; CVD, cardiovascular disease; BMI, body mass index; CAD/CHF, coronary artery disease/congestive heart failure; MASLD, metabolic dysfunction-associated steatotic liver disease

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diabetes and obesity continue to increase, the prevalence of NAFLD is forecasted to affect approximately 50% of the global population by 2040. In fact, the World Obesity Foundation recently released a report stating that without effective strategies to change the trajectory, over 50% of the world population will be overweight or obese by 2035 with an economic cost greater than $4 trillion, suggesting that the costs for NAFLD will continue to escalate as well. From the patient’s perspective, those with NAFLD reported fatigue, depression, lack of ability to physically perform their activities of daily living, and reduced work productivity.

In June 2023, a multi-society consensus was made to change the nomenclature from NAFLD to Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) with new diagnostic criteria focusing on cardiometabolic risk factors. Given the newly proposed diagnostic criteria and nomenclature change, concerns have been raised regarding the applicability of NAFLD research to this new definition. A study of patients from Hong Kong found 6/261 (2.3%) MR spectroscopy-diagnosed and 1/414 (0.2%) biopsy-proven NAFLD were unable to be classified as MASLD. Additionally, a population-based study from the United States utilizing data from the National Health and Nutrition Examination Survey found a 99% overlap between NAFLD and MASLD. Given the minimal discrepancy between MASLD and NAFLD, the findings from NAFLD studies will likely remain applicable under this new nomenclature.

Despite our knowledge of NAFLD prevalence, incidence, and its outcomes, incidence rate data for associated adverse outcomes is still sparse. Therefore, to provide more targeted interventions for people with NAFLD, it is vital to understand the incidence of adverse clinical outcomes in this population. This study aimed to fill this knowledge gap by identifying the incidence of the major health-related events occurring in people with NAFLD by systematic review and meta-analysis.

MATERIALS AND METHODS

Study design

We performed a systematic review and meta-analysis to evaluate the outcomes of NAFLD according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement for the conduct of meta-analyses of observational studies (http://www.prisma-statement.org/; Supplementary Table 1).

Search strategy and study selection

Our search strategy was developed in collaboration with a medical librarian (CDS, Stanford Lane Library). The search was conducted in three databases without language restriction (PubMed, EMBASE, and Cochrane Library from inception to June 22, 2021) using keywords such as “NAFLD” and “epidemiology.” Additional details can be found in the Supplementary File. Two authors independently performed the literature search and selected relevant articles. Discrepancies were resolved via consensus and/or discussion with a third author. Observational cohort studies of adult persons aged 18 years or older who had NAFLD at baseline, described the number of persons who reached an outcome of interest, and provided follow-up time data included. We excluded studies that included persons under the age of 18, special populations (e.g., viral hepatitis coinfection, hemodialysis patients), or studies that focused exclusively on a patient subgroup (e.g., elderly patients). Non-observational studies were excluded. For studies with overlapping patient populations, articles that provided the most data (largest patient sample, most subgroup data, most updated data) were selected.

Data extraction, study definition, and study quality assessment

Patients were considered to have NAFLD if they had evidence of hepatic steatosis in the absence of heavy alcohol use and other underlying liver diseases. Data on diagnostic method for NAFLD were collected (ultrasound, biopsy, non-invasive indices, and NAFLD diagnosis codes). Data were collected on several outcomes including mortality (all-cause, CVD-related, liver-related, and non-liver cancer-related), liver-related outcomes (fibrosis progression, cirrhosis, liver transplant, and HCC), decompensation (ascites, varices/variceal bleeding, hepatic encephalopathy), metabolic-related events (metabolic syndrome [MetS], hypertension [HTN], hyperlipidemia/dyslipidemia [HLD/DLD], DM), cardiovascular events (coronary artery disease/congestive heart failure [CAD/CHF], myocardial infarction [MI], ischemic/hemorrhagic stroke), renal impairment, depression/anxiety, and non-liver cancer. Additional data were collected on demographic, geographic
region, and presence of nonalcoholic steatohepatitis (NASH) at baseline. Study time was described via calculation of median study time from the recorded study initiation and end dates.

The primary endpoint was the incidence of adverse events in persons with NAFLD. Numerators were the number of persons with NAFLD at the start of the study. Denominators were the person-years of follow-up during the study period, which was determined via direct extraction of person-years follow-up time by the original studies, or by multiplying the total number of persons at risk with the mean/median year of study follow-up. We extracted the total number of persons with NAFLD at baseline within each cohort and the total number of persons who attained each outcome during the study period.

A specific case report form was developed for which two authors independently extracted data from eligible studies. Discrepancies in data extraction were resolved via consensus or consultation with a third author. A modified Newcastle-Ottawa scale with 5 main criteria and a maximum of 9 points was utilized for quality assessment (Supplementary Table 2). Studies with total scores of 7–9 were considered to have good quality, 4–6 as fair, and <4 as poor.

**Statistical analysis**

Random-effects models were used to estimate the pooled incidence of adverse events among NAFLD patients. The Cochran Q-statistic and I² statistic was used to assess heterogeneity with estimates associated with P-value of <0.05 in Q-statistic and I² ≥50% considered as having significant heterogeneity. The effects of heterogeneity on the incidence of adverse events were investigated via pre-planned subgroup analyses that included geographic region, sex, time-period, and presence of NASH at baseline, as per available data from individual studies. Standard Egger's test, DerSimonian-Lair and Sidik-Jonkman random-effects model adjustments, and funnel plot were used to assess for publication bias. P-values were generated using the standard DerSimonian and Laird random-effects models. Statistical analyses were conducted using the meta suite of commands in R statistical software (version 4.2.1; R Foundation for Statistical Computing, Vienna, Austria).
Liver-related events
Pooled incidence rates of liver-related events per 1,000 person-years were 24.28 overall, 3.39 for HCC, 10.85 for cirrhosis, 12.08 for hepatic decompensation (varices/variceal hemorrhage [4.72], ascites [6.68], hepatic encephalopathy [2.31]), and 11.99 for liver transplant (Table 2).

Metabolic-related Events
Pooled incidence rates for metabolic events were: MetS (25.40), HTN (25.84), HLD/DLD (26.43), and DM (19.01) (Table 2).

Other events
Pooled incidence rates for cardiovascular events were 24.77 overall (40.12 CAD/CHF, 7.07 for MI). Pooled incidence rates for other non-liver events were as follows: 30.28 for renal events, 29.10 for depression/anxiety, and 10.48 for non-liver cancer (Table 2).

Figure 1. Study selection flowchart and subgroups by NAFLD diagnostic method. NAFLD, nonalcoholic fatty liver disease.

Pooled incidence of adverse events - Subgroup analysis

By geographic region
Significantly higher rates of all-cause, CVD-related, and non-liver cancer-related mortality were observed in Europe and North America compared to Western Pacific/Southeast Asia with no differences observed for liver-related mortality (Fig. 2A). Significantly higher incidence of HCC was observed in Western Pacific/Southeast Asia compared to North America and Europe (Fig. 2B), though no differences were observed for other liver-related events (Fig. 2C).

For non-liver events, pooled incidence of CVD and HTN as well as non-liver events were highest in North America while Western Pacific/Southeast Asia had the lowest rates for both CVD and non-liver cancer (P=0.0059, <0.001, and <0.001, respectively, Fig. 2C–F, Supplementary Table 4).
By the presence of biopsy confirmed NASH
Analysis of 14,570 non-NASH NAFLD diagnosed via biopsy and 9,297 patients with biopsy-proven NASH showed those with NASH had a significantly higher incidence of HCC compared to those without NASH ($P=0.04$, Supplementary Table 5 and Fig. 3A–C). No statistically significant differences for liver-related and non-liver mortality ($all P>0.05$).

By NAFLD diagnostic method
Those with biopsy-diagnosed NAFLD had the highest rates of mortality ($P=0.0039$), fibrosis progression ($P=0.0004$), and liver transplant ($P=0.0096$) compared to NAFLD diagnosed via ultrasound, ICD, and other imaging. HCC incidence was also significantly higher among those with biopsy-diagnosed NAFLD compared to NAFLD diagnosed by ultrasound or ICD codes ($P=0.0002$). Meanwhile, NAFLD diagnosed via ultrasound had higher incidence of CAD/CHF and stroke compared to biopsy-diagnosed NAFLD (Supplementary Table 6).

By baseline advanced fibrosis and cirrhosis
There were no statistically significant differences in the pooled incidence of adverse clinical events among studies with a baseline prevalence of fibrosis less than the median (57%) and studies with a baseline prevalence of advanced fibrosis greater than the median (Supplementary Table 8). On the other hand, studies with a baseline prevalence of cirrhosis greater than the median (17%) had higher incidence of liver-related mortality, liver-related events, and HCC (Supplementary Table 9).

By time-period
By the median study year of 2007, there were significant decreases in the incidence rates of cardiovascular-related mortality ($P<0.0001$), non-liver cancer related mortality ($P<0.0001$), and non-liver cancer ($P=0.0012$), with a trending decrease in all-cause mortality ($P=0.0648$). However, there were significant increases in incidence rates of decompensated cirrhosis ($P=0.022$) (Supplementary Table 7). Notably, there was a decrease in baseline median age of the cohort by

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<th>Table 1. Baseline characteristics among participants with NAFLD</th>
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Data for random effects are reported as means (95% confidence interval [CI]). NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis. All Chi-square $Q<0.0001$. 
### Table 2. Incidence rate of adverse events among participants with baseline NAFLD

| Events                                      | Studies (n) | Participants (n) | Events (n) | Incidence rate per 1,000 person-years (95% CI)     | I² (%)
|---------------------------------------------|-------------|-----------------|-----------|---------------------------------------------------|-----
| **Mortality**                               |             |                 |           |                                                   |     |
| All-cause                                   | 37          | 513,409         | 75,371    | 14.57 (11.45–17.69)                                | 99.9|
| Cardiovascular disease-related              | 23          | 116,656         | 2,240     | 4.53 (2.92–6.15)                                  | 99.0|
| Non-liver cancer related                    | 22          | 116,949         | 2,239     | 3.27 (2.38–4.15)                                  | 98.7|
| Liver-related                               | 26          | 118,005         | 912       | 3.10 (1.96–4.24)                                  | 97.0|
| **Liver-related events**                    |             |                 |           |                                                   |     |
| Any event                                   | 40          | 603,907         | 108,512   | 24.28 (13.98–34.58)                                | 100.0|
| Fibrosis progression                        | 5           | 1,098           | 253       | 48.95 (22.86–75.04)                                | 92.8|
| Cirrhosis (compensated + decompensated)     | 21          | 309,694         | 5,370     | 10.85 (6.65–15.06)                                 | 99.5|
| Liver transplant                            | 10          | 3,503           | 126       | 11.99 (0.00–28.26)                                 | 92.3|
| HCC                                         | 30          | 336,845         | 1,025     | 3.39 (1.97–4.81)                                  | 94.6|
| **Cirrhotic decompensation**                |             |                 |           |                                                   |     |
| Any decompensation                          | 16          | 131,983         | 3,136     | 12.08 (6.80–17.36)                                 | 98.3|
| Ascites                                     | 13          | 4,764           | 201       | 6.68 (2.75–10.61)                                 | 90.7|
| Varices/variceal bleeding                   | 11          | 4,318           | 139       | 4.72 (2.57–6.87)                                  | 83.0|
| Hepatic Encephalopathy                      | 8           | 3,462           | 58        | 2.31 (1.07–3.55)                                  | 72.0|
| **Metabolic outcomes (in people without metabolic disease at baseline)** |             |                 |           |                                                   |     |
| Metabolic syndrome                          | 3           | 535             | 151       | 25.40 (0.00–68.87)                                 | 98.6|
| Hypertension                                | 12          | 5,591           | 922       | 25.84 (14.47–37.21)                                | 98.4|
| Hyperlipidemia/Dyslipidemia                 | 7           | 9,549           | 885       | 26.43 (11.38–41.47)                                | 98.4|
| Diabetes mellitus*                          | 24          | 39,562          | 5,025     | 19.01 (15.01–23.03)                                | 98.4|
| **Cardiovascular events (in people without cardiovascular disease at baseline)** |             |                 |           |                                                   |     |
| Any event                                   | 19          | 188,368         | 11,222    | 24.77 (8.46–41.08)                                 | 99.8|
| Coronary artery disease/congestive heart failure | 8   | 27,706          | 3,695     | 40.12 (2.13–78.11)                                 | 98.3|
| Myocardial infarction                       | 9           | 185,174         | 1,974     | 7.07 (0.00–14.90)                                 | 99.0|
| Stroke (ischemic/hemorrhagic)               | 10          | 148,571         | 3,602     | 8.05 (0.51–15.59)                                 | 92.5|
| Renal events                                | 6           | 330,872         | 14,712    | 30.28 (0.00–70.87)                                 | 99.8|
| Depression/anxiety                          | 1           | 19,871          | 5,783     | 29.10 (28.35–29.85)                                | N/A |
| Non-liver cancer                            | 11          | 29,243          | 3,165     | 10.49 (8.41–12.56)                                 | 96.5|

NAFLD, nonalcoholic fatty liver disease; HCC, hepatocellular carcinoma; N/A, not applicable.

*All Chi-square Q <0.0001.
the study year, with a regression coefficient of 0.19 per year (95% confidence interval 0.01–0.37) \( P = 0.039 \).

**By sex**

No significant differences in outcomes were noted between males and females with NAFLD (Supplementary Fig. 3).

**DISCUSSION**

In this study, using pooled data from 79 studies involving almost 1.4 million persons, we estimated the incidence rates for a comprehensive range of adverse events to include mortality, liver, and various non-liver outcomes for patients with NAFLD. The estimated pooled incidence rate for all-cause mortality, liver-related events, and various non-liver outcomes for patients with NAFLD.
mortality was 14.6 per 1,000 person-years. Among the cause-specific mortality, the rates were high for both liver as well as non-liver mortality (4.5, 4.5, and 3.1 per 1,000 person-years for cardiovascular, non-liver cancer, and liver related causes). This finding helps confirm previous reports that cardiovascular disease is among the primary causes of death among those with NAFLD especially among those with significant liver fibrosis or cirrhosis.23,24

In addition to mortality, we also estimated incidence rates per 1,000 person-years for liver-related events which were 24.3 overall, with notably lower rate of HCC (3.4) compared to other liver events (49.0 for fibrosis progression, 10.9 for cirrhosis, and 12.0 for liver transplant). The overall event rate for cardiovascular disease (24.8) was like that of liver-related events, and the non-liver cancer rate (10.5) was also similar to that of cirrhosis, while the rates of renal and depression/anxiety incidence were both about 30%. We estimated high incidence of cardiovascular events among those with NAFLD, especially CAD/CHF at a rate of 40 per 1,000 person-years. These findings further confirm the high cardiovascular burden of NAFLD identified by a prior meta-analysis on cardiovascular events.25 This current study further reinforces the significant associations between NAFLD and kidney disease that have been identified in the literature.26,27 A prior meta-analysis of middle-aged persons found a 1.2-1.5-fold increased risk of extrahepatic cancer among those with NAFLD.28 This current study further confirms these findings with an estimated incidence rate of 10.5 per 1,000 person-years among those with NAFLD. As this current study identified only one study on the incidence of depression/anxiety among those with NAFLD, further studies should be considered to better understand this association. We estimated the incidence of metabolic events to be high at a rate of approximately 20 per 1,000 person-years for DM, HLD/DLD, HTN, and MetS. Given that MetS is a major factor in the progression from NAFLD to NASH and significantly associated with increased risk for mortality, preventing development of MetS via a multidisciplinary approach is vital towards preventing both liver and non-liver adverse events among those with NAFLD.29,30

In our sub-analyses, we identified several differences in outcomes by region between North America, Europe, and Western Pacific/Southeast Asia. Compared to Europe and North America, Western Pacific/Southeast Asia had a lower risk for all-cause, cardiovascular-related, and non-liver cancer related mortality; however, there were no significant differences between the regions for liver-related mortality. Though non-liver cancer development was lower in Western Pacific/Southeast Asia, incidence of HCC was significantly higher compared to Europe and North America. Such differ-

Figure 3. Clinical outcomes among NAFLD participants with liver biopsy, stratified by biopsy proven NASH (additional details in Supplementary Table 5). (A) Mortality, (B) Liver-related events, (C) Non-liver events. NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; CVD, cardiovascular disease; HCC, hepatocellular carcinoma; HLD/DLD, hyperlipidemia/dyslipidemia; DM, diabetes mellitus.
ences in cardiovascular and non-liver cancer related mortality may be explained by the prevalence of the western diet in Europe and North America while the higher incidence of HCC development may be related to the higher prevalence of the PNPLA3 gene polymorphism in East Asia and its role in the development of HCC.31,32 Furthermore, the higher risk of HCC in Western Pacific/Southeast Asia may be due to competing risks for this outcome, as this population was found to also have the lowest risks for CVD-related, non-liver cancer-related, and all-cause mortality, thus allowing this population to live long enough to develop HCC. We excluded studies that included patients with known chronic hepatitis B but since hepatitis B virus is endemic in Asia, patients with NAFLD may have undiagnosed or occult HBV that can add to the risk for HCC development.

