LIVER FIBROSIS AND MAFLD: FROM MOLECULAR ASPECTS TO NOVEL PHARMACOLOGICAL STRATEGIES

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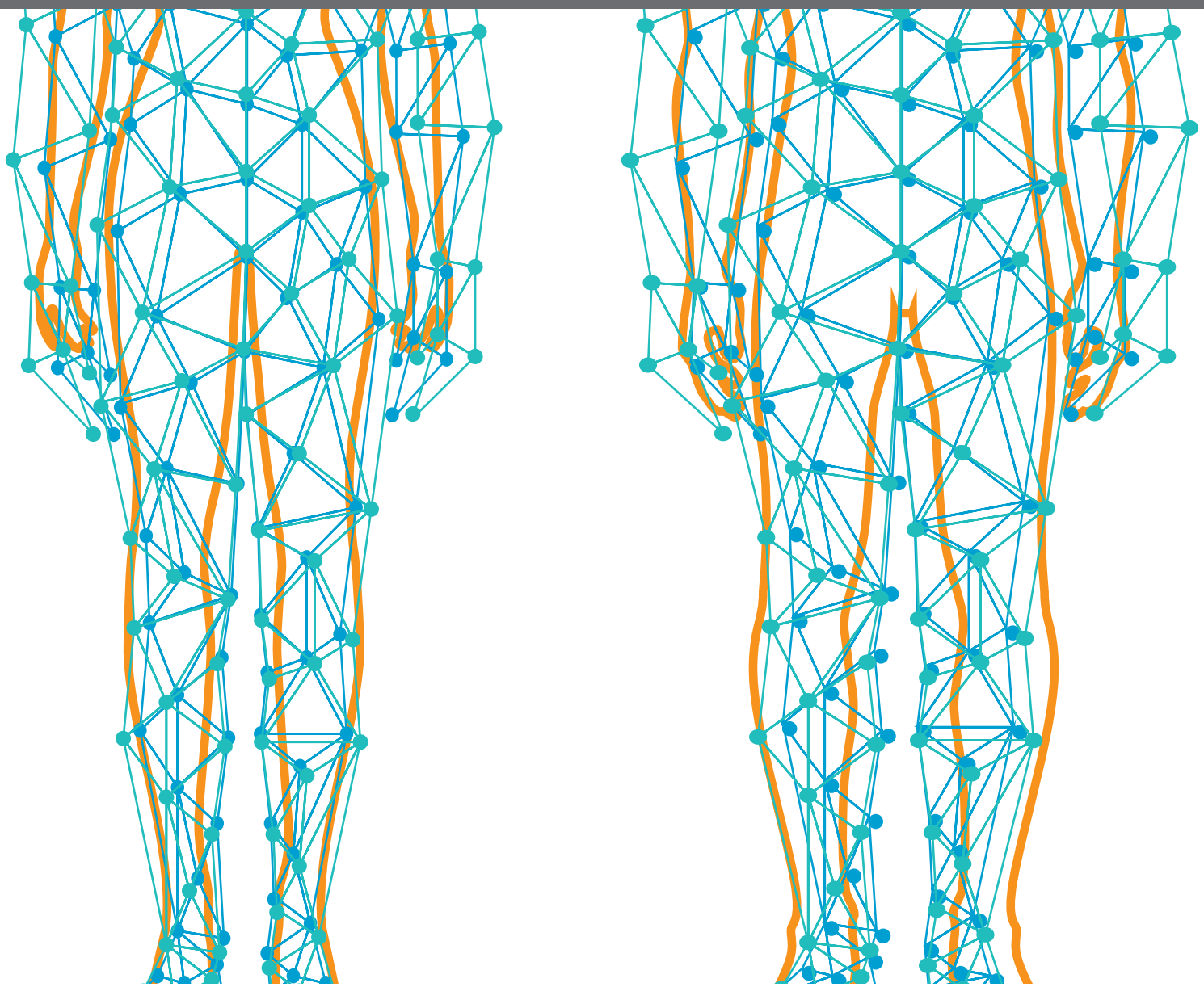

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LIVER FIBROSIS AND MAFLD: FROM MOLECULAR ASPECTS TO NOVEL PHARMACOLOGICAL STRATEGIES

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EDITORIAL
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Editorial: Liver Fibrosis and MAFLD: From Molecular Aspects to Novel Pharmacological Strategies

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The Editorial on the Research Topic

Liver Fibrosis and MAFLD: From Molecular Aspects to Novel Pharmacological Strategies

In this issue, Armendariz-Borunda and collaborators have recruited a group of talented researchers to review important and varied topics in liver fibrosis and recently defined MALFD. This issue of Frontiers in Gastroenterology addressed a range of approaches, basic and experimental to clinical practice, including some up-dated reviews in the field. The issue begins with an article that proposed 10 routine biochemical markers (AGE, ALPK, CHOL, GGT, AFP, APTT, PT, TT, PDW, and PLT) using multinomial logistic regression in a final model of a generic nomogram, covering mild-moderate fibrosis and severe fibrosis, and as stated by the authors, it can be effectively used to predict the degree of liver fibrosis in chronic hepatitis B-infected patients. The predictive value of the generic nomogram for liver fibrosis stage among HBV patients makes it reliable and convenient to use in wide populations (Xu et al.). However, the weakness of the study is that only HVB patients were included, thus the usefulness of the nomogram in other liver fibrosis etiologies must be validated.

It has been more than four decades since the term non-alcoholic fatty liver disease (NAFLD) was coined. NAFLD definition includes three main characteristics: confirmation of hepatic steatosis, non-existence of secondary causes for liver fat accumulation, and no coexisting causes of chronic liver disease (1). However, a great number of studies have emphasized that the disease is associated with metabolic dysregulations, leading an international panel of experts in 2020 to propose a change to the definition and arrive to a new consensus name: MAFLD—metabolic associated fatty liver disease—(2). Controversy regarding the utility of the MAFLD definition was raised; in this issue, the results from Huang et al. of the Third National Health and Nutrition Examination Survey (NHANES III), which included 14,797 participants, demonstrated that MAFLD and NAFLD overlapped, adding to the theory that this new definition miss patients with severe steatosis. Of 12,480 participants, 3,909 were diagnosed with MAFLD and 3,779 with NAFLD; 22.8% of participants were diagnosed with both NAFLD and MAFLD. Ultrasound grading of hepatic steatosis at baseline (1988 to 1994) was linked to mortality information through December 31, 2015, provided by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC). In univariable models, data emphasizes MAFLD patients had increased risk for all-cause mortality in a greater magnitude than patients with NAFLD, probably due to metabolic implications (Huang et al.). Race-ethnicity (non-Hispanic white) and presence of hepatic viral infection significantly increased the risks for overall mortality among patients with MAFLD, so these parameters should be taken into account in trials studying the outcome of MAFLD. Efforts

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from the research and clinical community to sub-phenotype the disease may contribute to the development of new specific treatments.

In the last decades, NAFLD has gained predominance among liver diseases and, as is known, chronic alcohol consumption must be discarded for its diagnosis. However, the effects of modest alcohol consumption on long-term clinical outcomes in NAFLD patients are not definite. Modest alcohol drinking in the study by Wongtrakul et al. was defined as consumption of <21 standard drinks (210 g) per week for men and <14 standard drinks (140 g) per week for women. The meta-analysis suggests that alcohol consumption should be avoided in patients with steatohepatitis or fibrosis (Wongtrakul et al.), since histological follow-up showed that modest alcohol use may diminish the resolution of NASH and increase risk of HCC in NAFLD patients with advanced fibrosis. On the contrary, NAFLD patients with low fibrosis risk may be allowed to engage in modest drinking because they had a lower mortality risk than lifelong abstainers. Data correlates with the cardiometabolic benefits of modest alcohol consumption and with the decreased risk of developing NAFLD in the general population (3–6). However, we should remember that alcohol intake is a risk factor for the development of HCC, both directly via DNA damage from toxic metabolites, oxidative stress, and inflammation and indirectly via chronic liver disease and cirrhosis (7, 8). Furthermore, obesity and DM2 are highly prevalent in the NAFLD population and this synergistic interaction could potentially augment the risk of HCC development (9).

An innovative prospective study conducted in a general Chinese population from 2013 to 2018 showed that 2,452 out of 14,154 participants developed NAFLD, diagnosed by liver ultrasonography. Muscle strength was assessed using a handheld dynamometer to measure HGS (hand grip strength). Hand grip strength was found to be inversely associated with NAFLD (Xia et al.). This result may not come as a surprise as skeletal muscle metabolism can influence insulin resistance and lipid metabolism; however, it is an ingenious way to associate these factors with muscle strength (easier to measure than muscle mass).

Some reviews in this Frontiers in Medicine issue elegantly cover the role of HIF (Hypoxia inducible factors) and epigenetics alterations in NAFLD and NASH development (Holzner and Murray; Rodríguez-Sanabria et al.). These latter reviews remind us of the importance of ROS, inflammation, and metabolic alterations in the development of these hepatic diseases and lead us to keep in mind the new definition of MAFLD. Hypoxia-inducible factor (HIF) are a family of transcription factors that represent a cellular oxygen-sensing system regulating cellular and systemic response to hypoxia (10). Liver hypoxia had been reported in high fat diet fed animals (11), but it remained uncertain what trigger liver hypoxia and HIF activation had in NAFLD. HIF signaling seems to be involved in several key aspects of NAFLD-like steatosis, inflammation, and fibrosis, while HIF2α antagonism in a HFD model of hepatosteatosis had shown promising results (12). In balance, HIF activation appears to be harmful in NAFLD, and may therefore be a useful therapeutic target. Rodríguez-Sanabria et al. describe in detail how DNA methylation processes, histone modifications, and miRNA expression have been closely associated with MAFLD progression. Since epigenetic changes are reversible, and lifestyle and environmental exposure can modify epigenetic patterns throughout life; a variety of epigenetic-based therapeutic interventions seem possible to be developed to modify MAFLD progress or resolution, including dietary microRNAs and supplementation with bioactive dietary compounds such as methyl donors, isothiocyanates, genistein, and resveratrol.

A third review by Qu et al. covers recent new targets and molecules involved in the pathophysiology of NAFLD metabolic dysregulation that could be involved in the progression to liver fibrosis. This review summarizes the therapeutic potential of a variety of molecules implicated in lipid metabolism, inflammation, cell apoptosis, oxidative stress, and extracellular matrix formation (Qu et al.). Such molecules include Fanitol X receptor, Glucagon-like peptide-1, and PPARs agonists, as well as Acetyl-CoA carboxylase, Stearoyl-CoA desaturase, fatty acid synthase, apoptosis signal-regulating kinase 1, and TGF-β-activated kinase 1 inhibitors, among others molecules like Vitamin E and LOXL2 (Lysyl oxidase-like 2) and TGF-β monoclonal antibody in experimental and clinical scenarios of NAFLD/NASH. Although none of the treatments achieved outstanding benefits without significant side effects in a large-scale trial, combinatorial therapies targeting multiple profibrotic pathways could be promising in achieving successful antifibrotic interventions in patients with MAFLD/NAFLD.

Torre et al. stylishly review how the liver immune system orchestrates a response driven by hepatic inflammation that precedes and accompanies fibrogenesis in the liver, where every kind of immune cell and every type of immune response plays a key role in NALFD/MALFD progression. Also, by reviewing therapeutic approaches they aimed to regulate the immune system in NAFLD/MALFD progression and to treat liver fibrogenesis, like CCR2 and CCR5 antagonist, galectin inhibitors, and modulation of macrophage polarization/differentiation (Torre et al.). This Frontiers in Medicine issue continues with a mini review focused on the fact that MALFD/NALFD is the most prevalent liver disorder worldwide and therefore non-invasive strategies for its diagnosis are needed to be developed and widely validated, especially in populations with co-variables like BMI, concomitant diseases, and ethnic background (Segura-Azuara et al.). This article starts defining five hepatic steatosis scoring systems and the reliability and categorization difficulties (Lipid Accumulation Product, NAFLD Liver Fat Score, HS Index, NAFLD Ridge Score, and Fatty Liver Index). Then, it goes on to describe NASH scoring systems and the most used hepatic fibrosis scoring systems so far, including fibromax and APRI. As it is known, liver biopsy remains the gold standard for diagnosis, followed by elastography studies. However, there are contraindications for liver biopsy and elastography requires specialized equipment and technicians; then due to the growing MAFLD pandemic alternatives for screening urge to be available for clinicians, especially for early diagnosis. Our Editorial issue continues by describing the association between cholecystectomy and NAFLD in adults by assessing a cross-sectional study of the National Health and Nutrition Examination Survey

in the USA (Xie et al.). Cholecystectomy was found to be positively associated with liver fibrosis and cirrhosis in this population. Gallbladder removal provokes changes in bile flow and concentration of bile acid in the bile duct (13), which may cause chronic cholestasis, NAFLD, and metabolic syndrome (14, 15); through time (<14 years, showed higher incidence of liver fibrosis) these changes act as risk factors. Thus, shunt of bile acids pathway should not be taken as a non-side effect intervention because of the diverse acute complications related to cholecystectomy. Zhang et al. (16) showed that sleeve gastrectomy procedure contributed to significant weight loss and reduced lipids in NAFLD patients and mice model. Molecular mechanisms involved in this effect include increased expression of DUSP1 -a phosphatase with dual specificity for tyrosine and threonine that can dephosphorylate MAP kinase MAPK1/ERK2- and reduce expression of miR-200c-3p. miR-200c-3p is known to regulate the MAPK-dependent signals (p-ERK1/2, p-p38, and p-JNK) that are linked to the promotion of hepatosteatosis via dual-specificity protein phosphatase 1 (DUSP1). This number includes the opinion of Zhang and Yang over T cells' subpopulations during NAFLD-related HCC. It is also described how proliferation of human CD4+ central and effector memory T cells can be affected by high-fat and high-calorie diet in diverse roles in NAFLD development. Likewise, authors end up with the suggestion that T cell manipulation regarding the stage of liver disease and microenvironment may provide a novel approach for HCC treatment, including those related to Gut-Microbiota and miRNA-mediated therapies. This issue explores the relationship between MAFLD and Chronic Kidney Disease (CKD) using Transient Elastography (TE), given the fact that MALFD definition includes metabolic dysfunction and almost all patients with CDK showed metabolic disorders in the form of an atherogenic dyslipidemia. In 335 patients with DM2 and MAFLD, 60.8% had CKD. Patients with CKD had higher mean liver stiffness measurements (LSM) than those without CKD. Surprisingly, steatosis appears to be a better predictor of CKD compared to LSM-assessed hepatic fibrosis (Marc et al.).