From the liver disease standpoint, non-NASH is felt to be mostly non-progressive whereas NASH can progress to cirrhosis and is not benign. We also explored incident events stratified by biopsy-diagnosed NASH compared to non-NASH patients. Interestingly, only liver-related mortality approached being significantly higher in those with NASH (10.2 per 1,000 person-years) compared to non-NASH patients (1.4 per 1,000 person-years, \( P = 0.0503 \)). On the other hand, those with NASH had a significantly higher incidence rate of HCC (14.8 per 1,000 person-years) compared to the non-NASH patients (0.79 per 1,000 person-years, \( P = 0.04 \)). The inability to find a significant difference in mortality and other events may be due to the non-significant difference in the incidence of fibrosis progression between the two groups. The leading predictor of adverse outcomes appears to be the progression of fibrosis. However, this suggestion needs further study as there was only one study used that reported on fibrosis progression.31 Additionally, as these studies included biopsy-diagnosed NAFLD, the study population likely had selection bias and may include those who are sicker and thus predisposed to adverse outcomes. As such, the results may underestimate the true difference in outcomes between NASH and non-NASH NAFLD. This is further evidenced in the comparison of adverse events by diagnostic modality, which found overall significantly higher rates of events, specifically liver-related events, among those diagnosed via biopsy compared to ultrasound; there could be misclassification in the diagnosis without liver biopsy. On the other hand, those that had NAFLD diagnosed via ultrasound had higher incidence rates for CAD/CHF and stroke, suggesting that NAFLD may have been found incidentally.34 Another important finding is the decreasing rates of non-liver events such as cardiovascular and non-liver cancer related events while liver-related events have increased between the pre-2007 and the post-2007 time-period, which may be due to a longer history of preventive efforts for cardiovascular disease and the cohort effect of the emerging NAFLD epidemic. The decreasing age of the study cohorts in the pre-2007 as compared to the post-2007 time period also suggests the effect of the increased awareness and diagnosis of NAFLD during the past three decades as well as the increasing prevalence of metabolic syndrome globally. Stratified analysis by sex found no significant differences in adverse events. Additionally, there was an increased incidence of liver-related events, HCC, and liver-related mortality among studies with higher baseline prevalence of cirrhosis though similar association was not statistically significant among studies with higher prevalence of advanced fibrosis. Together these results validate and extend the results from a recently published meta-analysis.35

A strength of our study is that we identified studies from multiple different regions. However, given that we only identified studies from Europe, North America, and Western Pacific/Southeast Asia, this current study does not include studies from Africa or South America, where NAFLD prevalence may be much higher.7 As such, further studies should be considered from other regions to further our understanding of the adverse effects of NAFLD on these populations. A limitation is that we did not include NAFLD diagnosed via non-invasive blood-based methods. However, given the non-standardized cutoffs across different populations and the fact that imaging is the recommended modality for NAFLD diagnosis, we felt it reasonable to include only those studies diagnosed via imaging or ICD-code.36 Additionally, as several studies did not report the person-years of follow-up, we used mean/median years follow-up to estimate the total person-years follow-up. This may have overestimated the denominator for the incidence rate, therefore underestimating the true incidence rate of adverse events associated with NAFLD. Despite non-significant Eggers’ test, DerSimonian-Laird and Sidik-Jonkman random-effects model adjustments, publication bias may remain and should be taken into consideration when interpreting this data. The results of our subgroup analysis identified that the effects of heterogeneity on incidence of adverse events rely on multiple factors across studies including region and baseline health status. Subgroup
data should be interpreted with caution as data were not available for all studies and sample size was smaller, thus leading to the potential introduction of additional bias. Additionally, we caution the interpretation of the results given the difficulty of confirming the temporal relationship between NAFLD and metabolic events as some patients may have had undiagnosed metabolic dysfunction at baseline, especially in those patients from retrospective cohort studies. Genetic polymorphism and the degree of severity as well as medical control of metabolic comorbidities may also have played a significant role in the development of MASLD, and should be examined in future studies. Finally, despite the minimal discrepancies between NAFLD and MASLD, the cautious interpretation of the results will be needed as the evaluation of outcomes associated with MASLD awaits future studies in this newly proposed disease category. 

To our knowledge, this is the first meta-analysis that identified the incidence rates of adverse events associated with NAFLD for a wide range of outcomes with detailed subgroup data. We estimated the incidence rates for mortality, liver-related events, non-liver related events, and provided estimates stratified by sex, baseline NASH status, diagnostic modality, time-period, and world regions. These incidence rates can be used to implement proper interventions targeted towards prevention and treatment, which is imperative as the prevalence of NAFLD increases. With the proposed changes in diagnostic criteria and nomenclature of NAFLD to MASLD, this study should guide future research on outcomes associated with MASLD.

Authors’ contribution

Study concept and supervision: MHN. Study design: MHL, DML, MHN. Data analysis: MHL. Data collection: All authors. Drafting of manuscript: MHL, DML, MHN. Data interpretation, review, and revision of manuscript: All authors.

Conflicts of Interest


SUPPLEMENTARY MATERIAL

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

REFERENCES


Identification of signature gene set as highly accurate determination of metabolic dysfunction-associated steatotic liver disease progression

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Graphical Abstract
Study Highlights

- We used a multi-omics approach to investigate the genomes, epigenomes, and transcriptomes of 134 MASLD patients and identified 1,955 MASLD-associated features. Then, we used machine learning to select the features that most accurately track MASLD progression. From this analysis, CAPG, HYAL3, WIPI1, TREM2, SPP1, and RNASE6 stood out as a signature gene set useful for discriminating the stages of MASLD progression. This signature gene set was verified using independent cohorts of MASLD, MASLD-associated cirrhosis, and liver cancer patients, suggesting it represents a group of biomarkers that apply to the full spectrum of MASLD-associated disease.

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Abbreviations:

ACC, accuracy; AI, artificial intelligence; ATAC-seq, assay for transposase-accessible chromatin sequencing; C, cytosine; DEGs, differentially expressed genes; DMRs, differentially methylated regions; FC, fold change; FFA, free fatty acid; FPR, false positive rate; GATK, Genome Analysis Tool Kit; GLM, generalized linear regression model; GO, Gene ontology; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; HFD, high-fat diet; HSI, hepatic steatosis index; KRGDB, Korean reference genome database; LFD, low-fat diet; MASLD, metabolic dysfunction-associated steatotic liver disease; MASH, metabolic dysfunction-associated steatohepatitis; NGS, next generation sequencing; PAS, periodic acid Schiff; PCA, principal component analysis; PoN, panel of normal; PPI, protein-protein interaction; qRT-PCR, quantitative real time polymerase chain reaction; RBF, radial basis function; ROC, receiver operating characteristic; SNP, single nucleotide polymorphism; SNV, single nucleotide variant; SVM, support vector machine; T, thymine; TPR, true positive rate; TSS, transcription start site; WES, whole exome sequencing; WGBS, whole genome bisulfite sequencing; WGS, whole genome sequencing

INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) is a metabolic disease characterized by fat accumulation in the liver.\(^1,\)\(^3\) MASLD includes simple steatosis, which is relatively early-stage and low risk, and metabolic dysfunction-associated steatohepatitis (MASH), which is late-stage disease characterized by serious liver inflammation and fibrosis.\(^4,\)\(^6\) Since MASH is often a precursor of cirrhosis, liver cancer, and liver failure, it is critical to discriminate between steatosis and MASH to guide patient treatment.\(^7,\)\(^9\) MASLD can be diagnosed using various non-invasive assessment methods, including imaging techniques, blood tests, and fibrosis assessment. However, a combination of these methods is required for a more accurate diagnosis and to assess the severity of MASLD.\(^10\)\(^-\)\(^12\) This underscores the need to identify novel molecular markers that would facilitate a faster and more precise MASLD staging.

Genome, transcriptome, and epigenome sequencing have already suggested potential biomarkers of MASLD in previous studies.\(^13\) Genetic variants, specifically single nucleotide polymorphism (SNPs) in PNPLA3, GCKR, TM6SF2, and AGXT2, have been associated with MASLD progression.\(^14,\)\(^15\) Comprehensive RNA-seq analyses have identified differentially expressed genes (DEGs) related to MASLD, providing insights into its severity involving processes such as the ablation of extracellular molecules, cytokine responses, and immune system functions.\(^16,\)\(^17\) In addition, epigenetic markers, particularly DNA methylation, have been explored. DNA methylation signatures related to age acceleration were correlated with MASLD severity, and hepatic fat-associated CpGs in peripheral blood samples of patients with type 2 diabetes revealed differentially methylated regions (DMRs), including ABCG1, CPT1A, and TMEM50B.\(^18,\)\(^19\) Despite these efforts uncovering significant markers associated with various stages of MASLD progression, securing the optimal gene set for accurately diagnosing a patient’s specific stage of MASLD progression remains an ongoing challenge.

Therefore, we decided to collect and analyze genomic, epigenomic, and transcriptomic data from a single cohort of patients progressing from steatosis to MASH, aiming to identify features that would enable an accurate diagnosis of MASLD stages. By feeding the MASLD-associated into a series of machine learning models that used linear regression methods, we were able to identify a set of 6 MASLD signature genes accurate enough to discriminate MASLD stage. We verified the utility of this gene set by using them to distinguish an independent cohort of MASLD and liver cancer patients from controls. Thus, this gene set will likely prove useful for the early diagnosis of MASLD and in guiding MASLD...
MATERIALS AND METHODS

Sample and sequencing library preparation

Pathologically proven biopsy-tissue and blood samples were obtained from a cohort of 134 MASLD patients, comprising 60 steatosis and 74 MASH patients in the study cohort who were recruited from the Dong-A University Hospital (Informed consent was obtained from all subjects, DAUHIRB-17-197) and Onhospital (Informed consent was obtained from all subjects, ONHIBR-19-001), Busan, Rep. of Korea. All fresh samples were frozen immediately after biopsy and stored at –70°C according to the protocols approved by the institutional review board for the human subject guideline that is in accordance with the principles of the Declaration of Helsinki. Hospital medical records and pathology reports of patients were reviewed by internal pathologist. The clinical features and the information of samples used for NGS analysis were provided in Supplementary Table 1 and Supplementary Table 2. For whole genome sequencing (WGS) and whole exome sequencing (WES), DNA was extracted from tissues and blood from MASLD patients. WGS libraries were generated using TruSeq Nano DNA (350), and 150-bp paired-end reads were sequenced on the Illumina platform. WES libraries were prepared using the SureSelectXT Library Prep Kit, and 100-bp paired-end reads were sequenced on the Illumina platform. For whole genome bisulfite sequencing (WGBS), samples were prepared using the Accel-NGS Methyl-Seq DNA Library Kit and the EZ DNA Methylation-Gold Kit. Then, 150-bp paired-end reads from the resulting libraries were sequenced on the Illumina platform. For total RNA-seq, RNA was extracted from the tissues of MASLD patients. Libraries were generated using the TruSeq Stranded Total RNA LT Sample Prep Kit, and 100-bp paired-end reads were sequenced on the Illumina platform (All sequencing was carried out by Macrogen, Inc., Seoul, Korea).

Detailed experimental procedures for histological diagnosis, genomic and epigenomic analysis, transcriptome analysis, machine learning, open chromatin accessibility analysis, statistics, high-fat diet (HFD) mouse model, hematoxylin and eosin (H&E) and with periodic acid schiff (PAS) staining, hepatocyte organoid culture, free fatty acid (FFA) treatment and Oil Red O staining and qRT-PCR are provided in supplementary information.

RESULTS

Identification of MASLD-associated somatic variants

To discover MASLD-associated markers, we took a multi-omics approach, looking at genomic, epigenomic, and transcriptomic data from WGS, WES, WGBS, and total RNA-seq using pathologically-proven biopsy tissue samples obtained from 134 MASLD patients (Fig. 1A). First, to limit our exploration to somatic markers that offer insights into genetic changes occurring in diseased cells, enhancing our understanding of the molecular basis of the disease, we eliminated any germline mutations by comparing WGS data obtained from liver biopsies with those obtained from blood samples (Fig. 1B). By integrating WES data screening for somatic variants in exon regions likely to affect the function of genes, we narrowed our search to 3,888 somatic variant callings. Of these, 79% (3,054) were classified as type of missense mutations. The most common type of somatic variant was the SNP, specifically the SNV in which a T nucleotide was altered to a C (Supplementary Fig. 1). Then, we focused on 504 different genes with 861 somatic variant sites detected in more than two of the 120 MASLD patient samples (Supplementary Table 3). These 504 genes with MASLD-associated somatic variants were broadly distributed throughout all chromosomes (Fig. 1C). Next, we asked whether the variants in these 504 genes were exclusive mutations (Fig. 1D). We found that 346 of 504 genes (69%) with the variants were exclusive, but the remaining 158 genes (31%) showed multiple, non-exclusive variants. Among them, genes mostly showed two variation sites. When we classified the various exclusive or non-exclusive variants in individual genes (Fig. 1E), we found missense mutations were the most common in both genes with exclusive and non-exclusive variants. Among them, genes mostly showed two variation sites. When we classified the various exclusive or non-exclusive variants in individual genes (Fig. 1E), we found missense mutations were the most common in both genes with exclusive and non-exclusive variants. In genes with non-exclusive variants, we observed cases in which two of the same type of variation appeared along with cases showing combinations of two or more different types. To determine the contribution of these MASLD-associated somatic variants to gene expression, we analyzed the expression levels of the 504 genes between their altered and non-altered groups...
Figure 1. MASLD-associated somatic variants identified through comprehensive WGS and WES analysis. (A) Overall research strategy for identifying MASLD-associated features via a multi-omics approach. (B) Pipeline for calling somatic variants. (C) Distribution of genes with MASLD-associated somatic variations across the chromosomes. (D) Pie chart showing genes with exclusive or non-exclusive variants. (E) Dot plot presenting the types of mutations in genes with exclusive or non-exclusive variants. (F) Dot plot showing gene expression changes between the altered and non-altered groups. MASLD, metabolic dysfunction-associated steatotic liver disease; WES, whole exome sequencing; WGBS, whole genome bisulfite sequencing; WGS, whole genome sequencing; DEGs, differentially expressed genes; DMRs, differentially methylated regions.
We found 16.76% (58) of the 346 genes with exclusive variations showed a statistically significant differences in their expression level, while only 9.49% (15) of the 158 genes with non-exclusive variants showed statistically significant expression changes.

Since variations of PNPLA3, TM6SF2, and AGXT2 have all been reported as genetic factors contributing to MASLD, we also examined the variations on these genes and detected in the WGS results from both liver tissues and in blood, suggesting they are instead germline mutations (Supplementary Fig. 2). In our cohort, a PNPLA3 variation (rs738409 C>G), a TM6SF2 variation (rs58542926 C>T), and an AGXT2 variation (rs2291702 T>C) were detected in 76.67%, 25.83%, and 67.50% of the samples, respectively (Supplementary Fig. 2A). We confirmed diminished expression levels of these genes in the steatosis and MASH altered groups, with remarkable reductions in homozygous variants (Supplementary Fig. 2B and 2C). Thus, these MASLD-related genetic variations were common in our cohort, but they were excluded because we were searching specifically for somatic mutations. From these results, we suggest MASLD-associated somatic variations in 504 genes.

Figure 2. Identification of differentially methylated regions associated with MASLD progression. (A) Scatter plot showing genes with a methylation ratio that is significantly different between steatosis and MASH samples. (B) Correlation between DNA methylation status and gene expressions. (C) Representative loci showing hypermethylation in the PACS2 and hypomethylation in the PEG10 promoter. MASLD, metabolic dysfunction-associated steatotic liver disease; MASH, metabolic dysfunction-associated steatohepatitis.

(Fig. 1F). We found 16.76% (58) of the 346 genes with exclusive variations showed a statistically significant differences in their expression level, while only 9.49% (15) of the 158 genes with non-exclusive variants showed statistically significant expression changes.

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Differentially methylated regions in MASLD

Next, to identify DMRs associated with MASLD, we performed WGBS in 104 MASLD patients (Fig. 2). We identified 87 DMRs with p-values less than 0.05 in the comparison between steatosis and MASH samples. 68 of 87 DMRs (78%) were located within known CpG regions and 58 of these DMRs (66.7%) were annotated to reference genes (Supplementary Table 4). Of these 58 DMRs, 13 DMRs were hypo-
methylated and 45 DMRs were hypermethylated in MASH (Fig. 2A). We next asked whether the differential methylation associated with MASLD progression also contributed to gene expressions (Fig. 2B). As results, of the 13 genes with hypomethylated CpGs and the 45 genes with hypermethylated CpGs, 38.4% (5) and 68.8% (31) showed inverse correlations with gene expression, respectively. Indeed, the correlation coefficients comparing methylation status and gene expression were statistically significant ($P$-value=$3.07E-03$). Figure 2C shows the hypermethylated promoter region of PACS2 and the hypomethylated promoter of PEG10 as examples of altered genes associated with MASH. Together, our results of epigenomic analysis provided MASLD-associated DMRs that could affect disease progression through the regulation of gene expression.

**Genes related to MASLD progression**

Next, to investigate genes related to MASLD progression, we performed a total RNA-seq analysis and found 1,393 DEGs in the comparison of steatosis and MASH (Supplementary Table 5). Among these, 645 steatosis- and 748 MASH-enriched genes were defined by a 1.3-fold or greater change in expression level between MASLD stages (Fig. 3A). To understand the function of steatosis- and MASH-enriched genes, we performed analysis of GO (Fig. 3B), motif search, and TRRUST enrichment (Fig. 3C). Results of these analysis showed DEGs were involved in terms of cell-cell adhesion, metabolic pro-

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cess, and cytokine signaling and were regulated by transcription factors such as NFKB, JUN, and SMAD3/4 have already been associated with MASLD progression.

**Integrated networks of MASLD-associated features within functional modules**

From MASLD-associated somatic variations, DMRs, and DEGs we identified, we designated 1,955 as MASLD-associated features. We next investigated whether these MASLD-associated features collaborate in functional modules (Fig. 4). Considering the proportion of MASLD-associated features in each module, frequency represented in terms for steatosis- or MASH-enriched genes, we found dominant 6 functional modules such as response to cytokine, regulation of immune system process, cell cycle, regulation of phosphorus metabolic process, inflammatory response, and lipid localization (Fig. 4A). MASLD-associated features accounted for about 10% of the list corresponding to genes annotated from the public database of functional modules. Since MASLD-associ-
ated features may simultaneously be MASLD-associated variations, DMRs, or DEGs, we categorized them in detail. Individual functional modules included genomic, epigenetic, and transcriptomic features (Fig. 4B). As an example, the 125 MASLD-associated features related to cytokine responses included 98 DEGs, 2 DMRs, 23 genes with variations, and 2 genes involved in DEGs/variations (Fig. 4C). We found MASLD-associated features appearing in one or more func-

Figure 5. Using machine learning modeling to select features that permit MASLD stage discrimination. (A) Feature selection via machine learning modeling. (B) 203 stacked features obtained from 16 independent models. (C) Designing the signature gene set consisting of the top-ranked genes that provided the highest accuracy. (D) Dot plot of signature gene sets of various sizes against their accuracy in discriminating MASLD stages. The chosen gene set is indicated (ACC=0.955). (E) ROC curve plots showing the accuracy of the 6 signature gene set and individual genes (6 signature set P-value=1.04E-19; CAPG P-value=2.48E-14; HYAL3 P-value=1.26E-11; WIPI1 P-value=1.57E-10; TREM2 P-value=3.64E-13; SPP1 P-value=1.28E-12; RNASE6 P-value=1.90E-09). (F) ROC curve plots indicating the accuracy of non-invasive indices and the signature gene set (6 signature set P-value=1.04E-19; FIB-4 P-value=4.48E-04; Hepatic Steatosis Index P-value=5.1E-14; NAFLD fibrosis score P-value=5.50E-02). MASLD, metabolic dysfunction-associated steatotic liver disease; ACC, accuracy; ROC, receiver operating characteristic.
tional modules, both specifically and sometimes redundantly (Fig. 4D). They also showed strong cooperation within MASLD-associated functional modules (Fig. 4E and Supplementary Fig. 3). Thus, MASLD-associated features were connected primarily to representative functional modules related to MASLD, collaborating with one another within their functional modules. This suggests MASLD-associated variations, differential methylated regions, and expression changes have a close relationship with one another.

Identification of signature genes through feature selection

To ascertain the signature gene set for diagnosis of MASLD stages, we established machine learning modeling with feature selection (Fig. 5A). To prevent bias in the feature selection process, we randomly divided the samples in our cohort: 70% were assigned to a training set and 30% to a testing set. We started with 14,396 genes and robust scaling was processed to individually normalize the expressions (Supplementary Fig. 4). Then, redundant features were eliminated by...
repeating linear SVM modeling until less than 1,500 features with optimum coefficient scores remained. The ~1,500 selected features were placed in a testing set to evaluate their accuracy through RBF kernel model with optimal parameters. Finally, we established 20 machine learning models for MASLD-stage discrimination and found 16 models with over 80% accuracy (ACC) (Supplementary Fig. 5A).

Next, to identify signature genes from the selected features through machine learning modeling, we first asked whether there are similarities between the selected features (Fig. 5B). We found that 203 features were shared across 16 individual models, and we designated these “stacked features”. Then, we looked at the features shared between the 203 stacked features and the 1,955 MASLD-associated features obtained from multi-omics analysis. We selected 64 features for further analysis and used them to discover an optimal combination of signature genes by generalized linear regression model (GLM) (Fig. 5C). First, after measuring the accuracy of the 64 features independently, we ranked them by an accuracy score. CAPG had the highest accuracy score (ACC=0.82) (Supplementary Fig. 5B and Supplementary Table 6). Then, we tried to identify the genes that gave the highest accuracy when paired with CAPG. This process was repeated with one feature after another, considering only those features that maintained a combined accuracy as high as the other models with more features (Fig. 5D and Supplementary Table 7). We found that the accuracy of combined gene set increased as features were added to it, but a combination of 6 genes saturated at the highest level of accuracy. In this way, we identified a set of 6 signature genes—CAPG, HYAL3, WIPI1, TREM2, SPP1, and RNASE6—that yielded the highest accuracy in MASLD stage discrimination. We improved the discriminability of steatosis and MASH samples by applying only the 6 signature genes compared with either data from whole transcriptome or 1,393 DEGs (Supplementary Fig. 6). Moreover, we confirmed that utilizing a set of genes enhances the ability to distinguish MASLD stages compared to individual genes (Fig. 5E), as well as non-invasive markers, such as non-alcoholic fatty liver disease (NAFLD) fibrosis score, FIB-4, and Hepatic Steatosis Index (HSI) (Fig. 5F).

Together, we propose that the 6 signature genes identified using machine learning modeling are essential molecular markers for assessing MASLD progression.

Application of the signature gene set to liver disease

To determine whether signature genes can be applied to the full spectrum of MASLD-associated disease and related histological features, we calculated its accuracy in diagnosing
an independent cohort of 216 samples comprising 10 normal, 51 steatosis, and 155 MASH samples (Fig. 6A, GSE135251) and a cohort of 78 samples comprising 6 normal and 72 MASLD samples, providing information on steatosis, inflammation, ballooning hepatocyte, and liver fibrosis stage (Fig. 6B, GSE130970).

When we plotted ROC curve plots, we found that the signature gene set discriminates between steatosis and MASH (F0-F4) with an AUC score of 0.795 (Fig. 6A). Since MASH samples were graded F0 to F4 according to disease progression, we further analyzed the early-stage MASH groups (F0-2) and the late-stage MASH groups (F3-4). When predicting the groups, steatosis samples from the early-stage MASH (F0-2) samples that were relatively close in disease progression, the performance was still high accuracy (AUC=0.767). Further, in distinguishing steatosis from late-stage MASH (F3-4), which show significantly different levels of disease progression, the signature gene set predicted with high AUC score (AUC=0.863). Next, we were interested in the possibility to extend the coverage of signature gene set from normal to whole spectrum of MASLD. By applying the combination gene set of signature genes, it was possible to distinguish between normal and whole MASLD with very precisely (AUC=0.968). Also, normal and steatosis tissues (AUC=0.947), and normal and MASH tissues (AUC=0.979) showed highly accurate results. Furthermore, we confirmed that the signature gene set accurately distinguished the degree of lobular inflammation (AUC=0.931) and steatosis grade (AUC=0.943) (Fig. 6B). Although the AUC for distinguishing cytological ballooning was about 0.765, the accuracy between fibrosis stages was over 0.838, with an AUC value of 0.857 confirmed for the NAFLD activity score. The expression level of each signature genes was confirmed for normal, steatosis, and MASH and as expected, their expression levels significantly increased with progression through the various stages of MASLD (Fig. 6C) and subgroups based on histological features (Fig. 6D). These results suggest that the discriminatory capacity of the signature gene set for distinguishing different stages of MASLD is comparable to that of histological features.

We further examined the expression levels of the signature genes in an in vivo model fed a HFD (Fig. 6E) and in hepatic organoids treated with 1 mM FFA (Fig. 6F). In the in vivo model fed a HFD, signature gene levels were significantly increased, consistent with a remarkable accumulation of fat in the liver when compared to controls (Fig. 6E). Moreover, we observed a similar increase in signature gene expression in...
organoids induced to accumulate lipid by treatment with 1 mM FFAs (Fig. 6F and Supplementary Fig. 7). These results demonstrate that our signature gene set not only differentiates steatosis from MASH in MASLD progression, but also normal tissue from steatosis. This means it can be used in the early-stage detection of MASLD.

Next, we asked whether the signature gene set could be applied to the detection of liver cancer—which often follow MASLD (Fig. 7). RNA-seq data from liver cancer patients reported in a previous study (GSE77314) were re-analyzed to validate the combination set of signature genes.23 The accuracy to distinguish between control and cancer was calculated by GLM. Soundingly, the accuracy between control and liver cancer was exceedingly high (ACC=0.970, Fig. 7A). In addition, we found high expression of signature genes showed correlation with poor overall liver cancer survival (Fig. 7B). Taken together, changes in signature gene expression can distinguish not only MASLD progression but also normal tissue from liver cancer. This means that our set of 6 signature genes can be used as biomarkers for the full spectrum of MASLD-associated disease.

Chromatin accessibility of signature genes

Since chromatin accessibility contributes strongly to gene expression, we further examined changes in chromatin accessibility at signature gene loci by analyzing ATAC-seq on representative steatosis (n=4) and MASH (n=4) samples (PRJNA725028, Fig. 8).24 Because the signature genes are also MASH-enriched genes, we first investigated the accessibility status for the promoters of MASH-enriched genes. We found accessibility enrichment at these promoters was significantly increased in MASH samples (Fig. 8A). Furthermore, we confirmed that the enrichment of open chromatin regions at signature gene loci was remarkably increased in MASH (Fig. 8B). We also estimated the combination of the chromatin accessibility scores for the signature genes using PCA and found that also could predict disease progression (Fig. 8C).
8D illustrated the increased enrichment of open chromatin regions at CAPG and HYAL3 loci in MASH compared to steatosis samples. These results indicate both signature gene expression and chromatin accessibility can act as biomarkers for MASLD progression.

**DISCUSSION**

This study identified MASLD-associated features through integrative genomic, epigenomic, and transcriptomic analyses of samples from 134 MASLD patients. We used machine learning modeling to select from these MASLD-associated features those that could be used to accurately distinguish the stages of MASLD, and then we validated this signature gene set in independent cohorts of MASLD and liver cancer patients. Thus, our results provide diagnostic biomarkers that can accurately discriminate the various stages of MASLD-associated disease.

As big data technologies continue to emerge, machine learning and artificial intelligence (AI) are increasingly being applied to diagnose various human diseases and make decisions regarding their treatment. In previous studies, histological images and/or clinical information have been used as inputs for deep learning or machine learning models designed to predict disease progression. In addition, MASLD researchers have used histological images to predict fibrosis scores in MASH patients and clinical information to distinguish healthy patients from those suffering from MASH. Recently, the researchers tried to apply machine learning for MASLD study. One study used lipidomics data and machine learning to detect MASLD and other study used public data (NIDDK NAFLD data and Optum data) to predict MASH. Although they had delivered interesting results, the possibility of clinical application may be limited because of either limited data source (lipidomics only or no omics data) or poor AUC (model with AUC 0.82 or 0.76). In our machine learning modeling approach using molecular features, we identified a signature gene comprising CAPG, HYAL3, WIPI1, TREM2, SPP1, and RNASE6, which can discern the various stages of MASLD with high accuracy (Fig. 6A, normal vs MASLD AUC=0.968; normal vs. MASH AUC=0.979). Additionally, the signature gene set demonstrated high accuracy in discriminating between histological feature-based subgroups related to MASLD, achieving effective performance (Fig. 6B, AUC=0.931 for lobular inflammation; AUC=0.943 for steatosis grade; AUC=0.838 for fibrosis stage). We also confirmed that the diagnostic performance of the signature gene set could accurately distinguish disease stages with high accuracy across various subgroups associated with MASLD, including obesity, PNPLA3 mutation, and diabetes, which are known to have close connections with MASLD (Supplementary Fig. 8). These results indicate that the signature gene set identified in this study could be applied to various subgroups related to MASLD, demonstrating its potential as a diagnostic marker.

There is no uncertainty regarding the distinct functional roles of individual genes within the signature gene set in human diseases, as their expression escalates with the progression of MASLD. However, the degree of expression alterations for individual genes exhibits variability among different subgroups associated with MASLD (Fig. 6C and 6D), and the diagnostic capabilities of individual genes diverge (Fig. 5E). This emphasizes the need for a signature gene set, rather than relying on individual genes, to diagnose the stage of the disease. Using the signature gene set to assess disease stages in diverse subgroups of MASLD yielded the highest accuracy (Supplementary Fig. 9), surpassing that of non-invasive assessments (Fig. 5F). Furthermore, we explored the potential use of the signature genes as non-invasive markers and confirmed their ability to discriminate with an AUC of 0.76 between normal and MASLD in cell-free RNAs in blood (Supplementary Fig. 10). This highlights the superior precision achieved with the signature gene set in evaluating MASLD progression.

In summary, using a multi-omics approach coupled with feature selection via machine learning modeling, we identified a signature gene set that can accurately predict the stages of MASLD. We found this signature gene set can be applied to the full MASLD spectrum, from normal tissue to MASLD-related cancer. Our current understanding of this signature gene set has provided markers for the diagnosis of MASLD, but further study will be required for clinical application with larger patient cohort and functional analysis of signature genes.

**Authors’ contribution**

S.O. contributed to the analysis of WGS, WES, WGBS, and total RNA-seq and establishment of machine learning modeling and interpretation of “omics” data; Y.-H.B., S.-Y.H., J.-S.J., J.-H.C., Y.-H.R., S.-W.L., G.-B.C. contributed to interpretate clini-
cal information and patients enrollment; S.J. and S.Y. contributed to the analysis and interpretation of the data; Y-S.L., Y-H. B., Y.S.L., and G.P. contributed to sample acquisition, sample processing, quality control, and data generation; S.H. contributed to the functional analysis; B.K. and W.K. contributed to the ATAC-sequencing; K.H.Y., R.H.S., Y-S.L., and J.H.P. conceived and designed the study; S.O., S.J., S.Y., and K.H.Y. drafted the manuscript; all authors read and approved 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).
The data are available from the Korean Nucleotide Archive (https://www.kobic.re.kr/kona; Accession ID: PRJKA210057).