As a final point, Fridén et al. designed a study to investigate associations between liver fatty acids measured in three different lipid fractions—cholesteryl esters (CE), phospholipids (PL), and triacylglycerols (TAG)—and liver fibrosis in patients with NAFLD. Also, they wanted to examine whether these associations between liver fatty acids and fibrosis could be confirmed in plasma-derived fatty acids. A positive association between liver PL 22:0 and inverse associations between liver PL 22:6n-3, TAG 18:1n-9, and TAG 18:1 and liver fibrosis were observed. These associations were confirmed in plasma TAG 18:1n-9 and 18:1, however an inverse association was observed for plasma PL 22:0. Total plasma TAG MUFA was inversely associated with liver fibrosis. This result suggests that plasma fatty acids could potentially be used as biomarkers for discriminating patients with NAFLD fibrosis (Fridén et al.).

This issue will engage the reader with an update of MAFLD molecular mechanism and clinics, as well as therapeutic approaches, traveling from oxidative stress, inflammation, fat accumulation and steatohepatitis, through to complications like liver fibrosis, cirrhosis, and hepatocellular carcinoma. This is a journey that could take years in patients, but is addressed far quicker in this issue.

AUTHOR CONTRIBUTIONS

AS-R and AT: manuscript writing. JA-B: review and writing of manuscript. All authors contributed to the article and approved the submitted version.

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NAFLD or MAFLD: Which Has Closer Association With All-Cause and Cause-Specific Mortality?—Results From NHANES III

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Background: The recent change of terminology from non-alcoholic fatty liver disease (NAFLD) to metabolic dysfunction-associated fatty liver disease (MAFLD) has raised heated discussion. We aim to investigate the association of MAFLD or NAFLD with all-cause and cause-specific mortality to compare the outcomes of the two diagnostic criteria in population-based study.

Methods: We recruited 12,480 participants from the Third National Health and Nutrition Examination Survey (NHANES III) with matched mortality data in 2015. Participants were divided into four groups for survival analysis: without NAFLD or MAFLD, with only NAFLD, only MAFLD. Cox proportional hazard regression was used to estimate multivariable-adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for all-cause and cause-specific mortality. Subgroup analysis were applied in MAFLD patients.

Results: The weighted prevalence of MAFLD and NAFLD was relatively 27.4 and 27.9%. Participants with NAFLD or MAFLD were largely overlapped (weighted Cohen's kappa coefficient 0.76). MAFLD increased the overall risk for total mortality in a greater magnitude than NAFLD [HR 2.07 (95% CI 1.86, 2.29) vs. 1.47 (1.20, 1.79)], However, the difference was non-significant after metabolic parameters were adjusted. Risks for cardiovascular, neoplasm, and diabetes-related mortality were similar between MAFLD and NAFLD. Referring to individuals without both NAFLD and MAFLD, individuals with only NAFLD showed reduced total mortality [HR 0.48 (0.34, 0.68)] and neoplasm mortality [HR 0.46 (0.24, 0.89)] in crude. Nevertheless, individuals with only MAFLD independently increased the risk for total mortality [adjusted HR 1.47 (1.22, 1.77)] and neoplasm mortality [aHR 1.58 (1.09, 2.28)]. The risk for overall mortality in MAFLD was consistent between subgroups except for race-ethnicity and whether secondary to viral hepatitis.

Conclusions: Participants with MAFLD or NAFLD were highly concordant. MAFLD showed greater risk for all-cause mortality and equal risk for cause-specific mortality referring to NAFLD. The new terminology excluded participants with lower mortality risk and included participants with higher risk. Drug development for MAFLD should consider ethnic differences.

Keywords: NAFLD, MAFLD, mortality, NHANES, diagnosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease affecting around one-quarter of the population worldwide, causing a global economic burden (1). The definition of NAFLD requires presence of fat on imaging to liver biopsy and exclusion of other liver diseases e.g., excess alcohol intake, drug-induced liver injury, and viral hepatitis (2). NAFLD is also regarded as a "metabolic disease" since it is closely associated with metabolic disorders including obesity, dyslipidemia, and diabetes mellitus (3), of which the common etiology is insulin resistance (4). Patients with NAFLD have a higher risk of cardiovascular events. The leading cause of mortality in NAFLD patients is cardiovascular disease and major excess mortality may result from extrahepatic cancer (1, 5, 6). A meta-analysis suggested that NAFLD was independently associated with increased absolute risk of all-cause mortality, but the risk for cardiovascular and cancer mortality was similar between NAFLD and non-NAFLD participants (1, 6–8).

The progression and prognosis of NAFLD are highly heterogeneous. Only 2–3% of participants progressed from steatosis to non-alcoholic steatohepatitis (NASH) and advanced fibrosis. Liver related mortality only explained 7% of deaths among NAFLD patients (9, 10). At the beginning of 2020, experts from the European Liver Patient's Association (ELPA) proposed a change of nomenclature from NAFLD to metabolic dysfunction-associated fatty liver disease (MAFLD), which was mainly defined as liver fat deposition along with obesity, diabetes, or combined metabolic disorders (11, 12). This change emphasized the importance of metabolic disorder complicated with fatty liver regardless of the heterogeneous etiology since the exclusion of other liver diseases was no longer required.

Intense dispute raised over the change of the terminology since whether the change from NAFLD to MAFLD can benefit clinical practice and drug development is largely unknown. Studies suggested that participants with NAFLD and MAFLD were highly compatible with each other, and patients with MAFLD were more likely to have worse metabolic profiles than NAFLD (13, 14). Other experts concerned that the change may exclude patients with worse outcome, such as participants with "lean NAFLD" who have lower BMI and better metabolic profile, and participants with severe hepatic steatosis who may have more liver fibrosis and elevated long-term comorbidities (13, 15). In addition, although MAFLD may reflect relevant risk factors as a metabolic disease, whether this change is necessary regarding biomarker identification, treatment strategy and prognosis is largely unknown (16).

A key question to be answered is whether the change from NAFLD to MAFLD could affect the association between fatty liver and clinical outcomes. A study from Japan suggested that individuals with NAFLD and MAFLD had similar metabolic traits at baseline as well as incidence for cardiovascular events after a 7-year follow-up (17). However, the association between MAFLD and mortality in the long run was largely unknown. Here we aimed to use the National Health and Nutrition Examination Survey III cohort and the follow-up mortality data to answer whether the terminology MAFLD is superior to NAFLD regarding their long-term mortality risk and cause-specific mortality risk.

MATERIALS AND METHODS

Study Design and Participants

The Third National Health and Nutrition Examination Survey (NHANES III) profiles health estimates of civilian non-institutionalized US population using a multistage, stratified sampling design from 1988 to 1994 (18). Ultrasound grading of hepatic steatosis was combined at baseline. Linked mortality information through December 31, 2015, was provided by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC).

In NHANES III, 14,797 participants aged 20–74 years with assessment of hepatic steatosis were recruited. Exclusion criteria included: (1) ungradable images of hepatic steatosis (N = 941); (2) participants without important covariates: body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), waist circumference, total cholesterol (TC), total triglyceride (TG), high-density lipoprotein cholesterol (HDL), fasting plasma glucose (FPG), fasting insulin and glycosylated hemoglobin (HbA1c) (N = 1,366); (3) participants with missing follow-up data (N = 10). After exclusion, 12,480 eligible participants were followed up for a medium of 22.8 years (interquartile range 20.7–24.7 years, **Figure 1**).

Laboratory Measurement and Index Calculation

Serum biochemistries were measured by the Hitachi 737 automated multichannel chemistry analyzer (Boehringer Mannheim Diagnostics, Inc., Indianapolis, Indiana). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was adopted to estimate the level of β-cell function. Methods for non-invasive fibrosis assessment, such as NAFLD fibrosis score (NFS score), AST-to-platelet ratio index (APRI), and fibrosis-4 index (FIB-4), were evaluated by original formulas (**Supplementary Table 1**).

Definitions and Subgroups

Categorized assessment of hepatic steatosis by ultrasound encompassed none, mild, moderate and severe, and only mild to severe hepatic steatosis was regarded as evidence of hepatic steatosis (19). NAFLD was diagnosed if an adult with steatosis confirmed by ultrasound without (1) high alcoholic consumption (over one drink daily among women or over two drinks daily among men); (2) presence of hepatitis B surface antigens or antibodies to hepatitis C; (3) iron overload (transferrin saturation $\geq$ 50% along with serum ferritin $\geq$ 400 μg/L in women and $\geq$500 μg/L in men) (20). MAFLD was defined by the international expert consensus statement in 2020 (12), including ultrasound confirmed hepatic steatosis plus one of the three criteria: overweight or obesity defined as BMI $\geq$ 25 kg/m^2, presence of type 2 diabetes mellitus, and metabolic disorders described by any two indicators: (1) waist circumference (WC) $\geq$ 102 cm in men or $\geq$88 cm in women; (2) blood pressure $\geq$140/90 mmHg or taking anti-hypertension drugs; (3) raised triglycerides (plasma

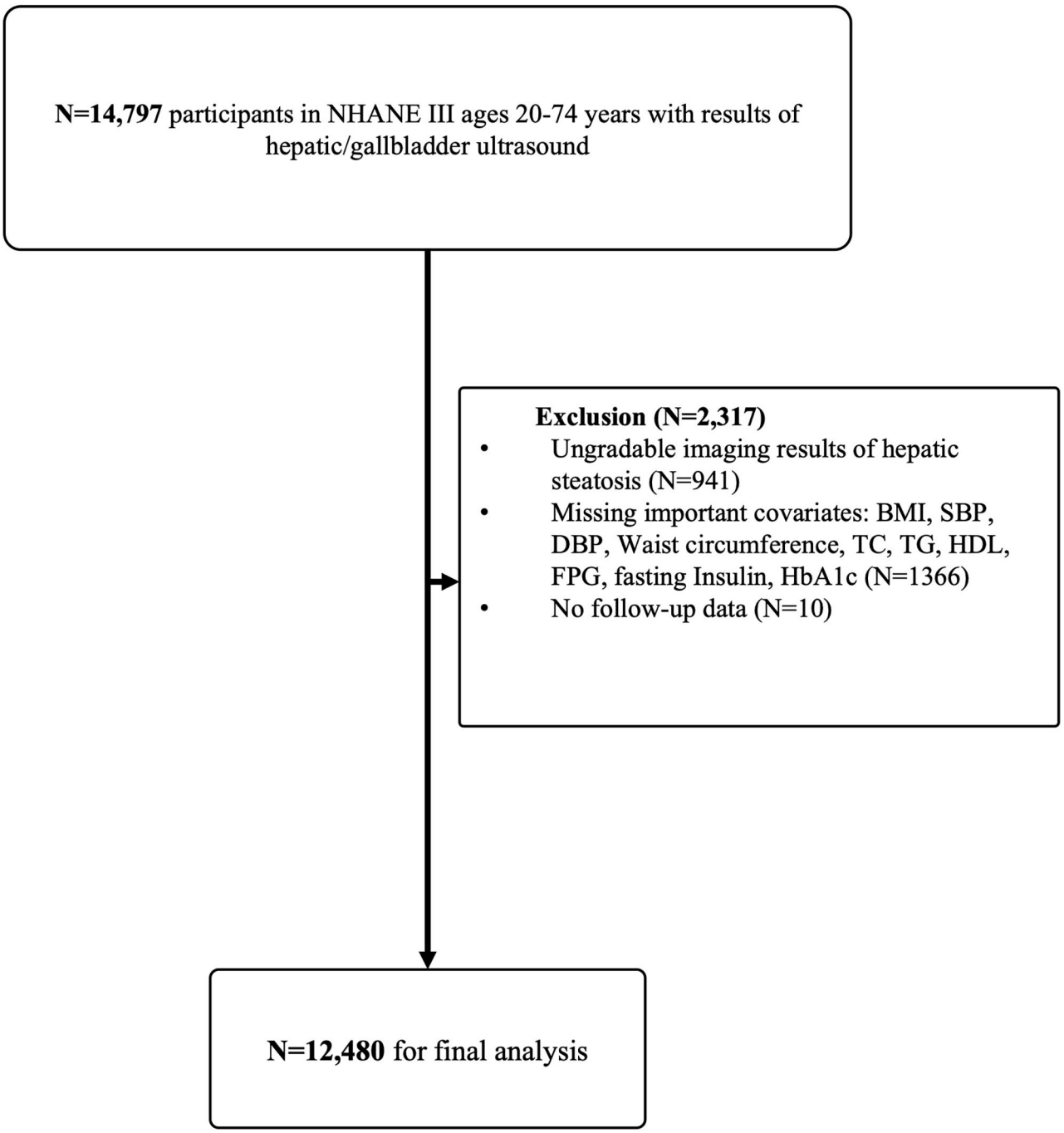

FIGURE 1 | Flow-chart of the Study. NHANE III, The National Health and Nutrition Examination Survey III (1988–1994). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin.

triglycerides ≥ 1.70 mmol/L or taking specific anti-lipid agents); (4) reduced HDL cholesterol (plasma HDL < 1.0 mmol/L for men and <1.3 mmol/L for women or taking specific agents); (5) prediabetes status (FPG 5.6–6.9 mmol/L, or 2-h post-load glucose levels 7.8–11.0 mmol or HbA1c 5.7–6.4%); (6) HOMA-IR ≥ 2.5; (7) plasma high-sensitivity C-reactive protein (CRP) level > 2 mg/L.

The presence of diabetes mellitus was defined as a self-report history of diabetes mellitus, fasting glucose levels (FPG) ≥ 7.0 mmol/L, 2-h post-load glucose levels ≥ 11.0 mmol (for

participants given an oral glucose tolerance test), HbA1c ≥ 6.5% or taking diabetes drugs. Hypertension was defined as BP ≥ 140/90 mmHg, or taking anti-hypertension drugs. The definition of metabolic syndrome was according to the joint interim statement in 2009 (21). Waist circumference criteria in ATP III (≥102 cm in male; ≥88 cm in female) was used for abdominal obesity in the United States. Elevated liver enzymes were defined as AST > 37 U/L in men and >31 U/L in women or ALT > 40 U/L in men and >31 U/L in women.

According to the NCHS, all-cause death recorded all deceased participants. Main causes of death following the guidelines of International Statistical Classification of Diseases, Injuries, and Causes of Death (ICD-9 before 1998 and ICD-10 afterwards) presented as cause-specific mortality (22), consisting of cardiovascular mortality recorded by heart and cerebrovascular diseases, neoplasm mortality recorded by malignant neoplasms in all systems, and diabetes-related mortality recorded by diabetes mellitus.

We further separated the cohort into the four mutually exclusive groups based on definitions of MAFLD and NAFLD. Group M+N: participants meet the diagnostic criteria for both MAFLD and NAFLD definitions were in group M+N; Group N: participants can be defined as NAFLD but not MAFLD; Group M: participants defined as MAFLD but not NAFLD in group N or M; participants excluded by both definitions were viewed as control group.

Statistical Analysis

All analysis was weighted by sample weights to reflect population-based estimates. Continuous data were presented as mean and 95% confidence intervals (geometric mean for variables without normal distribution). Categorical variables were displayed as percentages. The baseline characteristics of the participants among groups were compared by one-way ANOVA test when appropriate for continuous variables or chisq test for categorical variables.

For survival analysis, we used Kaplan-Meier methods to estimate cumulative hazard. To establish cox regression models, the following confounders were considered initially:

- Sociodemographic features: age, sex, race-ethnicity, smoking status.
- Hepatic assessment: alanine transaminase (ALT), aspartate transaminase (AST), CRP, alkaline phosphatase (ALP), FIB-4 score, NFS score, APRI.
- Metabolic assessment: BMI, WC, SBP, DBP, FPG, fasting insulin, HOMAIR, TC, TG.

LASSO regression with minimum mean 10-fold cross-validated error was applied for confounder selection to avoid multicollinearity. Among confounders above, we excluded the variables that were penalized to zero by LASSO model (**Supplementary Table 2**). The LASSO model suggested waist circumference and HbA1c, were stronger indicators than BMI and diabetes, so we used the former instead. Variables with no-zero parameter were classified as above and adjusted stepwise in cox regression models to estimate hazard ratios (HR) and 95% confidence intervals (95% CI) for overall and cause-specific mortality. Participants missing relevant covariates were excluded.

Finally, we assessed the association of MAFLD with all-cause, cardiovascular, and neoplasm mortality within subgroups by age (20–39 years, 40–55 years, >55 years), sex, race-ethnicity, diabetes, hypertension, dyslipidemia (raised triglycerides or reduced HDL cholesterol), metabolic syndrome, BMI (Underweight/normal weight: <25 kg/m^2, overweight: 25–30 kg/m^2 and obesity: >30 kg/m^2), severity of hepatic steatosis, NFS, APRI and FIB-4 score (≤weighted mean value, >weighted mean value), presence of other etiologies (alcohol, hepatitis virus, and iron overload), adjusting by age, sex, and race-ethnicity if appropriate. Bonferroni correction was applied and significance was defined as $p < 0.0033$.

All statistical analyses were conducted in R software version 4.0.2. The LASSO regression model was conducted by the R-package "glmnet" (23).

RESULTS

Baseline Characteristics of the Participants With MAFLD and NAFLD

Of 12,480 participants, 3,909 were diagnosed with MAFLD (weighted prevalence 27.4%) and 3,779 were diagnosed with NAFLD (weighted prevalence 27.9%) (**Table 1**). Correlation analysis suggested MAFLD was highly concordant with NAFLD (weighted Cohen's kappa coefficient 0.76).

22.8% of participants were diagnosed with both NAFLD and MAFLD (M+N), and the weighted prevalence of only NAFLD (N) and only MAFLD (M) was 5.1 and 4.6% (**Table 1**). At baseline, group N were youngest (mean age: 34.2 years) and complicated with fewest metabolic disorders and histories of cardiovascular diseases. Among these four groups, group M had the highest proportion of men (66.7%), ever smokers (70.5%), the highest prevalence of high alcohol consumption (75.4%), viral hepatitis (17.3%), iron overload (10.9%), hypertension (57.2%), severe hepatic steatosis (23.8%) and the highest level of blood pressure (mean SBP: 129 mmHg; mean DBP:79.2 mmHg), liver enzymes (mean AST: 33.3 U/L; mean ALT: 31.0 U/L; mean GGT: 69.1 U/L; mean ALP: 88.7 U/L), and fibrosis scores (mean NFS score: −1.45; mean APRI score: 0.47; mean FIB-4 score: 1.46). Group M+N had the highest prevalence of metabolic syndrome (58.2%), with highest level of blood lipid (mean TG: 1.89 mmol/L, mean TC: 5.42 mmol/L), blood glucose (mean HbA1c: 5.66%).

Associations of MAFLD/NAFLD With Mortality

We used LASSO regularization to preselect 11 covariates (**Supplementary Table 2**), of which age, sex, and race-ethnicity, hepatic assessment (FIB-4 score, NFS score, ALP, and CRP), metabolic parameters (WC, SBP, HbA1c, fasting insulin, TG) were selected for further adjustment. In univariable models, MAFLD increased the risk for all-cause mortality by one-fold compared with non-MAFLD participants. In reference to non-MAFLD participants, MAFLD enhanced the risk for all-cause mortality significantly when age, sex, race-ethnicity, FIB-4, NFS

TABLE 1 | The characteristics of the participants ($N = 12,480$)*.

	Overall	MAFLD vs. control		NAFLD vs. control		Separate groups			
		Non-MAFLD	MAFLD	Non-NAFLD	NAFLD	Control	N	M	M+N
N (%)	12,480 (100)	8,571 (72.6)	3,909 (27.4)	8,701 (72.1)	3,779 (27.9)	8,043 (67.5)	528 (5.1)	658 (4.6)	3,251 (22.8)
Age (years)	42.1 (41.8, 42.4)	40.1 (39.8, 40.4)	47.4 (46.9, 47.9)	40.9 (40.6, 41.3)	45.1 (44.6, 45.6)	40.5 (40.2, 40.9)	34.2 (33.2, 35.1)	46.9 (45.8, 47.9)	47.5 (47.0, 48.0)
Men (%)	5,865 (48.7)	3,897 (46.2)	1,968 (55.1)	4,164 (48.4)	1,701 (49.3)	3,702 (47.1)	195 (33.7)	462 (66.7)	1,506 (52.8)
Race-ethnicity (%)									
Non-Hispanic White	4,648 (76.0)	3,276 (76.7)	1,372 (74.2)	3,284 (76.4)	1,364 (75.1)	3,070 (76.6)	206 (78.5)	214 (73.5)	1,158 (74.4)
Non-Hispanic Black	3,544 (10.5)	2,658 (11.0)	886 (9.2)	2,668 (11.1)	876 (9.1)	2,495 (11.1)	163 (9.8)	173 (10.5)	713 (8.9)
Mexican-American	3,765 (5.5)	2,272 (4.8)	1,493 (7.4)	2,371 (4.9)	1,394 (7.0)	2,127 (4.8)	145 (4.8)	244 (7.3)	1,249 (7.5)
Others	523 (8.0)	365 (7.5)	158 (9.1)	372 (7.6)	151 (8.9)	351 (7.5)	14 (6.9)	27 (8.7)	131 (9.3)
Ever smoking (%)	6,408 (55.3)	4,326 (54.1)	2,082 (58.3)	4,554 (55.8)	1,854 (53.8)	4,101 (54.8)	225 (44.4)	453 (70.5)	1,629 (55.9)
BMI, kg/m^2	26.5 (26.4, 26.6)	25.0 (25.0, 25.1)	30.5 (30.3, 30.7)	25.6 (25.5, 25.7)	28.9 (28.7, 29.1)	25.3 (25.2, 25.4)	21.4 (21.2, 21.6)	29.9 (29.5, 30.4)	30.6 (30.4, 30.8)
Waist circumference (M), cm	95.0 (94.7, 95.4)	91.1 (90.7, 91.4)	104 (103, 104)	92.6 (92.2, 92.9)	101 (101, 102)	91.6 (91.3, 92.0)	81.0 (79.9, 82.2)	102 (101, 104)	104 (104, 105)
Waist circumference (F), cm	91.7 (91.4, 92.0)	84.5 (84.1, 84.8)	101 (101, 102)	86.2 (85.8, 86.6)	94.7 (93.9, 95.5)	85.5 (85.1, 85.8)	73.9 (73.3, 74.6)	102 (100, 105)	101 (101, 102)
SBP, mmHg	121 (120, 121)	118 (118, 118)	127 (127, 128)	119 (119, 120)	124 (123, 124)	119 (118, 119)	109 (108, 110)	129 (128, 130)	127 (127, 128)
DBP, mmHg	74.3 (74.1, 74.4)	72.9 (72.7, 73.1)	77.9 (77.6, 78.3)	73.6 (73.4, 73.8)	76.1 (75.7, 76.4)	73.2 (73.0, 73.4)	68.9 (68.2, 69.6)	79.2 (78.4, 80.1)	77.7 (77.3, 78)
HbA1C, %	5.32 (5.31, 5.34)	5.20 (5.19, 5.22)	5.64 (5.60, 5.67)	5.24 (5.22, 5.25)	5.54 (5.51, 5.58)	5.22 (5.20, 5.23)	5.02 (4.99, 5.05)	5.52 (5.43, 5.62)	5.66 (5.62, 5.70)
HOMA-IR	2.04 (2.02, 2.07)	1.70 (1.68, 1.72)	3.32 (3.25, 3.4)	1.81 (1.78, 1.83)	2.81 (2.74, 2.88)	1.73 (1.71, 1.75)	1.32 (1.28, 1.37)	3.35 (3.15, 3.55)	3.32 (3.24, 3.41)
TG, mmol/L	1.31 (1.30, 1.32)	1.14 (1.13, 1.16)	1.87 (1.84, 1.91)	1.2 (1.19, 1.21)	1.64 (1.61, 1.67)	1.17 (1.15, 1.18)	0.87 (0.85, 0.90)	1.79 (1.71, 1.87)	1.89 (1.85, 1.93)
TC, mmol/L	5.13 (5.11, 5.15)	5.03 (5.01, 5.05)	5.41 (5.38, 5.45)	5.09 (5.07, 5.11)	5.24 (5.21, 5.28)	5.07 (5.05, 5.1)	4.52 (4.45, 4.59)	5.37 (5.27, 5.46)	5.42 (5.38, 5.45)
HDL(M), mmol/L	1.13 (1.13, 1.14)	1.19 (1.18, 1.20)	1.02 (1.01, 1.04)	1.18 (1.17, 1.19)	1.03 (1.01, 1.04)	1.18 (1.17, 1.19)	1.29 (1.24, 1.33)	1.14 (1.11, 1.18)	1.00 (0.98, 1.01)
HDL(F), mmol/L	1.38 (1.37, 1.38)	1.43 (1.42, 1.44)	1.22 (1.21, 1.24)	1.42 (1.41, 1.43)	1.27 (1.25, 1.28)	1.42 (1.41, 1.43)	1.49 (1.46, 1.53)	1.35 (1.29, 1.4)	1.20 (1.19, 1.22)
AST, U/L#	21.4 (21.2, 21.6)	20.2 (20.0, 20.4)	24.5 (24.0, 25.1)	21.1 (20.8, 21.4)	22.2 (21.9, 22.6)	20.3 (20.1, 20.5)	19.9 (18.8, 20.9)	33.3 (31.1, 35.4)	22.8 (22.4, 23.2)
ALT, U/L#	18.0 (17.8, 18.3)	15.9 (15.7, 16.1)	23.6 (23.0, 24.2)	16.9 (16.7, 17.2)	20.8 (20.3, 21.3)	16.0 (15.7, 16.2)	14.9 (13.9, 15.8)	31.0 (28.9, 33.0)	22.1 (21.6, 22.7)
GGT, U/L#	29.4 (28.6, 30.1)	24.7 (24.1, 25.4)	41.6 (39.8, 43.4)	28.0 (27.1, 28.9)	32.8 (31.6, 34.0)	25.2 (24.5, 25.9)	18.4 (16.1, 20.8)	69.1 (61.1, 77.1)	36.2 (34.8, 37.6)
ALP, U/L#	80.9 (80.4, 81.4)	78.3 (77.7, 78.9)	87.6 (86.7, 88.6)	79.3 (78.7, 79.9)	84.8 (83.9, 85.7)	78.7 (78.1, 79.3)	72.9 (70.7, 75.2)	88.7 (85.9, 91.4)	87.4 (86.5, 88.4)
CRP, mg/L	3.93 (3.83, 4.03)	3.59 (3.47, 3.70)	4.83 (4.64, 5.02)	3.75 (3.63, 3.87)	4.38 (4.21, 4.56)	3.69 (3.56, 3.81)	2.29 (2.18, 2.41)	4.73 (4.22, 5.25)	4.85 (4.65, 5.06)
NFS score#	−2.23 (−2.26, −2.21)	−2.44 (−2.47, −2.41)	−1.68 (−1.73, −1.63)	−2.34 (−2.37, −2.30)	−1.97 (−2.02, −1.92)	−2.40 (−2.43, −2.36)	−3.03 (−3.12, −2.93)	−1.45 (−1.58, −1.32)	−1.73 (−1.78, −1.68)
APRI score#	0.22 (0.22, 0.22)	0.20 (0.20, 0.21)	0.26 (0.25, 0.28)	0.22 (0.21, 0.23)	0.22 (0.21, 0.23)	0.20 (0.20, 0.21)	0.21 (0.19, 0.23)	0.47 (0.39, 0.54)	0.22 (0.22, 0.23)
FIB-4 score#	0.91 (0.89, 0.92)	0.86 (0.85, 0.87)	1.03 (0.99, 1.06)	0.90 (0.89, 0.92)	0.91 (0.89, 0.93)	0.87 (0.85, 0.88)	0.77 (0.72, 0.81)	1.46 (1.27, 1.65)	0.94 (0.92, 0.96)
Severity of hepatic steatosis (%)									
None	7,940 (66.5)	7,940 (91.7)	0 (0)	7,940 (92.3)	0 (0)	7,940 (98.6)	0 (0)	0 (0)	0 (0)
Mild	1,695 (13.5)	387 (5.4)	1,308 (35.0)	270 (2.8)	1,425 (41.0)	62 (0.8)	325 (66.5)	208 (33.2)	1,100 (35.4)
Moderate	1,931 (13.6)	204 (2.4)	1,727 (43.0)	328 (3.3)	1,603 (40.1)	30 (0.6)	174 (27.4)	298 (43.0)	1,429 (43.0)
Severe	914 (6.4)	40 (0.5)	874 (22.0)	163 (1.6)	751 (18.8)	11 (0.1)	29 (6.1)	152 (23.8)	722 (21.6)
Diabetes (%)	1,852 (10.3)	767 (6.0)	1,085 (21.6)	957 (7.6)	895 (17.3)	767 (6.5)	0 (0)	190 (24.0)	895 (21.1)