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Dear Editor,

The consensus group, comprising patients from multiple societies, has updated the terminology for non-alcoholic fatty liver disease (NAFLD) to "metabolic dysfunction-associated steatotic liver disease" (MASLD) to more accurately reflect the underlying pathophysiology. This revision allows discernment of the underlying factors of steatotic liver disease (SLD) with increased precision and eliminates the potential for stigmatization. Nonetheless, modifications to the terminology can result in misunderstanding and temporarily impede progress. In addition, to optimize the utilization of valuable research resources, it is imperative to build upon previous investigations on NAFLD and extend the studies to MASLD. In this regard, several studies have been conducted to accumulate evidence.

The etiological associations of hepatocellular carcinoma (HCC) have undergone a significant transformation in the past decade due to a marked decrease in active hepatitis C virus (HCV) infection. The increasing prevalence of NAFLD-related HCC has become a major public health issue worldwide. In contrast to HCV-related HCC, the incidence of NAFLD-related HCC is relatively low. However, due to the significant number of individuals affected by NAFLD, challenges are posed from a cost-effective perspective to include and follow all patients to detect those affected at advanced stages. Recent breakthroughs in systemic therapy for advanced HCC, such as the use of molecular-targeted agents and immune checkpoint inhibitors, e.g., atezolizumab in combination with bevacizumab (Atezo/Bev), have resulted in significantly improved prognoses for patients with these conditions. The impact of NAFLD on the prognosis of patients is a subject of ongoing discourse as the efficacy of therapeutic interventions may be influenced by both favorable and unfavorable factors related to the underlying etiologies.

Consequently, effective management and treatment of patients with HCC necessitates comprehension of the prognostic implications of NAFLD in such patients. However, no studies have investigated the relationship between NAFLD and MASLD in individuals with advanced HCC who have undergone Atezo/Bev therapy.

We aimed to compare the prognosis of unresectable HCC
between patients with NAFLD and MASLD. This study included 216 consecutive patients with advanced HCC who received Atezo/Bev at one of 11 institutions in Japan between November 2020 and October 2023. All patients were of Asian origin. These participants were 78.7% male, median age 73 years, median body mass index 23.0 kg/m², 98% performance status ≤1, 94% Child-Pugh class A, and median alpha-fetoprotein level of 42.2 ng/mL (Table 1). SLD was diagnosed based on the presence of moderate or severe hepatic steatosis on ultrasonography. The most common chemotherapies were first-line (136/216, 63.0%) followed by second-line (64/216, 29.6%).

Of the 216 patients, 38 (17.6%) were diagnosed with NAFLD, including one patient who did not fulfill the cardiometabolic criteria for MASLD and was classified as cryptogenic SLD. (Fig. 1A). A significant proportion (97.4%) of the patients with NAFLD in our study were also diagnosed with MASLD. Thus, the backgrounds of the NAFLD and MASLD patients were nearly indistinguishable, a finding that is not surprising given that the difference was found only in one patient (Table 1). Our findings align well with previous studies reporting that more than 95% of NAFLD patients fulfilled the MASLD criteria.3,10 Figure 1B shows the Kaplan-Meier curve for overall survival. No significant difference was observed in the overall survival rate between the NAFLD and MASLD groups (median overall survival in patients with NAFLD and MASLD was 583 days [95% certificate index, 409–non-applicable days] and 472 days [95% certificate index, 404–non-applicable days], respectively, \( P=0.877 \)). The 1-year and 3-year survival rates were not significantly different between the patients with NAFLD and those with MASLD (1-year, 71.1% vs. 70.3%, \( P=0.941 \); 3-year, 26.3% vs. 24.3%, \( P=0.843 \)) (Fig. 1B).

In conclusion, these results indicate that the prognosis for patients with advanced HCC undergoing Atezo/Bev treatment with MASLD is comparable to that in patients with NAFLD. It is crucial to validate these findings through an international cohort comprising a greater number of patients.

Authors’ contribution
Hiroyuki Suzuki: study concept, design, and statistical analysis; Shigeo Shimose: data extraction, interpretation of data, and critical revision of the manuscript; Hideki Iwamoto: inter-

Figure 1. Prognosis of HCC under Atezo/Bev combination therapy in the NAFLD and MASLD groups. (A) The prevalence of NAFLD and MASLD in patients with HCC. (B) Probability of overall survival between the NAFLD and MASLD groups. HCC, hepatocellular carcinoma; Atezo/Bev, atezolizumab plus bevacizumab; NAFLD, nonalcoholic fatty liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease; NA, not applicable; mOS, median overall survival.

Abbreviations:
NAFLD, non-alcoholic fatty liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease; SLD, steatotic liver disease; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Atezo/Bev, atezolizumab plus bevacizumab
Table 1. Baseline patients characteristics

<table>
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<td>Sex, female/male</td>
<td>46/170</td>
<td>9/29</td>
<td>8/29</td>
</tr>
<tr>
<td>Etiology, NAFLD (MASLD)/HCV/HBV/Alcohol/Others</td>
<td>38/103/32/36/7</td>
<td>38/0/0/0/0</td>
<td>37/0/0/0/0</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
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<td>25.2</td>
<td>25.4</td>
</tr>
<tr>
<td>Performance status, 0/1/2</td>
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<td>30/8/0</td>
<td>29/8/0</td>
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<tr>
<td>Child-Pugh class, A/B</td>
<td>203/13</td>
<td>35/3</td>
<td>34/3</td>
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<tr>
<td>Alpha-fetoprotein (ng/mL)</td>
<td>42.2</td>
<td>34.6</td>
<td>32.9</td>
</tr>
<tr>
<td>BCLC B/C</td>
<td>108/108</td>
<td>21/17</td>
<td>21/16</td>
</tr>
</tbody>
</table>

Data are presented as medians.

NAFLD, non-alcoholic fatty liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease; HCV, hepatitis C virus; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer.

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 declare no conflicts of interest.

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Letter to the Editor

Similar respiratory function including chronic obstructive pulmonary disease between non-alcoholic fatty liver disease and metabolic dysfunction-associated steatotic liver disease

Tsubasa Tsutsumi¹, Dan Nakano¹, Machiko Kawaguchi¹, Hirokazu Takahashi², and Takumi Kawaguchi¹

¹Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Japan; ²Liver Center, Saga University Hospital, Saga, Japan

Keywords: Non-alcoholic fatty liver disease; Metabolic dysfunction-associated steatotic liver disease; Chronic obstructive pulmonary disease

Dear Editor,

We have read with great interest the multi-stakeholder consensus statement that redefined the nomenclature for fatty liver disease.¹ The transition from the term “Non-alcoholic Fatty Liver Disease” (NAFLD) to “Metabolic Dysfunction-Associated Steatotic Liver Disease” (MASLD) represents a pivotal moment in the understanding and communication of this prevalent condition.² However, it is necessary to ascertain whether the evidence accumulated in association with NAFLD can be applied in the same manner as the transition to MASLD.³

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death globally, yet there is no effective treatment. Similar to steatotic liver disease (SLD), it remains a condition with high unmet needs. Although smoking has been identified as a cause of COPD, investigation into the diverse clinical features of COPD has been limited.⁴ NAFLD is one of the comorbidities of COPD and is associated with its severity.⁵,⁶ In addition, metabolic dysfunctions, including hypertension and dyslipidemia, are associated with the prevalence of COPD.⁷ However, there is uncertainty about the differences between NAFLD and MASLD regarding respiratory function and the prevalence of COPD.

We investigated the respiratory function based on spirometry results in patients diagnosed with NAFLD and MASLD by abdominal ultrasonography. The health check-up database used in this study was derived from staff medical check-ups as required by the Occupational Health and Safety Law in Japan and as individual voluntary participation, not based on hospital data. We enrolled 34,073 Asian participants over 40 years of age, who underwent health check-up examinations from January 2010 to March 2020. Ultrasound examinations were performed by certified sonographers, and all images
were adequately recorded. The following information was obtained using a self-reported questionnaire: age, sex, current smoking habits, alcohol consumption, comorbidities, and medication use. We excluded 23,987 participants with multiple check-ups on the same subject, 173 participants with the presence of hepatitis B surface antigen or presence of anti-hepatitis C virus antibody, and 2,384 participants with moderate or heavy alcohol consumption (>20 g/day for female, >30 g/day for male) and a lack of data on alcohol consumption. Finally, we examined 7,529 participants who underwent abdominal ultrasound and spirometry tests (Supplementary Fig. 1).

Out of 7,529 individuals, 50% (3,755 out of 7,529) were diagnosed with steatotic liver by ultrasonography. NAFLD was diagnosed in 42.4% (3,192/7,529), including 6.1% (195/3,192) of patients who did not fulfill the cardiometabolic criteria for MASLD. MASLD was diagnosed in 39.8% (2,997/7,529). In our cohort, the overlap rate between NAFLD and MASLD was 93.9%. This result was nearly identical to previous studies that reported approximately 95% of MASLD cases meeting the diagnostic criteria of NAFLD. In our cohort, patients’ characteristics such as age, sex, body mass index, and habits of drinking and smoking were nearly identical for both NAFLD and MASLD cases.

We showed the spirometry results of patients with NAFLD and MASLD in Table 1. Vital capacity (VC) shows the maximum amount of air that can be expelled from the lungs. The median values for VC were almost identical in both NAFLD and MASLD groups, suggesting that neither condition significantly affects respiratory capacity. The values were compa-

<table>
<thead>
<tr>
<th>Variable</th>
<th>NAFLD</th>
<th>MASLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>42.4% (3,192/7,529)</td>
<td>N/A</td>
</tr>
<tr>
<td>Age</td>
<td>54 (47–63)</td>
<td>55 (48–64)</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>925/2,267 (29.0%/71.0%)</td>
<td>841/2,156 (28.0%/72.0%)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.5 (22.6–26.7)</td>
<td>24.8 (22.9–26.9)</td>
</tr>
<tr>
<td>Smoking habit (yes/no)</td>
<td>24.4%/75.6% (780/2,412)</td>
<td>24.3%/75.7% (727/2,270)</td>
</tr>
<tr>
<td>Pack years</td>
<td>0 (0–10)</td>
<td>0 (0–10)</td>
</tr>
<tr>
<td>Alcohol intake habit (none/yes)</td>
<td>56.1%/43.9% (1,792/1,400)</td>
<td>56.0/44.0% (1,678/1,319)</td>
</tr>
<tr>
<td>Spirometric values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC (L)</td>
<td>3.76 (3.11–4.33)</td>
<td>3.75 (3.11–4.32)</td>
</tr>
<tr>
<td>%VC</td>
<td>103.4 (94.6–113.2)</td>
<td>103.4 (94.6–113.2)</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.74 (3.08–4.32)</td>
<td>3.74 (3.08–4.31)</td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td>2.90 (2.41–3.41)</td>
<td>2.90 (2.40–3.40)</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>79.3 (75.0–82.7)</td>
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<tr>
<td>% FEV1</td>
<td>96.2 (87.3–105.3)</td>
<td>96.1 (87.1–105.2)</td>
</tr>
<tr>
<td>PEF at 25% (L)</td>
<td>0.9 (0.64–1.26)</td>
<td>0.89 (0.63–1.24)</td>
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<tr>
<td>PEF at 50% (L)</td>
<td>3.38 (2.54–4.28)</td>
<td>3.37 (2.53–4.28)</td>
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<tr>
<td>FEV1/FVC &lt;70% (yes/no)</td>
<td>7.9%/92.1% (253/2,939)</td>
<td>N/A</td>
</tr>
<tr>
<td>% FEV1 &lt;80% (yes/no)</td>
<td>11.8%/88.2% (377/2,815)</td>
<td>12.2%/87.8% (366/2,631)</td>
</tr>
</tbody>
</table>

**Abbreviations:**
NAFLD, non-alcoholic fatty liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease; IQR, interquartile range; VC, vital capacity; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; PEF, peak expiratory flow.
rable in both groups, indicating that the risk of restrictive pulmonary disease is not markedly elevated in either condition. Peak expiratory flow at 25% and 50% (PEF at 25% and PEF at 50%) are metrics assessing respiratory function during the middle of the exhalation process. Similar values in both NAFLD and MASLD cohorts suggest no significant specific impairment in respiratory function. Moreover, the presence of forced expiratory volume in 1 second/forced vital capacity (FEV1/FVC) <70% is a criterion for the diagnosis of COPD. This value was almost the same in both NAFLD and MASLD, 7.9% and 8.0%, respectively. In a large-scale cohort in Japan, the prevalence of COPD in individuals over 40 years of age has been reported to be 10.9%. Additionally, the National Health and Nutrition Examination Survey in South Korea also reported a COPD prevalence of 10.0% among patients with NAFLD. These results support the credibility of our health check-up data cohort.

In conclusion, the research evidence on respiratory dysfunction and COPD obtained in NAFLD could be applied to MASLD. The finding that one in ten patients with MASLD suffers from COPD, especially considering the widespread prevalence of MASLD, suggests that we should pay close attention to the potential for COPD in patients with MASLD.

Authors’ contributions
Conceptualization, T.T., and T.K.; methodology, T.T., and M.K.; software, D.N., and M.K.; validation, D.N., and M.K.; investigation, T.T., and T.K.; data curation, T.T.; writing—original draft preparation, T.T., T.K.; writing—review and editing, D.N. and M.K.; visualization, T.T.; supervision, H.T. and T.K.; project administration, T.T.; funding acquisition, T.T. and T.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

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

REFERENCES
Letter to the Editor

Letter regarding “Hepatitis B core-related antigen dynamics and risk of subsequent clinical relapses after nucleos(t)ide analog cessation”

Yun-Fan Liaw
Liver Research Unit, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taipei, Taiwan

Keywords: End-of-therapy; Quantitative HBsAg; HBV DNA

Dear Editor,

The multicenter cohort study conducted by Tsai et al. showed that hepatitis B core-related antigen (HBcrAg) <10^3 U/mL was significantly associated with lower clinical relapse (CR) and that time-varying HBcrAg level was a risk factor for subsequent CR following the cessation of nucleos(t)ide analogue (Nuc). The study also showed that only 3.5% of 203 patients experienced bilirubin elevation >2 mg/dL, and all fully recovered after retreatment, hence confirmed that discontinuing Nuc in HBV-suppressed patients is reasonably safe. However, there are several major points that require clarification and/or further discussion.

Multivariable analysis showed that the time-varying HBcrAg level was a significant factor for subsequent CR. However, Figure 5 presents a seemingly contradictory observation, depicting a cumulative CR incidence that remains comparable among patients with different HBcrAg kinetics. It suggests the necessity for a precise definition of “time-varying HBcrAg level” to reconcile this controversy. Their findings that there is no discernible difference in CR rate among patients with different patterns of HBcrAg changes over time contradicts their implication that dynamic serum HBcrAg measurement would be informative/helpful for off-Nuc monitoring.

Multivariable analysis also showed that the end-of-therapy (EOT) quantitative HBsAg (qHBsAg) level was a significant factor associated with the risk of CR, whereas EOT HBcrAg and time-varying HBsAg levels did not exhibit such associations. Together with the controversial results of HBcrAg in point 1, highly sensitive assays for HBsAg (HBsAg-HQ) and HBcrAg (iTACT-HBcrAg) could potentially exert an impact on the study results.

The results from the multivariable analysis have confirmed the findings of an earlier head-to-head comparison, demonstrating that HBcrAg was not a predictive factor for off-Nuc CR. Recalculation of their reported data, including Supplementary Figure 3, showed that the 1-year CR rate was 22.2% (12 out of 54) in patients with qHBsAg <10^2 IU/mL, which was nearly half of the 39.8% (39 out of 98) observed in those with HBcrAg <10^3 U/mL. These findings suggest that qHBsAg <10^2 IU/mL is more predictive than HBcrAg <10^3 U/mL for 1-year

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Editor: Han Ah Lee, Chung-Ang University College of Medicine, Korea
Received: Jan. 24, 2024 / Revised: Jan. 29, 2024 / Accepted: Jan. 31, 2024
CR following Nuc cessation. The present study could further engage in a head-to-head comparison to assess the predictive ability of different EOT qHBsAg and HBcrAg levels for off-Nuc CR. Furthermore, in contrast to the minimal fluctuation of HBcrAg during off-Nuc follow-up, qHBsAg decline was reported to be significantly accelerating from \(-0.095 \log_{10} \text{IU/mL per year during Nuc therapy to } -0.116 \log_{10} \text{IU/mL per year post-Nuc cessation}\). It would be more informative if the cumulative incidence over time was compared among patients with different EOT qHBsAg levels.

HBsAg loss, a hallmark of functional cure, is the main justification for the strategy of finite Nuc therapy.\(^5,6\) The duration of follow-up (16.7–67.1 months) of this study is sufficiently long to yield the cumulative incidence of HBsAg loss in this cohort, particularly among those who remained un-retreated, to test the predictive ability of HBcrAg for HBsAg loss.

Univariable analysis showed a hazard ratio (HR) of 1.36 (95% CI 1.15–1.61) for time-varying HBV DNA levels. The HR is almost identical to 1.36 (95% CI 1.14–1.63) for the time-varying HBcrAg level. This should be included in the multivariable analysis to provide an adjusted HR. Furthermore, it has been shown that off-Nuc HBV DNA levels >2,000 IU/mL during off-Nuc follow-up may predict subsequent CR.\(^7\) This factor should be considered or compared in the analysis. Additionally, the Asian-Pacific stopping rule recommended HBV DNA assay every 3 months in the first year, and more frequent monitoring for CR if virologic relapse (>2,000 IU/mL) was detected.\(^7\) As such, it appears HBcrAg may not play a complimentary role during off-Nuc follow-up.

In conclusion, EOT qHBsAg <10\(^2\) IU/mL is a superior predictor for CR compared to HBcrAg <10\(^2\) U/mL, at least in the first year post-Nuc cessation. Their findings do not support the conclusion that the dynamic HBcrAg measurement after Nuc cessation was more accurate than the dynamic HBsAg levels in the prediction of CR. Further studies on the role of HBcrAg in patients with low EOT qHBsAg levels or in combination with other biomarkers, such as HBV-RNA,\(^9\) are warranted. Finally, cost is an important concern in clinical practice. In our hospital, the cost of a qHBsAg assays is less expensive than that of HBcrAg assays and only less than 25% of the cost of HBV DNA assays.\(^10\) Unless the clinical utility of HBcrAg has a significant advantage over qHBsAg, it appears more advisable to utilize qHBsAg during and/or after antiviral therapy.

**Acknowledgements**

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**Conflicts of Interest**

The authors have no conflicts to disclose.

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**Abbreviations:**

CR, clinical relapse; EOT, end-of-therapy; HBcrAg, hepatitis B core-related antigen; HR, hazard ratio; Nuc, nucleos(t)ide analogue; qHBsAg, quantitative HBsAg
Aliment Pharmacol Ther 2022;56:310-320.
Dear Editor,

We would like to express our gratitude to Cheng and Yu for their insightful comments on our study. Tenofovir disoproxil fumarate (TDF) has been associated with lipid-lowering effects in chronic hepatitis B (CHB) patients. However, after switching to tenofovir alafenamide (TAF) from TDF, lipid profiles showed changes, including gradual increases in total cholesterol, low-density lipoprotein, and triglycerides, and a progressive decrease in high-density lipoprotein. Another study showed that switching from TDF to TAF was associated with weight gain, derangements of lipid profile, and increased insulin resistance in patients with CHB. The underlying mechanisms behind these differing effects remain elusive. Therefore, concerns have arisen regarding the long-term cardiovascular (CV) risk implications of these changes, motivating our study.

Cheng and Yu note that, while the association between long-term TDF or TAF treatment and changes in lipid profiles may contribute to CV risk to some extent, traditional CV risk factors such as smoking, hypertension, diabetes, and steatotic liver disease remain paramount determinants, as demonstrated in our study. We completely agree with this notion. Nevertheless, evaluating the long-term CV risk between the two treatments in CHB patients is essential due to the lack of conclusive evidence from previous studies, which primarily focused on surrogate markers rather than hard clinical outcomes, which was the primary outcome of our study.

As Cheng and Yu pointed out, it might be premature to conclude whether the CV risk in patients treated with either TDF or TAF is “beneficial”, “neutral”, or “detrimental” based on limited evidence. In particular, without knowing the exact underlying mechanism for outcomes, drawing firm conclusions may be challenging. However, a Korean study demonstrated that TAF treatment did not lead to significant changes in lipid profiles when compared to untreated patients with CHB and non-CHB patients, suggesting that TAF may not exacerbate lipid profiles. Additionally, in our study, we com-
pared total cholesterol levels in patients on TAF to untreated patients with CHB, revealing no significant difference between the two groups. This may indicate that concerns about changes in lipid profiles when switching from TDF (known for its lipid-lowering effects) to TAF (considered lipid-neutral) may be somewhat exaggerated, despite lipid profiles beginning to return to their baseline.

While acknowledging the necessity of long-term TDF or TAF use, controlling metabolic risk factors, such as steatotic liver disease, is crucial for reducing liver-related morbidity and mortality in these patients. Additionally, further research is needed to elucidate the underlying mechanisms driving differential metabolic impacts between the two treatments, emphasizing the importance of well-designed prospective studies to validate our findings.

Authors’ contribution

H Hong and J Choi were responsible for the acquisition, analysis, and interpretation of data, and statistical analysis. H Hong and J Choi drafted and approved the manuscript.

Acknowledgements

This study was supported by grants from the National Research Foundation of Korea (NRF) funded by the Korean government (Ministry of Science and ICT) (No. 2021R1G1A1009506). The interpretation and reporting of the data were the sole responsibility of the authors.

Conflicts of Interest

J Choi has received a research grant from Gilead Sciences. H Hong has nothing to disclose.

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Abbreviations:

TDF, tenofovir disoproxil fumarate; CHB, chronic hepatitis B; TAF, tenofovir alafenamide; CV, cardiovascular
Correspondence

Correspondence on Letter regarding “Toward hepatitis C virus elimination using artificial intelligence”

Ming-Ying Lu12 and Ming-Lung Yu123

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Keywords: HCV; DAA; AI; SDG 3

Dear Editor,

We are grateful for the comments1 from Professor Hur and Professor Lee on our recent publication. We developed artificial intelligence (AI) models for predicting direct-acting antiviral agents (DAAs) failure among a large chronic hepatitis C (HCV) cohort.2 In patients with decompensated liver cirrhosis, the AI model is beneficial in determining the optimal timing for the initiation of DAA therapy. We agreed that more intensive antiviral therapy beyond the current guidelines may be considered for HCV patients who are susceptible to DAA failure.

Due to the presence of overfitting in the training dataset, the current AI model needs further optimization to improve its generalizability. We have ever tried hyperparameter tuning and simplifying the input features through dimensional reduction to avoid overfitting. In such an imbalanced dataset, it is a challenge to maintain the accuracy of the AI model and avoid overfitting. Unsolved overfitting may imply there are unidentified risk factors regarding treatment response. Our study only incorporated 55 clinical host and virologic features before and after treatment in the current model. The diversity of host genetics, cytokine dynamics, immunity, metabolism, baseline or treatment-emergent resistance-associated substitutions of HCV, etc. may simultaneously affect the efficacy of DAA.3-6 A combination of multi-omics in the AI model may enhance the predictive accuracy of the validation datasets in the future. Furthermore, all the subjects were enrolled from a single ethnic population. It is necessary to validate this AI model in independent cohorts of various ethnicities. Seeking opportunities for international research collaboration to verify and optimize this AI model is mandatory.

Authors’ contribution
MY LU drafted the manuscript. ML Yu reviewed and finalized the manuscript.

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Conflicts of Interest

MY Lu has no conflicts to disclose. ML Yu disclosed the following: research grant from Abbvie, Gilead, Merck, and Roche diagnostics; consultant for Abbvie, BMS, Gilead, Roche, and Roche diagnostics; and speaker for Abbvie, BMS, Eisai, Gilead, Roche, and Roche diagnostics.

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Abbreviations:

AI, artificial intelligence; DAAs, direct-acting antiviral agents; HCV, hepatitis C virus

http://www.e-cmh.org https://doi.org/10.3350/cmh.2024.0152
Optimizing off-treatment outcome predictions: The potential of time-varying HBcrAg and the need for more research

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Keywords: Chronic hepatitis B; Antiviral treatment; Hepatitis B core-related antigen

Dear Editor,

We sincerely appreciate the editorial by Drs. Huang and Tseng,1 commenting on our recent study published in Clinical and Molecular Hepatology2 regarding the role of time-varying hepatitis B core-related antigen (HBcrAg) in predicting clinical relapse (CR) for chronic hepatitis B (CHB) patients who discontinued tenofovir or entecavir. We agree that nucleos(t)ide analogue (NA) cessation is generally safe with hepatitis B surface antigen (HBsAg) seroclearance,3 which is the only treatment endpoint widely acceptable across guidelines.4,5 Unfortunately, it rarely occurs during long-term NA therapy.6 Treatment discontinuation without first achieving HBsAg seroclearance invariably leads to recurrence of viremia and could precipitate hepatitis flare and even life-threatening acute on chronic liver failure.7 The strategy of finite NA therapy, therefore, cannot be practiced without thorough consideration of the conceivable benefits and potential risks for an individual patient.

Substantial research efforts have been devoted to identify suitable candidates for finite NA therapy. Nevertheless, most predictors reported in the literature were host factors or viral biomarkers at or prior to treatment cessation.3 Little is known about the risk assessment during the posttreatment monitoring. The risk could vary over time and the assessment may need to change accordingly to inform clinical management.

Our study demonstrated for the first time that serum HBcrAg levels as a time-varying predictor was more accurate than a fixed value in stratifying the risks of CR. This finding was further affirmed by multiple variable analyses adjusted for the serum level of HBsAg measured at treatment cessation, which was a validated biomarker associated with CR risks. Accordingly, our data suggest the most recent HBcrAg level is more predictive than a previous measurement to guide posttreatment monitoring. For the convenience of clinical application, the cutoff may be set at 1,000 U/mL to
stratify distinct risks of CR.

Serum HBcrAg can only come from the covalently closed circular DNA (cccDNA) of HBV, in contrast to HBsAg which may also be produced via transcription of fragments of viral DNA integrated to host genome, particularly in the setting of long-term NA therapy. This feature distinguishes the two circulatory viral markers with great implications for clinical utility. A growing body of literature has shown the potential of applying HBcrAg measurement in clinical practice as it may help predict HBeAg seroconversion, durability of NA therapy response, as well as the occurrence and recurrence of hepatocellular carcinoma. In fact, prior studies including our own work, have shown that serum HBcrAg level at treatment cessation was useful to predict off-therapy relapses. Our current study further expanded the potential utility of this biomarker, suggesting that serum HBcrAg not only be measured at treatment cessation but also during posttreatment follow-up.

As the editorialists point out, however, the commercial assay employed in the current study was not a sensitive one and unable to quantify serum HBcrAg levels below 1,000 U/mL. The current assay might fail to detect subtle fluctuations of serum HBcrAg after NA cessation. Consequently, it is not possible to appreciate the variability among HBcrAg levels lower than the detection limit and evaluate how it might correlate with clinical outcomes. This limitation could substantially compromise the usefulness of this biomarker particularly when more than half the patients in our study cohort had a serum HBcrAg level unquantifiable by the present assay. Although it is reasonable to classify patients with a HBcrAg level below 1,000 U/mL into a low-risk group, whether they can be more precisely risk stratified was unclear. Therefore, we agree with the editorialists that further research using more sensitive assay, such as the iTACT-HBcrAg, is needed to harness the full potential of HBcrAg measurement in clinical application.

We also concur with the editorialist’s insightful comment highlighting the importance of frequent HBV DNA measurement in the posttreatment monitoring. According to the updated Asian Pacific Association for the Study of the Liver (APASL) guidance, serum HBV DNA should be measured monthly for the initial 3 months after NA cessation, followed by bi-monthly or tri-monthly measurement, if viremia remains undetected. This intensive early monitoring aims to promptly identify emerging viral breakthrough and early forecasts imminent hepatitis flares or even severe acute exacerbations.

We did not propose to replace HBV DNA by HBcrAg in the management of patients who stopped NA therapy. In our opinion, these two biomarkers play different yet complementary roles during the posttreatment monitoring. Nevertheless, we acknowledge that it was beyond the scope of the present study to investigate how the various predictors can be combined to optimize posttreatment monitoring of patients discontinuing NA therapy. More research is clearly warranted.

In summary, our study demonstrated the clinical relevance of measuring serum HBcrAg during the follow-up of patients who stop NA therapy, and found that the most recent level was more accurate than a previous measurement to predict CR. Although the commercial assay lacks the sensitivity to detect nuances of serum HBcrAg below the lower limit of detection ranges, a HBcrAg level below 1,000 U/mL may still serve as a clinically useful cutoff for predicting CR. A more sensitive assays with a broader range of detection may improve the clinical utility of HBcrAg and refine the risk assessment based on its dynamic measurement. Finally, it remains largely unknown how to incorporate various risk predictor for optimizing posttreatment monitoring. Our novel findings should call for further studies.

Authors’ contribution
Manuscript drafting: Ying-Nan Tsai, Yao-Chun Hsu. Manuscript edition and final approval: all authors.

Conflicts of Interest
Ying-Nan Tsai reported no conflicts of interest. Jia-Ling Wu reported no conflicts of interest. Yao-Chun Hsu has received research grants from Gilead Sciences, lecture fees from Abbvie, Bristol-Myers Squibb, Gilead Sciences, and Novartis, and has served as an advisory committee member for Gilead Sciences.

Abbreviations:
CHB, chronic hepatitis B; CR, clinical relapse; EOT, end of treatment; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; NA, nucleos(t)ide analogue

http://www.e-cmh.org https://doi.org/10.3350/cmh.2024.0220
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Both liver parenchymal and non-parenchymal cells express JCAD protein under various circumstances

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Keywords: Junctional protein associated with coronary artery disease (JCAD); Hepatocytes; Hepatic stellate cells; Bile epithelial cells; Liver sinusoidal endothelial cells

Dear Editor,

In Volume 30, Issue 2 of Clin Mol Hepatol, Dr. Byoung Kuk Jang from the Department of Internal Medicine, Keimyung University School of Medicine, Daegu, Korea, provides an editorial¹ to summarize the major findings of our publication in the same issue.² All authors appreciate the positive comments to this original article. Regarding junctional protein associated with coronary artery disease (JCAD) expression in other cell types involved in cholestatic insults, the authors would provide additional evidence to clarify this concern.

As demonstrated in the publication, JCAD is expressed in hepatic stellate cells (HSCs), which are the critical effector cell type for cholestatic fibrosis through the Hippo-YAP signaling pathway. Bile epithelial cells (BECs) are often damaged, and the remaining cells may proliferate to respond to cholangioyte injury and develop bile duct reactions. This repair process is essential for the maintenance of bile duct integrity. Proliferative bile duct cells may release cytokines and other intermediates to portal fibroblasts and HSCs to coordinate the repair process. Therefore, it is of great interest to investigate whether JCAD participates in the proliferation of BECs. In serial sections of primary biliary cholangitis (PBC), immunohistochemical staining shows that bile duct epithelial cells are CK-19-positive, and JCAD is also positive in the structure of newly formed bile ducts in the same location. Moreover, transcription factor YAP is positive in some bile duct structures. In contrast, very faint staining in the portal triads was visualized in the control sections (Fig. 1). This piece of preliminary evidence demonstrates that JCAD is highly expressed in reactive bile duct epithelial cells and presumably may contribute to their proliferative response through the same underlying mechanism. In fact, the author’s team has acquired other preliminary data to support that CK-19-positive cells are overlapped with JCAD-positive cells in mouse models of cholestatic insults in an on-going project. In addition, JCAD

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was co-localized with F-actin in bile canaliculi in regenerative mouse liver, which implies that JCAD functions as a conjunction protein critical for the formulation of tight conjunction between hepatocytes. As a conjunction protein, it is unsurprising to demonstrate that JCAD is positive for liver sinusoidal endothelial cells (LSEC) in an on-going project. Whether inflammatory cells, such as Kupffer cells, macrophages or lymphocytes, express JCAD needs further investigation. In summary, so far, the author’s team has demonstrated that JCAD is expressed in parenchymal hepatocytes, non-parenchymal bile epithelial cells and hepatic stellate cells under different conditions and will further investigate its role in various modes of chronic injury and repair processes. Hopefully, in-depth investigation of this novel protein would facilitate the development of new molecular therapeutics for chronic injury, which may advance to hepatic fibrosis, end-stage liver disease, and malignancies.

Authors’ contribution
Li Xie, Li Zhang, Hui Chen and Yong-Yu Yang are responsible for data acquirement, and manuscript preparation. Jian Wu is for concept synthesis, manuscript preparation and finalization.