(Continued)

TABLE 1 | Continued

	Overall	MAFLD vs. control		NAFLD vs. control		Separate groups			
		Non-MAFLD	MAFLD	Non-NAFLD	NAFLD	Control	N	M	M+N
Hypertension (%)	4,046 (27.9)	2,225 (20.9)	1,821 (46.4)	2,541 (24.5)	1,505 (36.7)	2,202 (22.3)	23 (2.7)	339 (57.2)	1,482 (44.3)
Metabolic syndrome (%)	4,113 (28.3)	1,848 (17.2)	2,265 (57.8)	2,186 (20.6)	1,906 (47.6)	1,834 (18.3)	0 (0)	352 (53.4)	1,906 (58.2)
History of myocardial infarction (%)[#]	395 (2.5)	203 (1.7)	192 (4.7)	225 (1.9)	170 (4.1)	201 (1.8)	2 (0.2)	24 (3.1)	168 (5.0)
History of congestive heart failure (%)[#]	328 (1.5)	176 (1.0)	152 (2.8)	191 (1.1)	137 (2.5)	173 (1.1)	3 (0.4)	18 (2.1)	134 (3.0)
History of stroke (%)[#]	208 (1.3)	113 (1.0)	95 (2.0)	121 (1.0)	87 (2.0)	112 (1.1)	1 (0.2)	9 (0.3)	86 (2.4)
Elevated liver enzymes (%)[#]	1,192 (7.6)	540 (4.9)	652 (14.5)	756 (6.7)	436 (9.8)	511 (4.9)	29 (4.8)	245 (31.8)	407 (11.0)
High alcohol consumption (%)[#]	1,026 (16.5)	682 (15.4)	344 (19.8)	1,026 (21.5)	0 (0)	682 (16.6)	0 (0)	344 (75.4)	0 (0)
Viral hepatitis (%)[#]	383 (2.6)	269 (2.5)	114 (2.9)	383 (3.6)	0 (0)	269 (2.7)	0 (0)	114 (17.3)	0 (0)
Iron overload (%) [#]	407 (3.7)	306 (4.4)	101 (1.8)	407 (5.1)	0 (0)	306 (4.7)	0 (0)	101 (10.9)	0 (0)

[*]Continuous values were presented as mean (95% confidence interval) and categorical variables were presented as counts (percentages), weighted by sample weights. Percentages may not total 100 due to rounding.

[#] The values were missing for some participants.

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; Control, participants without MAFLD or NAFLD; N, participants only with NAFLD; M, participants only with MAFLD; M+N, participants with MAFLD and NAFLD; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1C, glycosylated hemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; AST, aspartate transaminase; ALT, alanine transaminase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; NFS, NAFLD fibrosis score; APRI, AST-to-platelet ratio index; FIB-4, Fibrosis-4 index.

score, ALP, and CRP was adjusted [HR 1.21 (1.09, 1.33)], but this increase was non-significant when waist circumference, HbA1c, SBP, TG, and fasting insulin were further adjusted [HR 1.03 (0.93, 1.15)]. In reference to non-NAFLD participants, NAFLD increased the risk for all-cause mortality by around 50%, and the significance was lost after age, sex and ethnicity related factors were corrected [HR 1.05 (0.87, 1.28)] (**Table 2**). Both MAFLD and NAFLD showed a relatively significant positive correlation with cardiovascular and neoplasm mortality, however, risks of these mortalities were equal between participants with and without MAFLD or NAFLD after age and sex were adjusted. The relative risk of diabetes-related mortality was markedly elevated in participants with either MAFLD or NAFLD even after all factors were adjusted.

We further divided the participants into four groups. In reference to the group without MAFLD and NAFLD (control group), group N reduced all-cause mortality by around 50%, and the association was non-significant after age, sex and race-ethnicity were adjusted; group M independently increased the risk of all-cause mortality by 47%; group M+N was significantly associated with elevated all-cause mortality unless waist circumference, HbA1c, SBP, TG and fasting insulin were adjusted [HR 0.96 (0.86, 1.07)] (**Figure 2A**, **Table 2**). For cardiovascular mortality, group M and group M+N both showed an increased risk than control in-crude, but this risk was unaltered in group M after FIB-4, NFS score, CRP and ALP score were adjusted and in group N+M after sex and age were adjusted (**Figure 2B**, **Table 2**). Group M independently increased the risk of neoplasm mortality after all confounders were adjusted. The risk of neoplasm mortality was reduced in group N and enhanced in group M+N in reference to control group in-crude (**Figure 2C**, **Table 2**). Group M and group M+N relatively enhanced risk of diabetes-related mortality unless corrected by metabolic factors, compared with the control group (**Figure 2D**, **Table 2**). The risk of group N in diabetes-related risk was unavailable without enough events.

Subgroup Analysis of MAFLD

The risk of the MAFLD for overall mortality was similar in subgroups with different age, BMI, severity of hepatic steatosis, diabetes, hypertension, dyslipidemia, metabolic syndrome, FIB-4, and other etiologies (**Figure 3**). Significant heterogeneity was only found in different ethnicities and presence of viral hepatitis (Bonferroni corrected). MAFLD increased risk for all-cause mortality in non-Hispanic white race [HR 1.37 (1.22, 1.54)], with viral hepatitis [HR 2.56 (1.56, 4.21)] or without viral hepatitis [HR 1.24 (1.13, 1.37)]. There was no difference in subgroups in cardiovascular and neoplasm mortality risk in MAFLD (**Supplementary Figures 1, 2**).

DISCUSSION

Compared with patients with NAFLD, patients with MAFLD had increased risk for all-cause mortality in a greater magnitude in spite of similar cardiovascular, neoplasm and diabetes-related mortality risk. The nomenclature changes excluded participants who were negatively associated with mortality and captured

TABLE 2 | Cox regression model for overall and disease-specific mortality of participants.

	Deaths	Unadjusted HR	Model 1	Model 2	Model 3
Overall mortality					
MAFLD	1,561	2.07 (1.86, 2.29)*	1.27 (1.16, 1.41)*	1.21 (1.09, 1.33)*	1.03 (0.93, 1.15)
NAFLD	1,326	1.47 (1.20, 1.79)*	1.05 (0.87, 1.28)	0.99 (0.81, 1.20)	0.81 (0.66, 1.00)
Cardiovascular mortality					
MAFLD	409	2.01 (1.66, 2.44)*	1.17 (0.96, 1.42)	1.10 (0.90, 1.34)	0.83 (0.68, 1.02)
NAFLD	352	1.53 (1.26, 1.86)*	1.07 (0.89, 1.30)	0.99 (0.81, 1.21)	0.80 (0.65, 0.98)*
Neoplasm mortality					
MAFLD	356	1.78 (1.45, 2.17)*	1.16 (0.94, 1.42)	1.12 (0.91, 1.39)	1.12 (0.88, 1.41)
NAFLD	307	1.31 (1.06, 1.61)*	1.01 (0.82, 1.25)	0.98 (0.79, 1.22)	0.96 (0.76, 1.21)
Diabetes-related mortality					
MAFLD	99	6.86 (3.94, 11.95)*	4.57 (2.63, 7.97)*	4.40 (2.49, 7.76)*	1.84 (0.97, 3.50)
NAFLD	78	3.26 (1.90, 5.59)*	2.54 (1.49, 4.34)*	2.72 (1.59, 4.63)*	1.38 (0.81, 2.37)
Overall mortality					
Control	2,139	Ref	Ref	Ref	Ref
N	73	0.48 (0.34, 0.68)*	0.92 (0.65, 1.31)	0.95 (0.65, 1.38)	1.09 (0.75, 1.58)
M	308	2.76 (2.28, 3.33)*	1.87 (1.57, 2.23)*	1.73 (1.44, 2.08)*	1.47 (1.22, 1.77)*
N+M	1,253	1.85 (1.65, 2.07)*	1.17 (1.05, 1.29)*	1.12 (1.00, 1.24)*	0.96 (0.86, 1.07)
Cardiovascular mortality					
Control	551	Ref	Ref	Ref	Ref
N	15	0.46 (0.20, 1.02)	1.01 (0.45, 2.30)	0.93 (0.36, 2.42)	1.24 (0.48, 3.25)
M	72	2.35 (1.60, 3.45)*	1.53 (1.03, 2.28)*	1.47 (0.98, 2.20)	1.05 (0.70, 1.58)
N+M	337	1.86 (1.51, 2.28)*	1.11 (0.91, 1.35)	1.03 (0.84, 1.27)	0.80 (0.64, 0.98)
Neoplasm mortality					
Control	530	Ref	Ref	Ref	Ref
N	21	0.46 (0.24, 0.89)*	0.81 (0.42, 1.56)	0.88 (0.46, 1.72)	0.89 (0.46, 1.72)
M	71	2.16 (1.50, 3.10)*	1.54 (1.08, 2.20)*	1.59 (1.12, 2.26)*	1.58 (1.09, 2.28)*
N+M	285	1.63 (1.31, 2.02)*	1.08 (0.87, 1.35)	1.04 (0.83, 1.31)	1.04 (0.81, 1.34)
Diabetes-related mortality					
Control	66	Ref	Ref	Ref	Ref
N	0	NA	NA	NA	NA
M	21	9.13 (4.15, 20.05)*	6.66 (3.03, 14.62)*	5.53 (2.61, 11.71)*	2.09 (0.71, 6.14)
N+M	78	5.86 (3.25, 10.58)*	4.01 (2.23, 7.22)*	4.02 (2.33, 6.94)*	1.78 (0.95, 3.35)

*$p < 0.05$.