Conflicts of Interest
All authors declare that no conflict of interest is involved in participation or contribution to the present work.

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Abbreviations:
JCAD, junctional protein associated with coronary artery disease; HSCs, hepatic stellate cells; BECs, bile epithelial cells; LSEC, liver sinusoidal endothelial cells; PBC, primary biliary cholangitis; YAP, Yes-associated protein
Correspondence on Letter regarding “Prognosis of biopsy-confirmed MASLD: A sub-analysis of the CLIONE study”

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Keywords: Cardiovascular disease; Liver biopsy; Cardiometabolic criteria

Dear Editor,

We thank Joo Hyun Oh and Dae Won Jun for commenting on our recent publication.¹ Their Letter comprehensively describes our analysis of liver biopsy–confirmed nonalcoholic fatty liver disease (NAFLD) patients in the Clinical Outcome Nonalcoholic Fatty Liver Disease (CLIONE) cohort² divided into metabolic dysfunction-associated steatotic liver disease (MASLD) and non-MASLD groups based on the presence or absence of cardiometabolic criteria (CM).³ We would like to focus our response on the following three issues raised by the authors:

Is there an increased incidence of cardiovascular disease (CVD) and/or mortality in individuals with MASLD compared to the general population?

The Suita study is a prospective cohort study of 6,475 patients aged 30–84 years in Japan. During a median observation period of 16.6 years, 590 patients developed CVD, 346 of whom had stroke events and 244 who had coronary heart disease.⁴ Within this cohort, female MASLD patients (Fatty Liver Index ≥60) had an elevated risk of CVD and stroke, with hazard ratios and 95% confidence intervals of 1.69 (1.16, 2.46) and 2.06 (1.31, 3.24), respectively.⁴ No corresponding associations were detected in men. Leung et al.⁵ reported the incidence of CVD in an Asian cohort of 307 liver biopsy–confirmed MASLD patients. These individuals were observed for a median of 49 months, with CVD noted in 23 cases and stroke occurring in eight patients. Most recently, Le et al. performed a meta-analysis of 79 articles and analyzed clinical...
events in 1,377,466 adult patients with NAFLD (MASLD). They found that the incidence rate per 1,000 person–years for CVD-related mortality and incidence of CVD were 4.53 and 24.77, respectively. Importantly, significantly higher rates of both CVD events and mortality were observed in North America and Europe than in Western Pacific/Southeast Asia. However, as the authors point out, it was not possible to determine whether MASLD patients had a clearly higher incidence or fatality rate of CVD than non-MASLD patients. Moreover, although a sub-analysis of the Le et al. paper showed no sex-associated differences in the incidence of CVD or mortality, it is true that there are conflicting data, such as the Suita study. Thus, we cannot answer the question definitively, and future studies with larger cohorts in Asia are needed to resolve this issue.

What are the differences in clinical background between Western and Eastern liver biopsy–confirmed MASLD patients?

The Nonalcoholic Steatohepatitis Clinical Research Network (NASH–CRN) cohort is a prospective study of 1,773 liver biopsy–confirmed MASLD patients followed up for a median of 4 years. In contrast, the CLIONE is a retrospective observation study, representing a major difference between the two studies. Comparing the two cohorts, we note that age was similar for participants of NASH–CRN and CLIONE (mean: 52 vs. 54.5 years), although the proportion of women was slightly higher in NASH–CRN (64% vs. 54%). Participants in CLIONE were 100% Asian, whereas those in NASH–CRN were 85% White and 12% Hispanic. The prevalence of both diabetes (42% vs. 36%) and hypertension (61% vs. 42%) was higher in NASH–CRN than in CLIONE. Histologically, the prevalence of advanced fibrosis (stages 3 and 4) was higher in the NASH–CRN than in the CLIONE (30% vs. 16%) cohort. Similarly, the prevalence of cirrhosis (stage 4) was also higher in NASH–CRN (9% vs. 2%). When limiting the comparison of patient background to those with stage 4 disease, age (57 vs. 62 years) and percentage of women (74% vs. 81%) were lower in NASH–CRN than in CLIONE. In contrast, platelet count and body mass index (BMI) were higher in participants of NASH–CRN than those in CLIONE (platelet count, 154,000 vs. 123,000 μL; BMI, 35 vs. 30 kg/m²). We believe that the reason stage 4 platelet count was lower in CLIONE than in NASH–CRN participants is because the diagnosis of stage 4 disease was made more rigorously in CLIONE. Focusing on cardiovascular events, the incidence of cardiac and vascular events was higher in NASH–CRN than in CLIONE (cardiovascular disease, 0.83 vs. 0.57; cerebrovascular disease, 0.40 vs. 0.27). Thus, as we can see, the NASH–CRN and CLIONE cohorts have different clinical backgrounds. One reason for these discrepancies may be that liver biopsy is still the gold standard for diagnosis in Japanese guidelines, whereas Western guidelines do not require this method.

What is the most important combination of CM in MASLD?

In the CLIONE cohort, we have found that steatotic liver disease (SLD) without the presence of CM is a mild condition, and no severe outcomes, such as carcinogenesis or cardiovascular events, occurred in this patient group during the observation period. However, the answer to the question of which specific CM most affect outcomes is more unclear. In our analysis, CM had no effect on long-term prognosis, including mortality or the incidence of liver-related events, when the cumulative number of risk factors was divided into low (0–2) and high (3–5) groups. However, each of the CM is quite broad, including a range of severities, and it is unclear whether meeting the criteria really has an impact on severe outcomes of SLD. Additionally, we understand that the reason why CM do not include Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) or high-sensitivity C-reactive protein (Hs-CRP) is to facilitate the identification of SLD for more clinicians around the world. However, given that there is currently no universally accepted biomarker of chronic in-

Abbreviations:
NAFLD, nonalcoholic fatty liver disease; CLIONE, Clinical Outcome Nonalcoholic Fatty Liver Disease; MASLD, metabolic dysfunction-associated steatotic liver disease; CVD, cardiovascular disease; NASH–CRN, Nonalcoholic Steatohepatitis Clinical Research Network; BMI, body mass index; SLD, steatotic liver disease; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; Hs-CRP, high-sensitivity C-reactive protein; SGLT-2i, sodium-glucose cotransporter-2 inhibitors; GLP-1RA, glucagon-like-peptide-1 receptor agonists
flammation in SLD, further research on the usefulness of indicators, including HOMA-IR and Hs-CRP, for diagnosis of MASLD pathophysiology is needed.

Recent studies have demonstrated the usefulness of the diabetes mellitus drugs sodium-glucose cotransporter-2 inhibitors (SGLT-2i) and glucagon-like peptide-1 receptor agonists (GLP-1RA) for treatment of MASLD. Thus, their potential as therapeutic agents is expanding and in actual clinical practice, there are many MASH/MASLD patients already taking SGLT-2i and GLP-1RA. In addition, the United States Food and Drug Administration recently approved the first oral thyroid hormone receptor-beta selective agonist for MASH/MASLD treatment. The world is therefore entering an era in which we will likely see a cure for MASH/MASLD. Moreover, with continuing advances in noninvasive diagnostic methods, we expect the need for liver biopsy to decrease in the future. In this historical context, the importance of the liver biopsy cohort may continue as an assessment of patients not taking oral therapeutic drugs.

Conflicts of Interest
The authors have no conflicts to disclose.

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In response to: Steatotic liver disease—know your enemies

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Keywords: NAFLD; Epidemiology

Dear Editor,

We thank Dr. Mak for her review of our manuscript on the incidence of adverse outcomes associated with nonalcoholic fatty liver disease (NAFLD) which is now known as metabolic dysfunction associated steatotic liver disease (MASLD).1,2 As noted in the editorial, the new terminology of MASLD was selected to align with the comorbidities most closely associated with this steatotic liver disease, in addition to recognizing the lack of alcohol use in certain cultures and in children. In addition to the change in terminology, the definition of MASLD was changed as well. Like NAFLD, MASLD requires steatosis to be present in ≥5% of hepatocytes and alcohol consumption to not be greater than 20 g for females and 30 g for males a day, in the absence of other liver disease associated with steatosis but in the presence of at least one cardiometabolic risk factor (CMR).3 As noted, the concordance between MASLD and NAFLD remains high even with the requirement for a CMR, which is promising given the vast amount of work that has already been completed to understand NAFLD and its burden.4,5

Interestingly, these same studies noted that the presence of being overweight or obese is by far the most common CMR among those with MASLD, as well as being a significant and independent predictor for having MASLD. However, it must be noted that the cutoff for meeting the body mass index (BMI) CMR is ≥25 kg/m² for non-Asians and ≥23.5 kg/m² for Asians, which may miss those that are lean and have not yet developed other comorbidities, especially those that are younger.3,4 None the less, being overweight or obese appears to play a large role in defining MASLD and its prevalence, and with the rising rates of obesity among children and adults as well as the co-occurring increasing rates of type 2 diabetes (T2D), we can expect MASLD to continue to increase, especially in the younger population.5,9 In fact, as highlighted in the editorial, we found that the age at which MASLD/NAFLD is being diagnosed is trending younger, which suggests that the adverse liver effects of obesity and T2D are already being seen in the increasing prevalence rates of NAFLD/MASLD. However, it is important to recognize that some of the increase in the number of cases could be due to the availability of non-invasive

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tests replacing the need for a liver biopsy. Despite this, the last forecasting model indicated that the prevalence of MASLD/NAFLD will be almost 60% by 2040 if nothing changes and that MASLD/NAFLD may no longer be considered just an older person’s disease.10

With these facts in mind, along with the current incidence of adverse outcomes described in our study, the burden of MASLD/NAFLD may quickly become overwhelming for the healthcare system. Fortunately, as noted above, there are better non-invasive tests available for the diagnosis of MASLD/NAFLD and high-risk MASLD/NAFLD (those at risk for fibrosis stage 2 or higher), which have enabled the development of risk stratification algorithms to help clinicians institute appropriate interventions.11

Although there are no direct pharmaceutical interventions yet for those with a more progressive disease, steatohepatitis (NASH/MASH), this position may soon change. In the meantime, diet and physical activity/exercise, which induce a weight loss of at least 5% to 10%, remain the cornerstones of treatment and have been better conceptualized to help clinicians intervene appropriately to assist in weight loss.12,13 New T2D medications (Sodium-Glucose Transport Protein 2 [SGLT2] inhibitors and glucagon-like peptide-1 receptor agonists [GLP-1RAs]) are also showing promise in not only controlling diabetes but also assisting in weight loss and have been reported to improve cardiovascular health.14 Bariatric surgery has also been shown to improve steatosis and maybe fibrosis if present and enough weight is lost.15 However, most of these therapies, outside of diet and exercise, are approved for adults and not children. Therefore, work must also continue on developing community interventions with community and school policy makers to create a healthy environment in which children can not only learn but be physically active and provided healthy food on a consistent basis. A recent study that reviewed the availability of resources for a healthy community using six domains (policies, guidelines, civil awareness, epidemiology and data, NAFLD detection, and NAFLD care management) found no country was well prepared to address the burden of MASLD/NAFLD.16

The lack of country preparedness is not surprising given that awareness of MASLD/NAFLD remains low.17 Another purpose for the renaming of NAFLD to MASLD was to address what was thought to be a main driver of low awareness—patients and clinicians perceived stigma associated with the words “non-alcoholic” and “fatty”—whereby their presence hindered patient and healthcare provider communication. However, a recent survey among patients with NAFLD and healthcare providers of patients with NAFLD found a discrepancy between what patients perceived as stigmatizing and what healthcare providers perceived. In fact, only a very small percent of patients reported that the use of the terms “fatty” and “non-alcoholic” was stigmatizing compared to a majority of healthcare providers who thought these words caused discomfort and stigma among their patients.18 As such, future research is needed to see if the name change will have helped to improve communication between patients and healthcare providers, with increased awareness as a result. Efforts must also continue to raise awareness of NAFLD/MASLD and its’ adverse outcomes, as outlined in our manuscript, among primary care physicians and endocrinologists where the majority of patients are seen.

Again, we thank Dr. Mak for her review, which brought attention to our work and allowed us the opportunity to expand the context of our work with the hope of bringing further awareness that MASLD/NAFLD is not a benign liver disease. As such, action is required among policymakers, healthcare providers, and the community to address this oncoming onslaught of liver disease, especially among the younger population.

**Authors’ contribution**

Michael H. Le: manuscript writing and editing. Linda Henry: manuscript writing and editing. Mindie H. Nguyen: manuscript writing and editing.

**Conflicts of Interest**

The authors have no conflicts to disclose.

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1. Mak LY. Steatotic Liver disease: Know your enemies. Clin Mol


Dear Editor,

We express our deep gratitude to Dr. Pirola and Dr. Sookoian for their interest in our research.\(^1\) The severity of metabolic-associated steatohepatitis liver disease (MASLD), which is characterized by the accumulation of fat in the liver, inflammation, and liver cell damage, is significant. If left untreated, MASLD can progress to more serious conditions, such as cirrhosis and liver cancer.\(^3\) This underscores the importance of disease detection and identifying pharmacological candidates with therapeutic potential. Various non-invasive biomarkers, obtained from serum and identified through imaging techniques, are employed to diagnose diseases and ascertain disease progression.\(^3\) However, in pursuit of more sensitive and precise diagnostics, omics-based biomarkers are rapidly gaining prominence in conjunction with these assessments.\(^5\)

Omics-based biomarkers for MASLD represent a compre-
hensive approach to understanding and diagnosing complex disease conditions by examining a vast array of biological molecules that reflect the state of a cell, tissue, or organism. In our previous study involving tissue samples from 134 patients with MASLD, we identified a set of genes (CAPG, HYAL3, WIP1, TREM2, SPP1, and RNASE6) that could collectively serve as a diagnostic panel to accurately distinguish MASLD progression through multi-omics analysis, including genomics, epigenomics, and transcriptomics.

We validated the effectiveness of the signature gene set in accurately differentiating discrete cohorts, revealing its substantial discriminatory capability. This gene set enables the stratification of subjects into severity-based subgroups within the examined populations. The ability to distinguish disease severity using signature genes is underpinned by the progressive elevation in their expression profiles, which correlates with the advancement of the disease state. Therefore, we propose that the signature gene set is better suited for assessing the overall severity of MASLD-associated diseases than for identifying a specific stage of MASLD.

Examining the applicability of a signature gene set identified by analyzing omics data obtained from patient tissues for non-invasive assessments is essential for transforming it into viable clinical biomarkers. We investigated whether the

**Figure 1.** Applications of the signature gene set discovered in tissues of patients with metabolic-associated steatohepatitis liver disease (MASLD) for developing non-invasive diagnostic markers. (A) Investigation of the expression levels of signature genes in sub-cell populations from peripheral blood mononuclear cells in patients with MASLD through scRNA-seq analysis. Dot plot describing the expression levels of features in each cell type. (B) The expression levels of signature genes in liquid biopsy samples from both healthy individuals and patients with hepatocellular carcinoma (HCC). (C) Identification of genes with variations and changes in expression in MASLD progression. Box plot showing the expression level of CLEC4M grouped into pathology (middle) and altered status (right). (D) Identification of genes with detected variations in both patient tissues and blood samples. Scatter plot showing the differentially expressed genes between steatosis and metabolic dysfunction-associated steatohepatitis (MASH). Blue dots denote MASH-downregulated genes, and red dots denote MASH-upregulated genes. The red dashed line represents the cutoff value (horizontal: *P*-value<0.05, vertical: |foldchange|>1.3). Pro-Myelocyte, progenitor myelocyte; Precursor B cell; NK, natural killer; MEP, megakaryocyte–erythroid progenitor cell; HSC, hematopoietic stem cell; GMP, granulocyte monocyte progenitors; DC, dendritic cells; CMP, common myeloid progenitors; BM, bone marrow. *P*-values are estimated using the Student’s t-test, *P*-value: <0.05, **<0.01, ***<0.001.