Values were presented as hazard ratio (95% confidence interval). Model 1: adjusted by age, sex and race-ethnicity (N = 12,480). Model 2: Model 1+ adjusted by FIB-4 score, NFS score, CRP, ALP (N = 12,281). Model 3: Model 2 + adjusted by waist circumference, HbA1c, SBP, TG, fasting insulin (N = 12,281). HR, hazard ratio; MAFLD, metabolic dysfunction-associated fatty liver disease, compared with non-MAFLD participants; NAFLD, non-alcoholic fatty liver disease, compared with non-NAFLD participants; Control, participants without MAFLD or NAFLD; N, participants only with NAFLD; M, participants only with MAFLD; M+N, participants with MAFLD and NAFLD; NA, not applicable.

participants who had higher all-cause mortality risk. The risk of mortality was similar among MAFLD subgroup except for non-Hispanic white race and viral hepatitis comorbidity.

Our study identified that patients diagnosed with new definition would have greater all-cause mortality risk in a medium follow-up time of 22.8 years. The risk for cardiovascular and neoplasm mortality was similar between MAFLD and NAFLD. Similarly, a previous study suggested that the fatal and non-fatal cardiovascular outcomes were similar between NAFLD and MAFLD after a 7-year follow-up (17). This indicated that the term MAFLD emphasized total mortality risk but did not affect the major outcomes of fatty liver. Adjusting confounders for mortality step by step, we found the association between NAFLD and all-cause mortality was non-significant after age and sex were adjusted and the risk of MAFLD on all-cause mortality was largely attributable to the dysregulated metabolic profile. The impact of metabolic disorder on mortality was more prominent in MAFLD compared with NAFLD. The risk of fatty liver on cardiovascular and neoplasm mortality was mainly owning to age, sex and race and our study showed cause-specific mortality was similar between NAFLD and MAFLD.

Some researchers were concerned that this new definition may lose some participants, especially those with severe steatosis (24). However, our study suggested in the patients excluded after the name switch, only 6% had severe steatosis. The excluded patients were mainly participants with NAFLD without apparent metabolic disorder, who had a "cardio-protective"

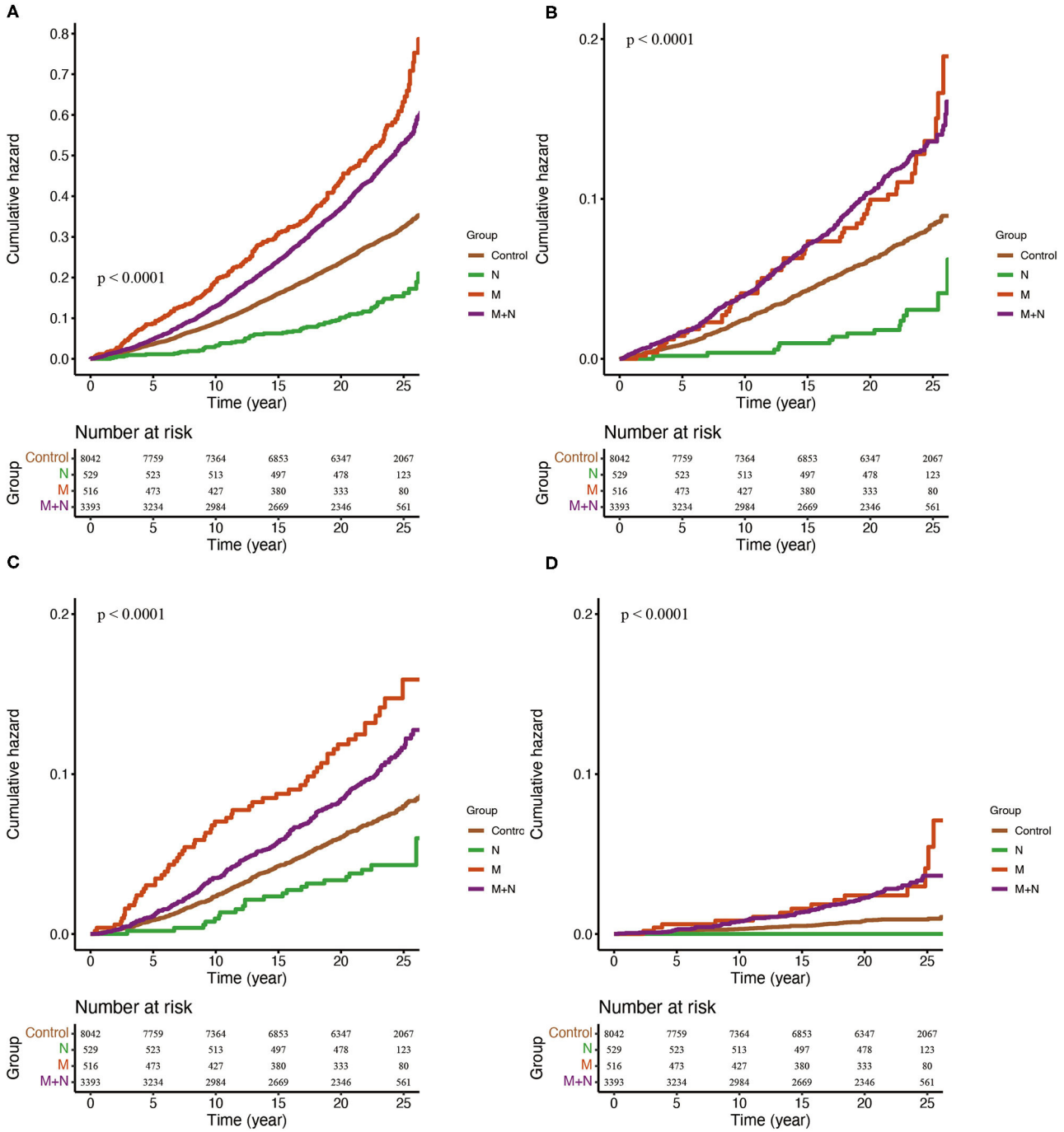

FIGURE 2 | Kaplan-Meier estimates of overall **(A)**, cardiovascular **(B)**, neoplasm **(C)** and diabetes-related **(D)** mortality. Control, participants without MAFLD or NAFLD. N, participants only with NAFLD; M, participants only with MAFLD; M+N, participants with MAFLD and NAFLD.

metabolic profile as well as significantly lower liver enzymes and hepatic fibrosis scores. More importantly, they showed reduced risk with mortality possibly owing to their young age and low levels of metabolic disorder. Therefore, the participants excluded might not be the priority for clinical intervention and drug development.

Other researchers found that the change in terminology included more patients with metabolic disorders (14), these patients were included in group M in our study. This group was independently associated with all-cause mortality, especially with neoplasm mortality. They were identified with the worst metabolic profile and advanced

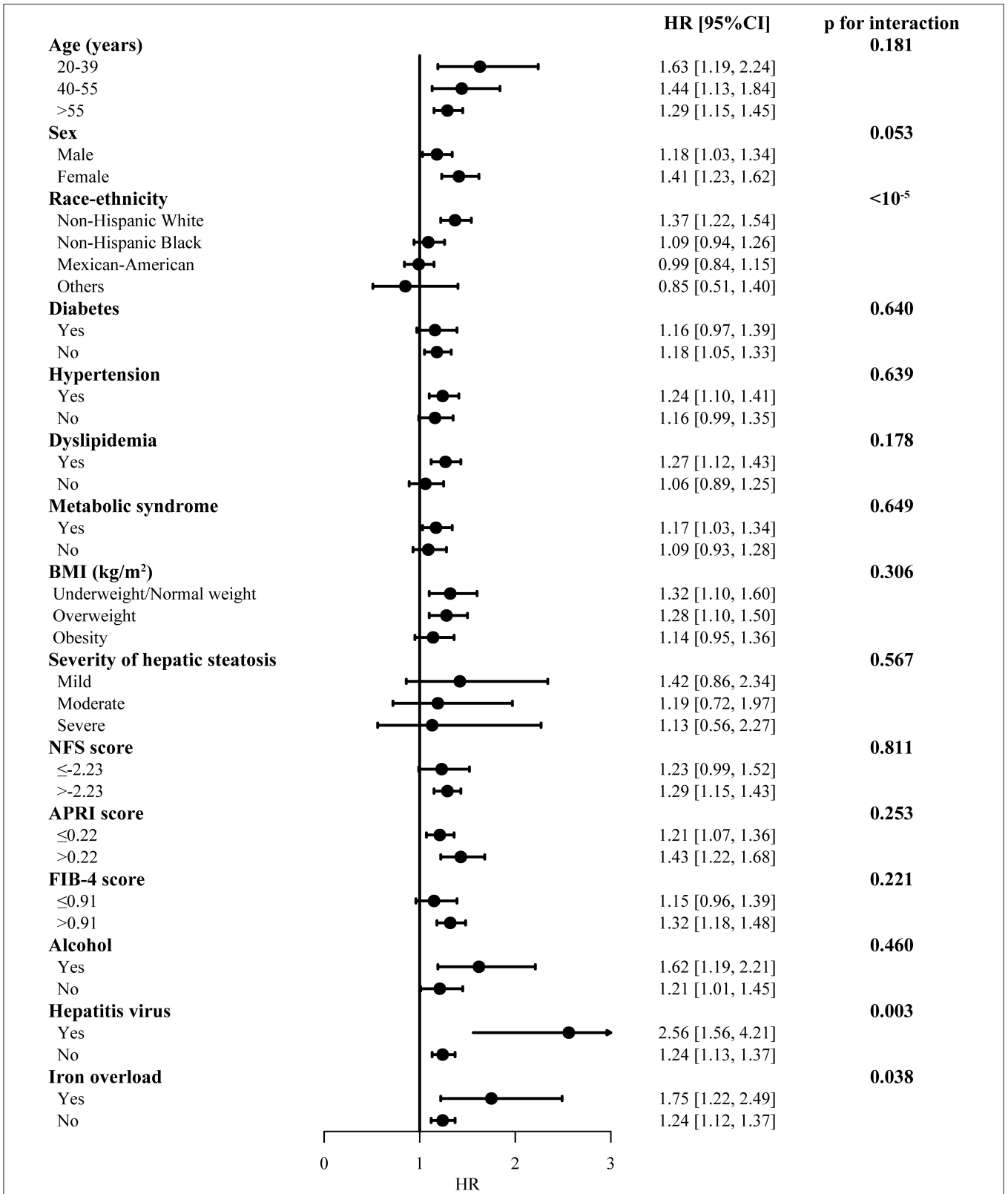

FIGURE 3 | Subgroup analysis for the overall mortality in participants with MAFLD. The model was adjusted by adjusted by age, sex and race-ethnicity. MAFLD, metabolic dysfunction-associated fatty liver disease, compared with non-MAFLD participants; BMI, body mass index; HR, hazard ratio; CI, confidence internal. Significance was determined as $p < 0.0033$ (Bonferroni correction applied).

hepatic inflammation and fibrosis, indicating possible worse liver outcomes (25, 26). These patients ignored by previous criteria of NAFLD were mainly patients with alcohol abuse, hepatic viral infection and iron overload, which tended to speed up the progression of extracellular and hepatocellular cancer (27). The risk for total mortality and cardiovascular mortality was similar between group M and group M+N, indicating the drugs developed for MAFLD may also be applicable for group M. Also, MAFLD with or without other etiology showed no heterogeneity in subgroup analysis regarding all-cause mortality risk. In this scenario, patients in group M may also benefit from drugs developed for MAFLD.

Similar HRs were observed in subgroups of age, sex, smoking status, metabolic dysfunction, hepatic steatosis, FIB-4, and different etiologies. We only detected significant heterogeneity of race-ethnicity and presence of hepatic viral infection in all-cause mortality, after Bonferroni correction ($p_{\text{interaction}} < 0.05/13$) was applied. The hazard ratio of MAFLD was highest in non-Hispanic white, followed by non-Hispanic black, Mexican-American and other races. As non-Hispanic whites accounted for over three fourth in the United States, they may be the group most affected by this disease. The design for clinical trials could possibly consider stratify patient recruitment according to ethnicities. We also observed significantly greater risks for overall mortality among patients with MAFLD secondary to hepatic viral infection, whereas failing tested the heterogeneity in cause-specific mortality. As the seventh leading cause of death globally and an increasing epidemic trend (28), hepatic virus infection may primarily accelerate the course of liver-related especially with comorbidity of fatty liver disease. Our results suggested drug development for patients with MAFLD should take racial difference and viral hepatitis infection into consideration in the future.

Our study used a large population-based prospective cohort with long follow-up to analyze the association between MAFLD/NAFLD and mortality. However, there are several limitations to our current study. The liver outcomes, especially fine categorization of liver cancer and advanced cirrhosis, were still needed for a comprehensive vision on the natural history of fatty liver disease. We were unable to perform the analysis due to data acquisition limitations. However, the incidence rate of cardiovascular mortality was around 5 times higher than liver-specific mortality in NAFLD (1). Extrahepatic neoplasm may be a primary source for extra mortality in NAFLD (5). In the NHANES III cohort, NAFLD showed similar liver-related mortality with non-NAFLD controls and liver-related mortality only account for <2% of total mortality (19, 20). Liver-related mortality become more relevant when the stages of steatosis progressed, however, this required precise categorization of fatty liver stages which we were unable to perform. The liver-related outcomes may change when other etiology, e.g., alcoholic liver disease (AFLD) and viral hepatitis, was included in MAFLD. Nevertheless, one study suggested in a fatty liver cohort with mixed background of NAFLD and AFLD, mortality from cardiovascular disease and total neoplasm still surpassed liver cirrhosis (29). In addition, the new definition emphasized the presence of metabolic derangements which mainly leads to elevated cardiovascular risk. By this means, we used total mortality, cardiovascular mortality and cancer mortality as our outcomes should still provide robust information to reveal the impact of nomenclature change. Secondly, hepatic steatosis in adults was detected by imaging techniques instead of liver histology, possibly weakening the reliability of the diagnosis of NAFLD. But one qualified meta-analysis showed high sensitivity and specificity in the detection of moderate-severe hepatic steatosis by ultrasound (30). With the improvement of ultrasound, imaging techniques still had limited sensitivity to detect mild steatosis (31). The study only used ultrasound results 30 years ago and the sensitivity of ultrasound detection was greatly improved in recent years (32). Thirdly, we did not excluded the drug-induced hepatotoxicity in the NAFLD definition since we were unable to establish causal relationship between drug use history and fatty liver in an epidemiological survey. One study reported very small portion of participants taken drugs related to hepatotoxicity, and there was no significant difference in mortality after excluding them (33). Finally, some non-statistically significant findings may be related to the limited sample size especially in subgroup analysis, indicative of lower power of the study. More similar studies should be designed and integrated to reduce type 2 error.

In conclusion, using baseline and follow-up data from the cohort of NHANES III, we found MAFLD had an enhanced risk for mortality and similar risk for cause-specific mortality with NAFLD. The definition MAFLD emphasized the role of metabolic disorder on the outcomes of fatty liver since the risk of MAFLD for mortality was largely attributable to its metabolic disorder. The switch from NAFLD to MAFLD captured participants with higher mortality risk regardless of losing some patients with reduced mortality risk. Ethnic differences and the presence of virus hepatitis should be taken into consideration when trials investigating outcomes for MAFLD were implemented.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/nh3data.htm.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NCHS Ethics Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QH, XZo, and LJ designed the research. QH and XZo collected, analyzed the data, and drafted the manuscript. XW and XZh

revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.693507/full#supplementary-material

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SYSTEMATIC REVIEW
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The Effects of Modest Alcohol Consumption on Non-alcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis

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Background and Objective: There is no consensus regarding modest alcohol consumption in patients with non-alcoholic fatty liver disease (NAFLD) due to conflicting results. The aim of this meta-analysis was to examine the effects of modest alcohol consumption on histological severity, histological course, hepatocellular carcinoma, and long-term clinical outcomes in NAFLD patients.

Methods: We searched MEDLINE and EMBASE databases from inception to October 2020 for studies evaluating the effects of modest alcohol consumption among patients with NAFLD. A random-effects meta-analysis using pooled odds ratio (OR) and hazard ratio (HR) was calculated with 95% confidence interval (CI). Study quality was assessed with the Newcastle-Ottawa Scale.

Results: Fourteen cross-sectional or cohort studies with aggregate data on 14,435 patients were included in the analysis. Modest alcohol consumption resulted in lower risks for steatohepatitis (OR 0.59; 95% CI 0.45–0.78; $I^2 = 12\%$) and advanced fibrosis (OR 0.59, 95% CI 0.36–0.95; $I^2 = 75\%$). Histological follow-up data showed that modest alcohol use was associated significantly with less steatohepatitis resolution but not with fibrosis progression. The HR for developing hepatocellular carcinoma was 3.77 (95% CI 1.75–8.15; $I^2 = 0\%$). NAFLD patients with modest alcohol intake had a lower mortality risk than lifelong abstainers (HR 0.85; 95% CI 0.75–0.95; $I^2 = 64\%$).

Conclusion: This meta-analysis suggests that medical advice for modest alcohol drinking should be made cautiously in caring for an individual patient based on the clinical context. Practically, patients with steatohepatitis or advanced fibrosis should avoid alcohol use, whereas patients with low fibrosis risk may be allowed for modest and safe drinking.

Keywords: non-alcoholic fatty liver disease, modest alcohol, histology, hepatocellular carcinoma, mortality, NAFLD, meta-analysis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disorder affecting approximately a quarter of the adult population worldwide (1, 2). NAFLD comprises a continuum of disease severities from steatosis to non-alcoholic steatohepatitis (NASH). It can evolve into an advanced disease that progresses to cirrhosis, liver failure, and an increased risk of hepatocellular carcinoma (HCC) (3). Moreover, NAFLD patients have increased risk of cardiovascular events, other malignancies, and mortality (2, 3). Insulin resistance is a common feature in NAFLD patients, and it is considered the major contributor to the development and progression of the disease (4).

Alcohol consumption in modest quantity is believed to improve insulin resistance, lipid metabolism, and inflammatory status, thereby exerting cardiovascular and metabolic benefits (5). These effects have been shown to reduce the risk of diabetes, cardiovascular disease incidence, and mortality in a J-shape dose-response (6–8). Specific types of alcohols such as red wines and certain drinking patterns, for instance, modest consumption, not binge drinking displayed superior cardiometabolic benefits (9). The benefits of modest alcohol consumption also decrease the risk of developing NAFLD in the general population (10–12). However, recommendations on alcohol consumption among patients with pre-existing NAFLD, where metabolic syndrome and established cardiovascular disease are common comorbidities, remain a topic of vigorous debate, given that the evidence supporting the protective benefits of modest alcohol on liver-related outcomes is less consistent. To date, studies have reported varying results on histological severity, the natural course of liver disease, as well as liver-related outcomes, particularly the development of HCC (13–27). Consequently, there is no current consensus in clinical practice for counseling patients with NAFLD regarding modest alcohol consumption.

Therefore, this systematic review and meta-analysis were performed to comprehensively assess the effects of modest alcohol intake on histological severity, histological progression, and the risk of significant clinical outcomes, namely, the development of cirrhotic complications, HCC, and all-cause death among patients with NAFLD.

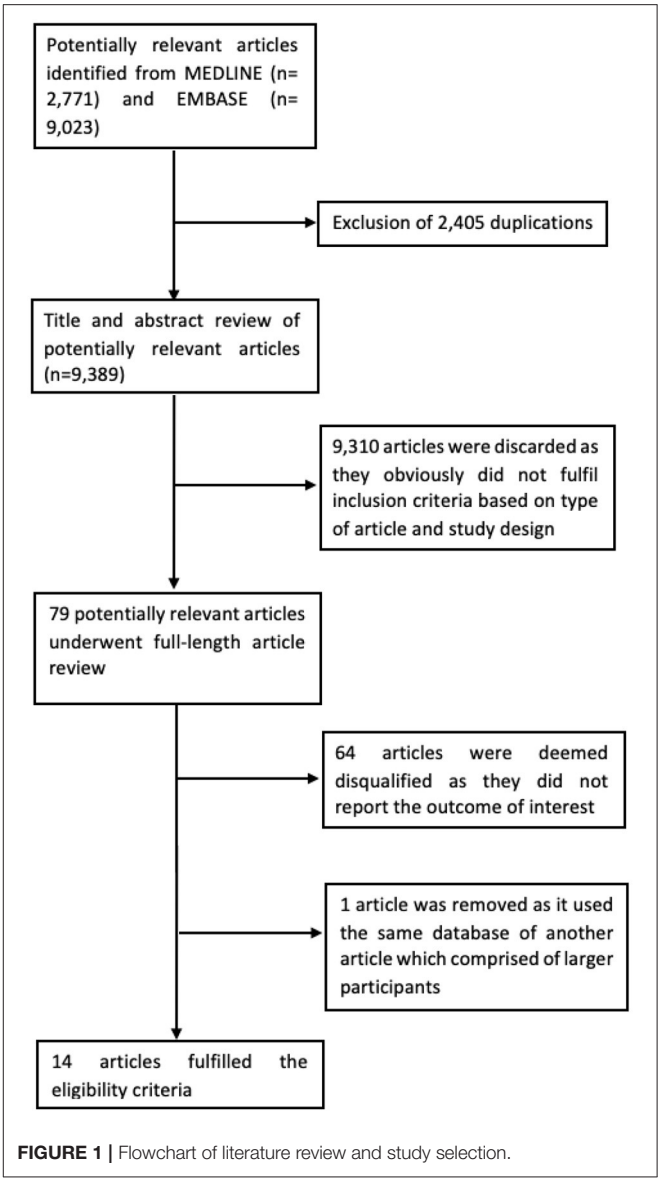

FIGURE 1 | Flowchart of literature review and study selection.

METHODS

Search Trials

Systematic literature review of EMBASE and MEDLINE databases from inception to October 2020 to identify all published studies that evaluated the effects of alcohol consumption on histological severity, histological progression, or clinical events in patients with NAFLD was independently conducted by two investigators (WW and SN). The search strategy that included the terms for "modest alcohol consumption" and "non-alcoholic fatty liver disease" is available in **Supplementary Data 1**. To ensure the comprehensiveness of eligible studies, the literature review was also conducted from the bibliography of the eligible studies initially retrieved from EMBASE and MEDLINE. This study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement (**Supplementary Data 2**).

Inclusion and Exclusion Criteria

Eligible studies must be full-text English articles. To evaluate the effects of modest alcohol consumption on histological severity, an eligible study had to be a cross-sectional study of biopsy-proven NAFLD patients and had to report whether modest alcohol intake was associated with NASH or advanced fibrosis compared to

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; OR, odds ratio; HR, hazard ratio; CI, confidence interval; RR, relative risk; IRR, incidence rate ratio; HR, hazard risk ratio; SIR, standardized incidence ratio; NHANES III, National Health and Nutrition Examination Survey III; AUDIT-C, Alcohol Use Disorder Identification Test–Consumption; DM, diabetes mellitus; NAS, NAFLD Activity Score; N/A, Not available; SAF, Steatosis Activity and Fibrosis; SLDA, Skinner Lifetime Drinking Assessment.

TABLE 1A | Characteristics of cross-sectional studies assessing histological severity between non-drinkers and modest drinkers included in the review.

Author, year	Country	Participant number (Non-drinker/ modest drinker)	Age	Sex, % men	DM, %	Definition of modest alcohol drinking	Method of assessing alcohol intake	Histology scoring system	Newcastle Ottawa Scores (selection /comparability /outcome)
Dixon, 2001	Australia	48/17	41	21	18	<200 g/week	Questionnaire	NAS	3/2/3
Cotrim, 2009	Brazil	57/75	37	31	12	<40 g/day	Interview	Matteoni	4/0/2
Dunn, 2012	USA	252/331	48	34	16	<20 g/day	AUDIT-C, SLDA	NAS	5/2/3
Kwon, 2014	South Korea	25/52	47	44	N/A	<40 g/week	SLDA	NAS	5/2/3
Hagstrom, 2017	Sweden	60/60	56	69	41	<168 g/week	AUDIT-C	NAS, SAF	5/2/3
Ajmera, 2018	USA	117/168	47	30	34	< 20 g/day (men) <10 g/day (women)	AUDIT-C	NAS	4/0/3
Kimura, 2018	Japan	208/93	56	45	37	<20 g/day	Questionnaire	NAS, SAF	4/0/3
Mitchell, 2018	Australia	74/91	52	38	41	<210 g/week (men) <140 g/week (women)	Questionnaire	NAS	5/2/3
Yamada, 2018	Japan	101/77	50	52	67.0	<20 g/d	Self-report	NAS	4/0/2
Tan, 2020	Malaysia	55/16	N/A	N/A	N/A	<21 units/week (men) <14 units/week (women)	Self-report	NAS	3/0/3

AUDIT-C, Alcohol Use Disorder Identification Test–Consumption; DM, diabetes mellitus; NAS, NAFLD Activity Score; N/A, Not available; SAF, Steatosis Activity and Fibrosis; SLDA, Skinner Lifetime Drinking Assessment.