**Abbreviations:**
MASLD, metabolic-associated steatohepatitis liver disease; DC, dendritic cells; GMP, granulocyte monocyte progenitors; HCC, hepatocellular carcinoma; MASH, metabolic dysfunction-associated steatohepatitis; DMRs, differentially methylated regions; DEGs, differentially expressed genes
signature genes could also be applied to liquid biopsy samples from MASLD-associated disease by examining scRNA-seq data from blood samples of patients with MASLD (Fig. 1A). In our previous study, we had already established that the expression of signature genes was elevated in cell-free RNAs of blood from patients with MASLD compared to that in healthy individuals. Consistent with this, we found that, except for SPP1, the signature genes were also detectable in scRNA data from blood samples of patients with MASLD. Notably, they were predominantly found in the dendritic cells, granulocyte-monocyte progenitors, monocytes, and pre-B cells. Further, we observed that signature gene expression was discernible in blood samples from both patients with hepatocellular carcinoma (HCC) and healthy control individuals, with notable upregulation in the HCC cohort (Fig. 1B). These findings support the potential of the signature genes identified in the biopsied tissues to serve as viable biomarkers for clinical applications. Furthermore, genes such as SPP1, which are intrinsically implicated in diseased tissues, have significant potential as critical markers for diagnosis. However, their applicability as biomarkers for non-invasive assessments may present limitations. Consequently, entities unearthed from multi-omics analysis of patient-derived tissue samples should undergo rigorous subsequent validation phases for their efficacious deployment as clinical biomarkers.

Exploring MASLD-associated features, encompassing genomic variations, differentially methylated regions (DMRs), and differentially expressed genes (DEGs), from comprehensive omics datasets in previous studies requires thorough investigation. In a previous study, genomic variations, specifically somatic variations, were investigated, and approximately 5% of these variations (23/504 genes) were examined for changes in gene expression during disease progression (Fig. 1C). Among them, the gene expression levels of C-type lectin domain family 4 member M (CLEC4M), a gene involved in the immune response in the liver, substantially decreased in metabolic dysfunction-associated steatohepatitis compared to those in steatosis. Additionally, the decrease in gene expression was more pronounced in the altered group than in the unaltered group. Therefore, understanding the specific mechanisms and pathways of CLEC4M in MASLD is crucial for gaining insights into its potential as a therapeutic target or biomarker.

Building on this, we explored potential biomarkers by identifying genomic variations that were concurrently present in both the tissues and blood samples of patients with MASLD. Analysis of the WGS data obtained from tissue samples of patients revealed variants in approximately 5,700 genes, with 78% of these genes also present in the WGS data obtained from blood samples (Fig. 1D). Among these, 6% exhibited expression changes associated with MASLD progression. To establish a direct link between these variations and changes in gene expression, further detailed investigations, such as eQTL analyses, are necessary. Nonetheless, the significance of our previous study lies in providing a consolidated dataset from a singular cohort, which offers a unique resource with substantial value for further research.

In conclusion, advancements in sequencing technologies have revealed an extensive repertoire of genetic and epigenetic determinants associated with MASLD. Nonetheless, rigorous validation of the pathogenic roles of these entities is imperative to identify key elements that influence disease etiology and therapeutic intervention and to ensure continuous commitment to advancing research and improving patient outcomes. Selecting signature gene sets with potential as biomarkers from a vast pool of MASLD-associated features, as achieved in previous research, conserves time required for experimental validation while maximizing the understanding of pathogenic effects during MASLD progression.

Authors’ contribution
S.J. and S.Y. contributed to the WGS, total RNA-seq, and scRNA-seq analyses; J.H.P., Y-S.L., and K.H.Y. conceived and designed the study; S.J., S.Y., and K.H.Y. drafted the manuscript; all authors have read and approved the manuscript.

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Conflicts of Interest
The authors have no conflicts to disclose.
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Reply to: “Evaluation of the histological scoring systems of autoimmune hepatitis: A significant step towards the optimization of clinical diagnosis”

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Keywords: Autoimmune hepatitis; Histology; Diagnosis

Correspondence

Dear Editor,

We would like to thank Dr. Atsumasa Komori for his interest in our study entitled “Comparison of four histological scoring systems for autoimmune hepatitis (AIH) to improve diagnostic sensitivity” and for the kind comments.¹,²

As highlighted in the editorial, the histopathological diagnosis of AIH poses challenges. This is especially true in cases of acute presentation, because the classical features of AIH (dense lymphoplasmacytic portal infiltrates with moderate/severe interface hepatitis) are not always seen in such cases. Moreover, the degree of lobular necroinflammation may significantly outweigh that of interface hepatitis or portal lymphoplasmacytic infiltration, potentially leading to a completely different histopathological interpretation. In our study, combining the simplified International AIH Group scoring system (“2008 IAIHG”) with the newly proposed histological scoring systems either by Balitzer et al.³ (“2017 UCSF”) or by the International AIH Pathology Group (“2022 IAHPG”)⁴ enhanced the sensitivity in diagnosing AIH compared to using the 2008 IAIHG alone. Similar results were observed in the subgroup analysis of AIH cases with acute presentation. Thus, unlike the 2008 IAIHG criteria, where histological scores are assigned only if there is evidence of interface hepatitis, emperipolesis, and hepatocytic rosettes (“typical”), or at least a chronic hepatitis picture with lymphocytic infiltration (“compatible”), the 2017 UCSF and 2022 IAHPG systems encompass cases with lobular hepatitis patterns that are more frequently observed in AIH with acute presentation. However, it is noteworthy that although the presence of interface hepatitis and portal lymphoplasmacytic infiltration is not mandatory for diagnosing AIH according to the two recent systems, there is still a component of at least mild interface hepatitis in the lobular hepatitis-predominant acute AIH cases. Indeed, all cases in our study demonstrated at least a mild degree of interface hepatitis, including

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those with acute hepatitis patterns.

We would also like to highlight another diagnostic conundrum, which is the differentiation between AIH and drug-induced liver injury with AIH-like features (DI-AIH). The histological differences between AIH and DI-AIH remain poorly characterized. Recently, Alkashash et al. compared nine cases of AIH and six cases of DI-AIH and found a higher degree of portal and interface inflammation and more prominent plasma cell infiltration in AIH compared to DI-AIH, while central perivenular inflammation was present in both scenarios. In addition, fibrosis has been shown to be more common in AIH compared with DI-AIH. However, it should be noted that fibrosis may not be as prominent in acute AIH cases. Our study did not include a control group consisting of other etiologies, such as DI-AIH or chronic viral hepatitis. As clinically verified liver biopsy cases of DI-AIH are relatively rare, a multicenter or multinational study would be necessary to evaluate the histological differences between AIH and DI-AIH and the applicability of existing histological scoring systems for diagnosing DI-AIH.

Finally, clinicopathological correlation and active communication between the pathologist and hepatologist are crucial in optimizing the histological diagnosis of AIH. The current IAIHG systems include histology scores, suggesting that histology is pivotal in making a clinical diagnosis of AIH. For the pathologist, having access to clinical information, including laboratory findings and medication history, facilitates the histopathological interpretation of liver biopsies in this context.

Authors’ contribution
Original draft: H.K.; Critical revision: H.K., S-H.J.

Conflicts of Interest
The authors have no relevant financial or non-financial interests to disclose.

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Abbreviations:
AIH, autoimmune hepatitis; IAIHG, International Autoimmune Hepatitis Group; IAHPG, International Autoimmune Hepatitis Pathology Group
Harnessing hepatitis B core-related antigen measurement to optimize posttreatment monitoring

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Keywords: Hepatitis B core-related antigen; Hepatitis B surface antigen; Finite antiviral therapy; Time-dependent analysis

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Dear Editor,

We thank Professor Liaw for taking the time to comment on our study\textsuperscript{1,2}. His expertise in the field and insightful feedback allow us to clarify important aspects that may have been confusing or inadequately explained.

REGARDING THE ASSAY SENSITIVITY FOR HEPATITIS B CORE-RELATED ANTIGEN

We agree that the commercial assay used in this study was not sensitive enough to precisely quantify serum hepatitis B core-related antigen (HBcrAg) levels when the serum levels were lower than 1,000 U/mL. We were aware of this limitation and discussed it in our article. Additionally, our observation that serum HBcrAg levels only fluctuated mildly after nucleos(t)ide analogue (NA) cessation could be related to the insensitivity of the method of measurement. More sensitive assays, like the iTACT-HBcrAg,\textsuperscript{3} may uncover more subtle fluctuations in serum HBcrAg and thus reveal different patterns of posttreatment changes. Moving forward, utilizing or developing more sensitive HBcrAg assays will be crucial for fully leveraging this biomarker’s potential predictive utility across a wider range of values.

A TIME-VARYING VARIABLE IS DIFFERENT FROM DIFFERENCES BETWEEN TIME POINTS

While the assay might lack sensitivity at low HBcrAg levels for detecting subtle kinetic differences, this does not negate our major finding that the most recent HBcrAg measurement outperforms the end-of-treatment (EOT) value for predicting clinical relapse (CR). The similar CR incidence observed between patients with decreasing versus stable or increasing annual HBcrAg trajectories should not be misinterpreted as in conflict with the superiority of the most proximate HBcrAg.
level. As a matter of fact, modeling a time-varying explanatory variable is distinctly different from analyzing changes between repeated measurements over time, as time-varying variables preserve the exact data points with time indices. In short, our analysis demonstrates that the most recent HBcrAg value relative to CR is most predictive of risk, regardless of prior values. We are confident that this novel finding broadens our current understanding of the application of HBcrAg in optimizing the care of patients discontinuing NA therapy.

COMPARISONS TO QUANTITATIVE HEPATITIS B SURFACE ANTIGEN

Professor Liaw astutely observed that the proportion of CR in patients with quantitative hepatitis B antigen (qHBsAg) <100 IU/mL was numerically lower compared to those with HBcrAg <1,000 U/mL during off-therapy monitoring. Nonetheless, there was no significant difference in this comparison. In fact, our observation that the time-varying HBcrAg levels outperform the time-varying qHBsAg levels in predicting CR among relapse-naïve patients did not result from sporadic comparisons of this kind. Rather, our finding is supported by the results of a multivariable Cox proportional hazards model specifically examining the associations of these biomarkers with CR risk. Moreover, our study does not argue against the predictive value of EOT qHBsAg, which has been shown in prior studies, including our own.

Seroclearance of HBsAg may indicate a functional cure and is an acceptable endpoint for NA treatment. However, HBsAg loss occurs relatively infrequently compared to CR. Thus, larger samples are essential to exploring predictors of HBsAg seroclearance. In our study population, HBsAg loss was observed in 21 patients during post-treatment follow-up. Following Professor Liaw’s recommendation, we estimated a cumulative incidence of 19.9% (95% confidence interval: 12.8–25.6%) for HBsAg seroclearance at 8 years, with antiviral therapy resumption as a competing risk event to avoid overestimation. Investigating dynamic HBcrAg levels for predicting HBsAg loss compared to other biomarkers was beyond this study’s scope. Further dedicated research in larger cohorts is warranted to examine associations between fluctuating HBcrAg trajectories and subsequent HBsAg clearance or seroconversion after NA cessation.

EARLY AND INTENSIVE HBV DNA MEASUREMENT IS ESSENTIAL AFTER TREATMENT CESSATION

We appreciate Professor Liaw underscoring the importance of frequently monitoring serum HBV DNA levels after NA cessation. The updated guidance from the Asian Pacific Association for the Study of the Liver (APASL) recommends monthly screening for the first 3 months, then bi-monthly or tri-monthly in the year thereafter, should viremia remain undetected. This intensive early monitoring intends to swiftly identify emerging viral breakthrough, as rapidly rebounding viremia predicts impending hepatitis flares or even severe acute exacerbations.

Distinct roles of HBV DNA versus anti-Genemia in Posttreatment Monitoring

In our opinion, the role of HBcrAg or HBsAg levels versus HBV DNA is distinctly different for relapse prediction. First, HBV DNA levels are uniformly very low or practically undetectable when NA therapy ceases per eligibility guidelines. In contrast, antigen levels often remain detectable at variable levels despite meeting cessation criteria. Besides this, the rebound of viremia can rapidly spike from undetectable to extremely high levels within weeks, whereas HBcrAg and HB-

Abbreviations:
HBcrAg, hepatitis B core-related antigen; NA, nucleos(t)ide analogs; EOT, end of treatment; CR, clinical relapse; qHBsAg, quantitative hepatitis B antigen; HBsAg, hepatitis B surface antigen

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sAg fluctuate mildly after NA cessation in most patients.
Therefore, we posit that serum HBV DNA provides a forecast for imminent active hepatitis and is especially useful right after treatment cessation.

On the other hand, the levels of antigenemia may better assess the relapse risks in the long term.

In response to the comment, we performed a comparative analysis examining all three biomarkers as time-dependent variables along with their interaction terms. The time-varying HBcrAg level emerged as the sole factor independently associated with CR risk (Table 1), as compared to the time-varying HBsAg and HBV DNA levels, all of which were measured annually. However, this result should not be misinterpreted as indicating that HBcrAg could replace HBV DNA monitoring. Unlike HBsAg or HBcrAg, HBV DNA needs to be measured much more frequently than once a year especially within the first year after NA cessation. What our findings suggest is that including serum HBcrAg measurement may complement HBV DNA testing for optimized posttreatment monitoring of patients stopping antiviral treatment.

Further research should continue investigating how best to integrate both biomarkers clinically.

SUMMARY

In summary, while the HBcrAg assay limitations restrict detecting subtle kinetic fluctuations posttreatment, this does not refute that the most recent level outperforms the EOT measurement for CR forecasting, irrespective of prior trajectories. Still, high-sensitivity assays may uncover finer fluctuations and patterns, so further optimization is indispensable. Besides this, our multivariate modeling demonstrated an important outcome; larger studies are needed to investigate time-varying HBcrAg and its more predictive utility as compared to HBsAg.

High-sensitivity assays might uncover finer fluctuations and patterns, so further optimization is indispensable. Besides this, our multivariate modeling demonstrated an important outcome; larger studies are needed to investigate time-varying HBcrAg and its more predictive utility as compared to HBsAg.

HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; CI, confidence interval; HR, hazard ratio.

Table 1. The regression model including all three time-varying biomarkers

<table>
<thead>
<tr>
<th>Variables</th>
<th>Adjusted HR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-varying HBsAg level, log IU/mL</td>
<td>3.72</td>
<td>0.60–23.08</td>
<td>0.158</td>
</tr>
<tr>
<td>Time-varying HBcrAg level, log U/mL</td>
<td>5.14</td>
<td>1.78–14.86</td>
<td>0.003</td>
</tr>
<tr>
<td>Time-varying HBV DNA level, log IU/mL</td>
<td>2.09</td>
<td>0.71–6.16</td>
<td>0.182</td>
</tr>
<tr>
<td>Time-varying (HBsAg level*HBcrAg level), log U/mL</td>
<td>0.75</td>
<td>0.52–1.09</td>
<td>0.131</td>
</tr>
<tr>
<td>Time-varying (HBsAg level*HBV DNA level), log U/mL</td>
<td>0.93</td>
<td>0.65–1.33</td>
<td>0.681</td>
</tr>
<tr>
<td>Time-varying (HBcrAg level*HBV DNA level), log U/mL</td>
<td>0.92</td>
<td>0.78–1.08</td>
<td>0.303</td>
</tr>
</tbody>
</table>

The time-dependent Cox regression model was deliberately carried out to compare the time-varying levels of HBcrAg, HBsAg, and HBV DNA, all of which were measured annually, for the association with clinical relapse. These three variables and their interaction terms were entered in the model to demonstrate the comparison, regardless of the respective statistical significance. No other variables were examined.

HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; CI, confidence interval; HR, hazard ratio.
Authors’ contribution

Manuscript drafting: Ying-Nan Tsai, Yao-Chun Hsu. Manuscript edition and final approval: all authors.

Conflicts of Interest

Ying-Nan Tsai reported no conflicts of interest. Jia-Ling Wu reported no conflicts of interest. Yao-Chun Hsu has received research grants from Gilead Sciences, lecture fees from Abbvie, Bristol-Myers Squibb, Gilead Sciences, and Novartis, and has served as an advisory committee member for Gilead Sciences and Sysmex.