TABLE 1B | Characteristics of longitudinal follow-up studies on histological progression between non-drinkers and modest drinkers included in the review.

Author, year	Country	Participant number	Age	Sex, % men	DM, %	Definition of modest alcohol drinking	Method of assessing alcohol intake	Histology scoring system	Follow-up duration (year)	Newcastle Ottawa scores (selection/ comparability/ outcome)
Ekstedt, 2009	Sweden	71	47	72	7	<140 g/week	AUDIT, SLDA	NAS	13.8	4/2/3
Ajmera, 2018	USA	285	47	30	34	< 20 g/day (men) <10 g/day (women)	AUDIT	NAS	3.9	4/2/3

AUDIT-C, Alcohol Use Disorder Identification Test–Consumption; SLDA, Skinner Lifetime Drinking Assessment.

abstainers. The pre-requisite outcomes included odds ratio (OR) with 95% confidence interval (CI). For histological progression, eligible cohorts must include serial follow-up liver biopsy to examine how modest alcohol consumption altered the natural history of NAFLD liver histology. These studies must report relative risk (RR), incidence rate ratio (IRR), hazard risk ratio (HR), or standardized incidence ratio (SIR) with 95% CI. To elucidate the effects of modest alcohol on long-term clinical outcomes, eligible studies had to be cohorts reporting RR, IRR, HR, or SIR with 95% CI comparing the risk of the following major clinical events: development of cirrhotic complications (ascites, variceal bleeding, spontaneous bacterial peritonitis, and hepatic encephalopathy), HCC, and all-cause deaths between the two NAFLD cohorts of modest drinkers and abstainers. Modest alcohol drinking was defined as consumption of <21 standard drinks (210 g) per week for men and <14 standard drinks (140 g) per week for women, although some variations were accepted. Two reviewers (WW and SN) independently determined study eligibility. In the first round of screening, titles and abstracts were reviewed to exclude articles that did not fulfill the eligible criteria. The second round of screening involved a full-text review to ensure that the eligible studies fulfilled all inclusion criteria. Disagreements were resolved by discussion with the senior investigator (PC).

Data Extraction

Extracted data included author, the country where the study was conducted, study design, year of publication, number of participants, recruitment or identification of NAFLD participants, methods used to identify and verify the definition of modest drinkers and abstainers, clinical outcomes, histological classification utilized to diagnose NASH and advanced fibrosis, baseline characteristics of participants, the average duration of follow-up for cohort studies, confounders adjusted in multivariate analysis and adjusted effect estimates with corresponding 95% CI. The appraisal of the quality of the eligible cohort studies was performed according to Newcastle-Ottawa Scale (28). The modified version of this scale was used to appraise cross-sectional studies (29). The quality of each study was evaluated by two investigators (WW and SN), and any differences in opinions were settled by the senior investigator (PC).

TABLE 1C | Characteristics of cohort studies comparing the risk of major clinical events between non-drinkers and modest drinkers included in the review.

Author, year	Country	Participant number (non-drinker/modest drinker)	Age	Sex, % men	DM, %	Definition of modest alcohol drinking	Method of assessing alcohol intake	Follow-up duration (year)	Outcomes	Newcastle Ottawa scores (selection/ comparability/ outcome)
Ascha, 2010	USA	120/68	57	44	73	<30 g/day	Self-report	2.7	HCC	4/0/3
Kimura, 2018	Japan	93/208	56	45	37	<2 units/day	Self-report	6	HCC	4/2/3
Hajifathalian, 2019	USA	3318/1250	49	53	26	<1.5 units/day	Self-report	5.8	Death	4/2/3
Aberg, 2020	Finland	993/6638	54	60	14	<20 g/week	Self-report	11.1	Death	4/1/3

HCC, Hepatocellular Carcinoma.

Statistical Analysis

All data analyses were conducted using Review Manager 5.3 software from the Cochrane Collaboration (London, United Kingdom). The generic inverse variance method of DerSimonian and Laird was employed to pool point estimates of all eligible studies, in which the weight of each study for the pooled analysis was in reversal to its standard error (30). Random-effects model was utilized as the eligible studies had different background populations and protocols. The Cochran's Q test and The I^2 statistic were employed to determine statistical heterogeneity. An I^2 value of >75% represented high heterogeneity, 51–75% moderate heterogeneity, 26–50% low heterogeneity, and 0–25% insignificant heterogeneity (31). Publication bias was evaluated with a funnel plot.

RESULTS

A total of potentially relevant 11,794 articles (9,023 from EMBASE and 2,771 from MEDLINE) were retrieved. After removing 2,405 duplicated articles, 9,389 articles remained for the first-round review. We then excluded 9,310 articles because they did not fulfill the inclusion criteria based on study design and types of articles resulting in 79 remaining articles for the second round full-text review. Fifteen studies fulfilled the inclusion criteria and were included in this study (13–27). However, Younossi et al. (27) and Hajifathalian et al. (25) used the identical database of National Health and Nutrition Examination Survey III (NHANES III); therefore, we selected Hajifathalian et al. due to the larger number of participants. **Figure 1** provides an overview of the literature review and study selection process. **Tables 1A–C** summarizes the study design, characteristics of participants, and Newcastle-Ottawa Scale of the included studies.

The definition of both abstainers and modest alcohol drinkers varied considerably across included studies from lifetime abstainers to 0.5 standard drink per day and 20 g of alcohol per week to 21 standard drinks (210 g) of alcohol per week, respectively. The definition for maximum amount for modest drinking in men was higher than that of women in three studies (13, 17, 22). All cross-sectional studies were biopsy-based studies. We did not find any studies examining the effects of modest alcohol on the development of cirrhotic complications in the NAFLD population.

Risk of Steatohepatitis Among NAFLD Patients With Modest Alcohol Consumption

Six cross-sectional studies investigating the association between modest alcohol drinking and NASH are shown in **Figure 2A** (13–18). Modest alcohol consumption had a lower prevalence of biopsy-proven NASH among NAFLD patients with a pooled OR of 0.59 (95% CI, 0.45–0.78; $I^2 = 12\%$).

Risk of Advanced Fibrosis Among NAFLD Patients With Modest Alcohol Intake

Eight cross-sectional studies comparing modest alcohol drinking to non-drinker were identified (13, 16–22). The pooled OR for advanced fibrosis among NAFLD patients with modest alcohol intake was 0.59 (95% CI, 0.36–0.95; $I^2 = 75\%$) (**Figure 2B**). Additionally, we conducted a sensitivity analysis for advanced fibrosis based on Newcastle-Ottawa scores of those studies. Studies with full Newcastle-Ottawa scores (16, 19, 21, 22) were regarded as high-quality studies and were included in the first sensitivity analysis. Four studies were considered high-quality and yielded a pooled OR of 0.45 (95% CI, 0.33–0.60; $I^2 = 0\%$) (**Figure 2C**). The other four studies (13, 17, 18, 20) were regarded as low-quality studies and were included in the second sensitivity analysis. Its pooled OR was 0.95 (95% CI, 0.53–1.70; $I^2 = 63\%$) (**Figure 2D**).

Systematic Review of the Histopathological Progression of the NAFLD Population With Modest Alcohol Consumption

Two studies investigating the histopathological progression of NAFLD populations were identified and included in the narrative review, but not in quantitative analysis because the results of each study varied significantly and could not be combined (13, 26). Ajmera et al. found that modest alcohol drinking was associated with less NASH resolution among NAFLD patients, with an OR of 0.32 (95% CI, 0.11–0.92) (13). Ekstedt et al. have shown that modest alcohol intake was not significantly associated with risk of a significant fibrosis progression in NAFLD (OR 0.93, 95% CI, 0.10–9.06) (26).

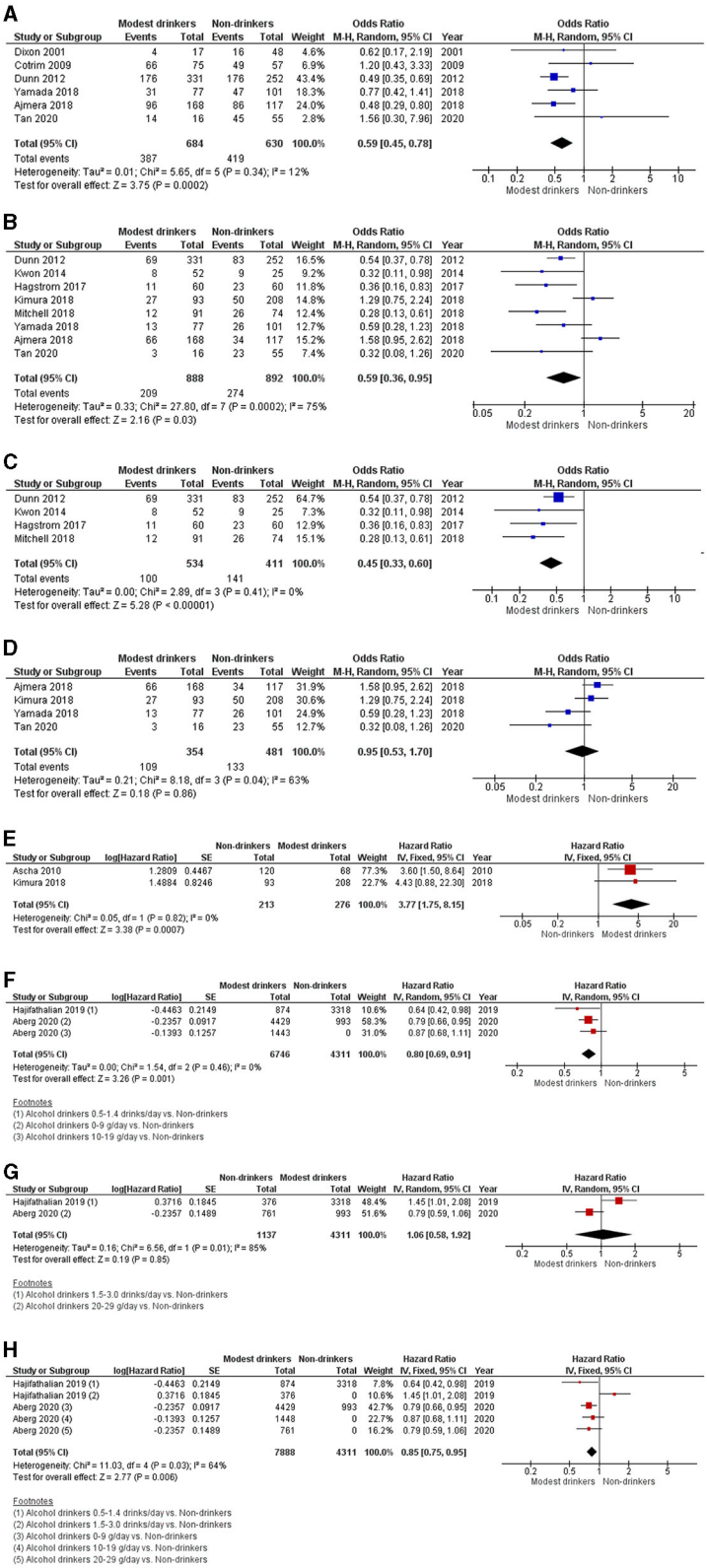

FIGURE 2 | Forest plot of meta-analyses for the association between modest alcohol consumption in NAFLD patients and **(A)** steatohepatitis; **(B)** advanced fibrosis; **(C)** advanced fibrosis among high-quality studies; **(D)** advanced fibrosis among low-quality studies; **(E)** the development of HCC; **(F)** mortality for light alcohol consumption; **(G)** mortality for moderate alcohol consumption; and **(H)** light-to-moderate alcohol consumption.