REFERENCES

Dear Editor,

I am grateful to Xie and his colleagues for providing additional new results to my editorial comments. The authors showed that junctional protein associated with coronary artery disease (JCAD) and YAP were positive in CK-19-positive cells known as bile duct epithelial cells (BECs) in newly formed bile ducts through immunohistochemical staining in liver tissue of primary biliary cholangitis patients. This result suggests that high expression of JCAD in reactive bile duct epithelial cells may be involved in the proliferative response of BECs. Additionally, although the authors did not show the results, they found co-localization of F-actin and JCAD in the bile canaliculi of the regenerative mouse liver, suggesting that JCAD may function as a binding protein important in forming tight junctions between hepatocytes. However, it is difficult to accurately determine its function simply by the level of expression in the tissue, so additional research will be needed to determine the role of JCAD expression in BECs in the future. The role of JCAD expression in hepatocytes was well demonstrated in a recently published paper by the authors. In a partial hepatectomy mouse model, JCAD deficiency was shown to cause delayed liver regeneration through the Hippo-Yap signaling pathway. Taken together, these results suggest that JCAD is expressed in various cells present in the liver and is involved in chronic injury and repair processes. However, on the one hand, increased expression of JCAD promotes the regeneration of hepatocytes and bile duct epithelial cells, but also promotes the progression of nonalcoholic steatohepatitis to hepatocellular carcinoma and activates hepatic stellate cells in cholestatic liver disease. However, since most diseases progress through an overlap of injury and repair, there are concerns that it may require more work to apply it directly for therapeutic purposes. Therefore, through further research on these issues in the future, we hope to accumulate knowledge about the mechanism and role of JCAD in various liver diseases and apply it to treatment.

Conflicts of Interest

The authors have no conflicts to disclose.

REFERENCES


Abbreviations:

JCAD, junctional protein associated with coronary artery disease; YAP, Yes-associated protein; BECs, bile epithelial cells
Immunopathogenesis of liver fibrosis in steatotic liver disease

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Keywords: Fibrosis; Hepatic stellate cells; Inflammation; Immunologic factors; Fatty acids
Recently, a new term, steatotic liver disease (SLD), has been introduced to the field of hepatology to encompass the various etiologies of steatosis.\(^3\) Hepatic steatosis, having an excessive fat accumulation, has been considered a key factor in the progression of serious liver diseases such as hepatic inflammation, fibrosis/cirrhosis, and hepatocellular carcinoma.\(^3\) It could be initiated by an overload of nutrition (e.g., glucose and fatty acids), alcohol and endocannabinoid-mediated metabolic dysfunction, or genetic factors.\(^3\) Interestingly, lines of evidence have suggested that beyond its role as a metabolic organ, the liver is considered an immunological and neurological organ due to its diverse metabolic functions, enriched immune cells (e.g., innate and adaptive immune cells), and production and release of hepato- transmitters such as glutamate or 2-arachidonoylglycerol (2-AG).\(^3\) So, these factors might affect the progression of SLD, from simple steatosis to steatohepatitis and fibrosis, by regulating the activation of hepatic stellate cells (HSCs).

It has been reported that inflammation and subsequent liver fibrosis occur due to various immune-mediated activations of HSCs through eating habit-mediated metabolic dysfunction (e.g., obesity, type 2 diabetes, or chronic alcohol consumption), sedentary behavior, and genetic predisposition.\(^4,5\) Steatohepatitis may be triggered by prolonged multiple stresses in fat-storing hepatocytes, such as endoplasmic reticulum (ER) stress, mitochondria dysfunction, and oxidative stress, including alcohol-induced reactive oxygen species (ROS) and lipotoxicity.\(^4,6\) These cellular stresses, combined with diverse harmful factors, such as inflammatory cytokines, adipokines, short-chain fatty acids, and pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) originated from adipose tissue (AT) and the intestine, may cause hepatocyte death and inflammation.\(^7\) Recently, an interesting study suggested glutamate-mediated cross-talk between AT and liver, in which hypoxic adipocytes excreted glutamate through the xCT transporter and the resulting production of interferon-γ (IFN-γ) in natural killer (NK) cells by metabotropic glutamate receptor 5 (mGluR5), subsequently leading to the augmentation of adipose macrophage activation and steatohepatitis in obese mice and patients.\(^8\)

Through these cellular responses and organ cross-talks, inflammatory immune cells migrate nearby damaged hepatocytes and stimulate the transformation of quiescent HSCs (qHSCs) into activated HSCs (aHSCs) by producing various mediators, including cytokines, chemokines, and extracellular vesicles. Injured fat-storing hepatocytes produce cytokines, such as C-C motif chemokine 2 (CCL2) and CXC motif chemokine ligand 1 (CXCL1), to recruit pro-inflammatory macrophages and neutrophils, respectively. After migration, macrophages, neutrophils, and resident Kupffer cells are stimulated by DAMPs (e.g., ATP, mitochondrial DNA, or double-stranded RNA), PAMPs (e.g., LPS, CpG DNA, Flagellin), or cytokines through specific receptors, including P2X purinoceptor 7 and toll-like receptors (e.g., TLR2, TLR3, TLR4, TLR5, and TLR9).\(^7\) Then, the production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, platelet derived growth factor (PDGF), IL-17A, and myeloperoxidase (MPO), transforming growth factor (TGF)-β1, and ROS by these cells leads to the activation of qHSCs.\(^9,10\) Moreover, several types of lymphocytes, such as Th17 cells, γδT cells, and B cells, augment HSC activation by producing IL-17, TNF-α, and IL-6.\(^9,11,12\)

In contrast, several types of cells, such as NK cells and restorative macrophages, can kill aHSCs or suppress HSC activation by producing death ligands, including TNF-related...

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**Abbreviations:**

SLD, steatotic liver disease; 2-AG, 2-arachidonoylglycerol; HSC, hepatic stellate cell; ER, endoplasmic reticulum; ROS, reactive oxygen species; PAMP or DAMP, pathogen- or damage-associated molecular pattern; AT, adipose tissue; IFN-γ, interferon-γ; NK, natural killer; mGluR5, metabotropic glutamate receptor 5; qHSC, quiescent HSCs; aHSC, activated HSCs; CCL2, C-C motif chemokine 2; CXCL1, CXC motif chemokine ligand 1; TLR, toll-like receptor; TNF, tumor necrosis factor; IL, interleukin; PDGF, platelet-derived growth factor; MPO, myeloperoxidase; TGF, transforming growth factor; TNF-related apoptosis-inducing ligand, TRAIL; Fas ligand, FasL; MMP, metalloproteinase; RAE-1, retinoic acid early inducible 1; SOCS1, suppressor of cell signaling 1

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apo"tosis-inducing ligand (TRAIL) and Fas ligand (FasL), IFN-γ, anti-inflammatory IL-10, and matrix metalloproteinases (MMPs). Once activated HSCs express retinoic acid early inducible 1 (RAE-1), a specific ligand for NKG2D, NK cells specifically produce IFN-γ, TRAIL, or FASL through the NKG2D-RAE-1 interaction. In addition, Ly6C<sup>low</sup>F4/80<sup>+</sup>CD11b<sup>+</sup> macrophages can induce fibrosis resolution by producing MMPs or Gr1<sup>+</sup>CD11b<sup>+</sup> bone marrow cells, which inhibit inflammation by IL-10 production in the early stage of liver fibrosis. However, prolonged injuries (e.g., chronic alcohol consumption) cause HSCs to have the ability to avoid this cytotoxicity by increasing TGF-β production and suppressor of cell signaling 1 (SOCS1) expression in aHSCs. Moreover, ROS might deplete NK subpopulations or suppress NK cytotoxicity in advanced liver fibrosis. A recent study suggested an interesting solution for how to overcome HSC evasion against NK cytotoxicity, in which mGluR5 activation in NK cells through the tail vein improved liver fibrosis in mice, suggesting a therapeutic intervention of liver fibrosis in patients.

In the past, SLD was somewhat neglected, but as viral hepatitis has recently been overcome, the possibility of SLD progression to serious chronic liver disease has emerged. Despite pro-inflammatory immune responses for SLD progression to liver fibrosis, it is noteworthy that certain immune cells (e.g., NK cells or restorative macrophages) may have strong anti-fibrotic functions. In particular, new therapeutics can be developed by carefully looking at intrahepatic neurological signaling (e.g., 2-AG production in HSCs and mGluR5 activation in NK cells). Therefore, precise molecular pathways related to the immunopathogenesis of liver fibrosis should be extensively investigated. Notably, further research will be needed to determine how the neuro-metabo-immune axis, still minimally investigated, influences SLD and liver fibrosis.

**Author’s contribution**

C.W. concept of the work, manuscript draft, figure generation. W.I.J. concept of the work, writing, revision and approval of the manuscript.

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**Conflicts of Interest**

The authors have no conflicts to disclose.

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Robust regimen with some forgiveness to non-adherence\(^{7,8}\)

1 tablet, once a day, with no food requirement\(^{2,6}\)

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2. EPCLUSA® Prescribing Information. Gilead Sciences Korea (2022. 2. 17.).
8. Cunningham EB et al. Adherence to sofosbuvir and velpatasvir among people with chronic hepatitis C in a large-scale 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.\(^{1}\) Consistent outcomes defined as SVR2 between 91% and 100% in all patient subpopulations.\(^{11}\)

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Our heartfelt wish for curing HBV, we present Vemlia.

- Comparable antiviral efficacy vs. TDF
- Improved safety profile in renal and bone parameters
- Increased affordability with lower price, 2,474/tablet
- Improved patients' compliance with daily pill bottle

   *The data above are clinical data conducted with Tenofovir alafenamide hemifumarate.
   *$95 won lower price than Original drug (June 2023)
Remarkable Response

The ORR was more than three times higher with lenvatinib versus control group.¹
Based on the masked IIR according to mRECIST, about 41% of patients* showed ≥ 30% decrease in tumor size.¹²

40.6%* Response Rate
(Masked IIR according to mRECIST)

Lenvatinib (n=478) Sorafenib (n=476)

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<th>Objective response (%), 95% CI</th>
<th>Effect size (95% CI)</th>
<th>P value</th>
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<td>Investigator response according to mRECIST</td>
<td>115 (24.1%, 20.2-27.9)</td>
<td>44 (9.2%, 6.6-11.8)</td>
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<td>Masked independent imaging response according to mRECIST</td>
<td>194 (40.6%, 36.2-45.0)</td>
<td>59 (12.4%, 9.4-15.4)</td>
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*ORR is one of the secondary endpoints and this is the result of the post-hoc exploratory tumor assessments using mRECIST by masked central independent imaging review. For more information, please refer to the full text of the article. (Kudo M, et al. 2018)

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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

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

Together for a better healthcare journey

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.

†Cure (sustained virologic response SVR12), defined as HCV RNA less than the lower limit of quantification at 12 weeks after the end of treatment. GT 1-6 treatment-naive, non-cirrhotic and compensated-cirrhotic patients. MAVIRET is not indicated in decompensated cirrhosis.

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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.

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Imported/Distributed: Miilat Co., Ltd. 6th Floor, Samhee Building, 427, Yeongdong-dong, Gangnam-gu, Seoul, Korea. Tel: (02) 3479-9100. www.abbv.ie.kr

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VEMLINO, 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

Restoration of Hepatic Mitochondrial Dysfunction by Carnitine Complex

Rapid Normalization of ALT Level

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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 25mg, Pyridoxine HCl 25mg, Riboflavin 0.5mg, Cyanocobalamin 0.125mg, Biphenyl dimethyl 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. 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. (P/T)

Storage: Tight 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, Nonalcoholic steatohepatitis
K77.0 Liver disorders in disease classified elsewhere
Gilead Liver Commitment
Exploring for Complete Understanding of Liver Disease
VEMLIDY-for the flow of life with chronic hepatitis B

평생 관리해야 하는 만성 B형 간염
환자의 생애주기를 고려한다면, Vemlidy®로 시작해 주세요.

References
1. 2018 대한간학회 만성 B형 간염 진료 가이드라인
3. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B viral infection
The new wave of GERD Treatment, P-CAB

FEXUCLUE
Fexuprazan hydrochloride

Excellent nocturnal symptom control: Longest half-life

Rapid and superior heartburn symptom relief

Full and fast onset of effect with the first dose

Significantly improved chronic cough of EE

Take once a day regardless of meal

Less affected by CYP2C19. Low potential of DDI individual variations
RECOGNIZE & KILL CANCER CELLS

Paradigm Shift in Cancer Treatment

Recognize & Kill the cancer cells

Immuncell-LC
Anticancer cellular Immunotherapeutics

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Received approval for cancer immunotherapy ‘Immuncell-LC’ from MFDS in 2007


Efficacy-Effect: Adjuvant therapy for patients whose tumor has been removed after curative resection for Hepatocellular Carcinoma (Operation, Radio Frequency Ablation, Percutaneous Ethanol Injection Therapy)

Dosage and Administration: Mix the settled cells and suspension fluid three or four times prior to administration. The interval and times of administration are as follows: 4 times, once a week; 4 times, once every two weeks; 4 times, once four weeks; 4 times, once every eight weeks 16 times in total.
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!  
(Sorafenib tosylate(Micronized))  
(since December 1st, 2020.)

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

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ATMEG COMBIGEL

Korea’s first COMBIGEL type of Product for a treatment of mixed dyslipidemia

* Incrementally Modified Drug

A New Therapy for Mixed dyslipidemia

ATMEG COMBIGEL® Soft caps
Atorvastatin 5mg, 10mg / Omega-3 1g
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% [INJECTION]
[CONTENTS] Each 100 mL contains 5 g and 20 g of Human Serum Albumin as active ingredient, for 5% Inj. and 20% Inj. respectively
[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.

CONTRAINDICATIONS: Patients with a history of hypersensitivity reactions to this drug and its components.

MANUFACTURER: SK Plasma Co., Ltd. 13494, 117 Gwangyang-ro, Andong-si, Gyeongsangbuk-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.mds.go.kr

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The non-invasive gold standard solution for comprehensive management of liver health

CAP 신의료기술 고시
보고복지부 고시 제2021-163호, 2021.6.7

New! Fibroscan 630 Expert
Spleen Stiffness Measurement (SSM by VCTE) Surrogate marker of PH

Scores (Agile 3+ & 4) by Echosens
LSM과 혈액 바이오 마커(ALT,AST,PIt) 결합 및 계산하여 NAFLD 환자의 F3/F4를 식별합니다

Surrogate marker of liver fibrosis
- Measurement of liver stiffness (expressed in kPa)
- Relevant in all Chronic Liver Diseases (CLD)

Surrogate marker of liver steatosis
- Measurement of ultrasound attenuation (expressed in dB/m)
- Relevant in Fatty Liver Diseases: AFLD, NAFLD, NASH

Surrogate marker of portal hypertension (PH)
- Shear wave frequency of 100Hz
- Relevant in the diagnosis of large esophageal varices
- Relevant for the risk stratification of cirrhotic patients

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**Besivo**

Besifovir dipivoxil

A safe journey for lifelong HBV treatment

The first developed nucleotide analogue in Korea.

**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.

---

* TDF : Tenofovir disoproxil fumarate, ** Knodell necroinflammation score

---

**REFERENCE**


---

**BE2304A1-2504**

**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 660mg 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 once 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.

**WARNINGS AND PRECAUTIONS**

1) Lactic acidosis and severe hepatomegaly with steatosis, including fatal cases, have been reported with the use of nucleoside analogs in combination with other antiretrovirals. Treatment should be suspended in any patient who develops clinical or laboratory findings suggestive of lactic acidosis or pronounced hepatotoxicity (which may include hepatomegaly and steatosis even in the absence of marked transaminase elevations). 2) Discontinuation of anti-HBV therapy may be associated with severe acute exacerbations of hepatitis. Patients infected with HBV who discontinue Besivo should be closely monitored with both clinical and laboratory follow-up for at least several months after stopping treatment. If appropriate, re-initiation of anti-hepatitis B therapy may be warranted. 3) HIV-1 antibody testing should be offered to all HBV-infected patients before initiating therapy with Besivo. Limited clinical experience suggests there is a potential for the development of HIV if Besivo is used to treat chronic hepatitis B virus (HBV) infection in patients with HIV infection that is not being treated. Therapy with Besivo is not recommended for HIV/HBV co-infected patients. 4) Since this drug contains lactose, it should not be administered to patients with genetic problems such as galactose intolerance, Lapp lactase deficiency, or glucose-galactose malabsorption.
STRIKE FIRST

Your precision strike.
Arming you to target HCC tumors directly and hit them hard with high-dose radiation therapy.

Proven.
Personalized.
Precise.
WE

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DO IT

Motility improvement from stomach to large intestine through peripheral D₂ Anagonist and AChE inhibitor mode of action¹,²

Clinical improvement and efficacy in functional dyspepsia proved in NEJM³
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 pharma companies measured by market cap
- Supply of nearly 50% of the world's insulin
- Over 30M people use our diabetes care products

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