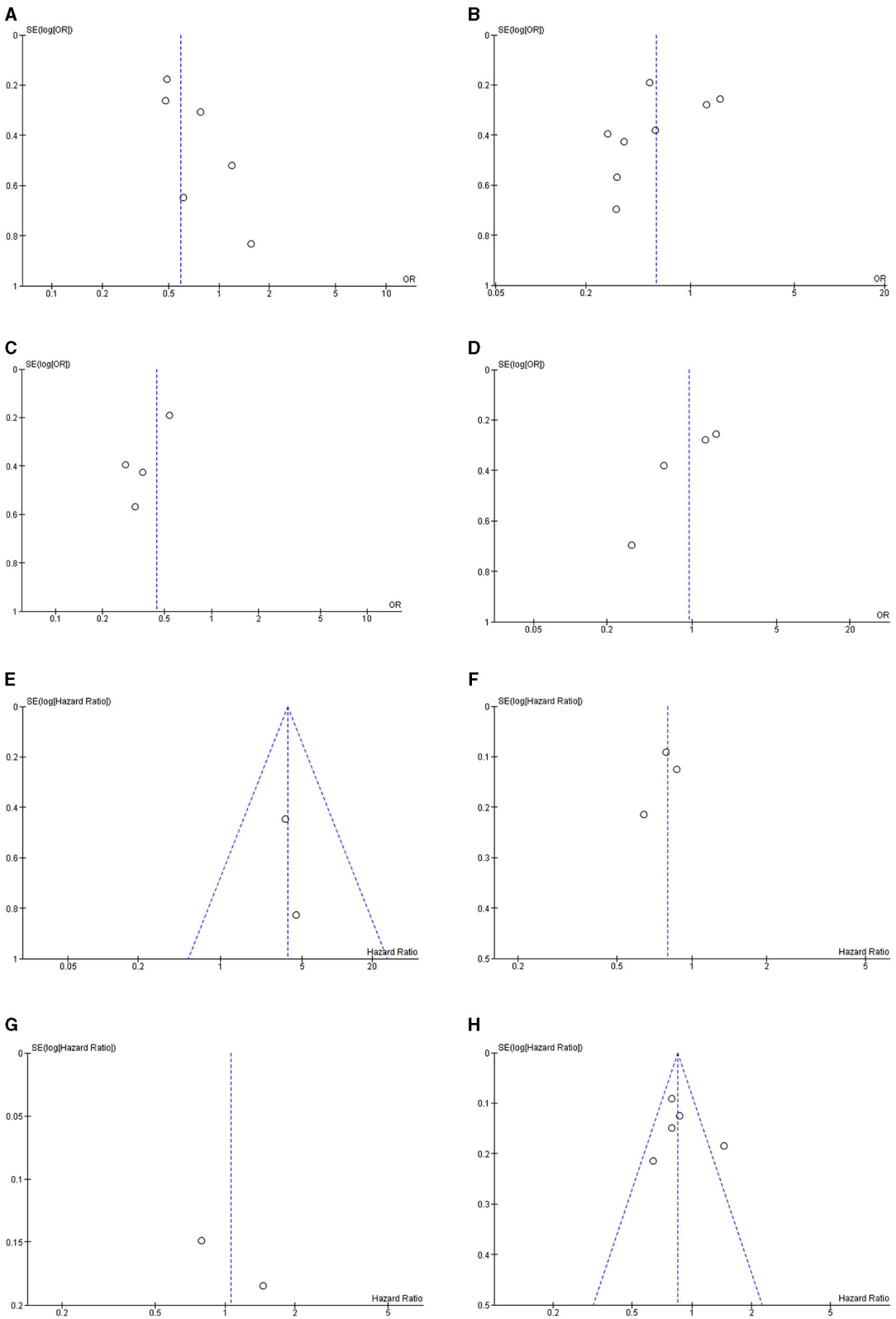

FIGURE 3 | Funnel plot of meta-analyses for the association between modest alcohol consumption in NAFLD patients and **(A)** steatohepatitis; **(B)** advanced fibrosis; **(C)** advanced fibrosis among high-quality studies; **(D)** advanced fibrosis among low-quality studies; **(E)** the development of HCC; **(F)** mortality for light alcohol consumption; **(G)** mortality for moderate alcohol consumption; and **(H)** light-to-moderate alcohol consumption.

Risk of HCC Among NAFLD Patients With Modest Alcohol Consumption

The pooled HR from two cohort studies of HCC development was 3.77 (95% CI, 1.75–8.15; $I^2 = 0\%$) (**Figure 2E**) (20, 23).

Overall Mortality Among NAFLD Patients With Light, Modest, and Light-To-Modest Alcohol Consumption

The pooled analysis of two cohort studies showed an HR of 0.80 (95% CI, 0.69–0.91; $I^2 = 0\%$) for light consumption ($\leq$19 g/day or $\leq$1.4 drink/day), 1.06 (95% CI, 0.58–1.92; $I^2 = 85\%$) for modest consumption (1.5–3.0 drinks/day or 20–29 g/day) and 0.85 (95% CI, 0.75–0.95; $I^2 = 64\%$) for light-to-modest alcohol consumption ($\leq$30 g/day) on mortality in NAFLD populations (**Figures 2F–H**) (24, 25).

Risk of Publication Bias

Funnel plots of all meta-analyses demonstrated asymmetry and suggested the presence of publication bias (**Figures 3A–H**).

DISCUSSION

This comprehensive systematic review and meta-analysis suggest a possible association between modest alcohol consumption and decreased NASH and advanced fibrosis. However, moderate alcohol use may diminish the resolution of NASH and increase risk of HCC in NAFLD patients with advanced fibrosis. In contrast, the data from population-based samples show a protective effect of low-to-moderate alcohol consumption on mortality in patients with NAFLD.

The present meta-analysis observed that modest alcohol drinking was associated with a lower risk of having steatohepatitis and advanced fibrosis in biopsy-proven NAFLD patients. This phenomenon may be explained by the modulation of insulin sensitivity and anti-inflammatory effects of modest alcohol intake, resulting in the attenuation of intrahepatic lipid synthesis, accumulation, and subsequent hepatic steatosis (32–37). The improvement of insulin resistance could moderate lipotoxicity, organelle stress, and hepatocyte injury caused by toxic reactive oxygen species generated by lipid metabolism (32, 38). Interestingly, the effect on advanced fibrosis was only observed in higher-quality studies where ascertainment of alcohol consumption was conducted using validated tools, primarily AUDIT-C and Skinner Lifetime Drinking History. In contrast, the remaining studies involved some degree of self-reported questionnaires or interviews. This could potentially lead to recall bias in the latter group of studies and implies its results since under-reporting may be as high as 40–50% in an alcohol consumption survey and remains a barrier to accurate quantification (39).

The increased risk of HCC among patients with NAFLD who consumed a modest amount of alcohol in our analysis was not unexpected and further emphasized the potentially harmful effects of alcohol. It is known that alcohol is an independent risk factor for the development of HCC both directly via DNA damage from toxic metabolites, oxidative stress, and inflammation and indirectly via chronic liver disease and cirrhosis (20, 40, 41). Furthermore, alcohol in conjunction with diabetes and obesity, as are highly prevalent in the NAFLD study population, also exhibits a synergistic interaction and potentially augments the risk of HCC development (20, 23, 41). Our finding was consistent with a previous study by Kawamura et al. demonstrating that the elevated risk of hepatocarcinogenesis started trending with the daily consumption of 20–39 g of ethanol. However, Kawamura et al. used a light drinker of $<$20 g per day as the baseline comparator, which differed from our baseline group consisted of abstainers (42). It is also worth noting that all of the patients in Ascha et al., which was weighted at 77% in our analysis, were cirrhotic and were referred for liver transplantation listing due to hepatic decompensation. Therefore, there could be confounding factors for an increased risk of developing HCC via the omission of non-cirrhotic and compensated cirrhosis populations (23). In addition, Kimura et al. found in the multivariate analysis that HCC was associated with fibrosis but not with a mild drinking habit and that all HCC patients had advanced fibrosis (fibrosis stage 3–4) (20). As a result, the interpretation of the risk of HCC development should be made with caution due to the limitations of the NAFLD population. Furthermore, this analysis consisted of only two eligible studies in which HCC was identified exclusively in patients with advanced fibrosis/cirrhosis. Therefore, further research to clarify the actual effect of modest alcohol drinking on the development of HCC across the spectrum of NAFLD patients is needed.

It is well-established that light to moderate alcohol consumption is associated with lower mortality for all-cause, cardiovascular, and cerebrovascular deaths via moderation of metabolic profiles (43, 44). However, studies demonstrating these protective effects were primarily conducted in the general population in national surveys (44). In line with these data, our analyses focused on patients diagnosed with NAFLD and found that modest alcohol consumption was associated with a reduction in all-cause mortality. This outcome could be driven by the decrease as mentioned earlier in the prevalence of advanced fibrosis, which was a significant predictor for long-term overall mortality among biopsy-proven NAFLD patients (45). Consequently, as a knock-on effect of advanced fibrosis reduction, mortality from cirrhosis as the leading cause of death (46) might be attenuated as a result. In addition, cardiovascular death is the leading cause of deaths among NAFLD patients, given the shared atherosclerotic risk factors such as age, diabetes, hypertension, dyslipidemia, insulin resistance, and metabolic syndrome (46, 47). Hence, NAFLD is unsurprisingly considered a risk for cardiovascular disease (48), and it is possible that the cardiometabolic benefits of modest alcohol consumption extended from the general population onto this particular group of NAFLD patients accompanied by atherosclerotic risks. This is particularly evident in Aberg et al. that cardiovascular outcomes, albeit not death-exclusive, were lower among very light drinkers (24). Similarly, we found that all-cause mortality benefits only persisted in light drinkers when patients were grouped according to light or moderate drinking habits. This finding implies that alcohol may not confer its protective effects

when consumed beyond a very low threshold. Different types of alcoholic beverages would also need to be accounted for, and further studies to prove this causal relationship in the NAFLD population.

Our meta-analysis has some limitations, which are inherent to the design of the included studies. First, the cross-sectional design of the studies evaluating the effects of alcohol use on the severity of liver disease limits our ability to establish causality of the observed associations. Several studies displayed that moderate alcohol drinkers tended to have higher socioeconomic status, increased physical activity, and less obesity than abstainers (49, 50). These factors have been demonstrated to influence drinking patterns and may affect the severity of liver disease, thereby confounding the association between alcohol use and NAFLD. Second, some of these studies reported incomplete adjustments for potential confounders, and thus reliability of the findings is diminished. Third, although longitudinal cohort studies provided the high quality of the prognostic relevance of modest alcohol use on clinical outcomes in NAFLD, these studies failed to obtain lifetime drinking histories to evaluate past heavy alcohol use. Thus, the population abstaining from alcohol drinking may be enriched for former heavy drinkers, leading to selection bias and more severe liver disease. Fourth, another potential limitation of population-based studies is that NAFLD diagnosis was made using serum biomarkers of steatosis such as fatty liver index and hepatic steatosis index. Accordingly, it is inevitable to have misclassified some of the participants in these studies concerning the presence or absence of NAFLD. Finally, although a random-effects model was applied in this meta-analysis, some findings need to be interpreted cautiously, given the high heterogeneity observed. From the results of the sensitivity analyses, it is assumed that high heterogeneity reflects differences in the tools used for alcohol assessment and characteristics of study populations.

Despite these considerations, this meta-analytic study also has important strengths. First, we believe that the topic of our meta-analysis is clinically relevant, given the conflicting literature on the effects of modest alcohol use in NAFLD and emerging data regarding possible mechanisms of modest alcohol protection for NAFLD. Second, we included studies that performed a liver biopsy to diagnose NASH and assess the liver fibrosis stage, and thus, the histological severity association was ascertained by the gold standard. Third, for the clinical outcomes, the included cohorts had long-term follow-up duration for the pre-specified outcomes to occur adequately. Follow-up time for HCC development, especially in patients with advanced fibrosis and cirrhosis, was as long as six years, while the mean follow-up for mortality was up to 11.1 years. Finally, we used standardized risk estimates from all eligible studies to combine estimates across studies.

CONCLUSIONS

Conflicting results from high heterogeneity of studies and evidence on whether modest alcohol consumption is detrimental or beneficial make clinicians uncertain for counseling abstinence or allowing modest alcohol drinking for potential health benefits. Thus, medical advice should be made cautiously in the context of individual clinical implications. Undoubtedly, patients with NASH and advanced fibrosis should be considered as high-risk groups for progressing to end-stage liver disease; hence, alcohol drinking should be avoided. On the contrary, NAFLD patients with low fibrosis risk may be allowed for modest and safe drinking. Thus, there is an urgent need to clarify possible variable impacts of modest alcohol use across the different stages of NAFLD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

WW, SN, and PC were involved in the study design, data search and collection, and writing of the manuscript. PC performed statistical analysis. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.744713/full#supplementary-material

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A Generic Nomogram Predicting the Stage of Liver Fibrosis Based on Serum Biochemical Indicators Among Chronic Hepatitis B Patients

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Introduction: Liver fibrosis staging is of great importance for reducing unnecessary injuries and prompting treatment in chronic viral hepatitis B patients. Liver biopsy is not suitable to act a screening method although it is a gold standard because of various shortcomings. This study aimed to establish a predictive nomogram as a convenient tool to effectively identify potential patients with different stages of liver fibrosis for patients with chronic hepatitis B.

Methods: A nomogram for multinomial model was developed in a training set to calculate the probability for each stage of fibrosis and tested in a validation set. Fibrosis stages were subgrouped as followed: severe fibrosis/cirrhosis (F3–F4), moderate fibrosis (F2), and nil-mild fibrosis (F0–F1). The indicators were demographic characteristics and biochemical indicators of patients. Continuous indicators were divided into several groups according to the optimal candidate value generated by the decision tree.

Results: This study recruited 964 HBV patients undergoing percutaneous liver biopsy. The multinomial model with 10 indicators was transformed into the final nomogram. The calibration plot showed a good agreement between nomogram-predicted and observed probability of different fibrosis stages. Areas under the receiver operating characteristics (AUROCs) for severe fibrosis/cirrhosis were 0.809 for training set and 0.879 for validation set. For moderate fibrosis, the AUROCs were 0.75 and 0.781. For nil-mild fibrosis, the AUROCs were 0.792 and 0.843. All the results above showed great predictive performance in predicting the stage of fibrosis by our nomogram.

Conclusion: Our model demonstrated good discrimination and extensibility in internal and external validation. The proposed nomogram in this study resulted in great reliability and it can be widely used as a convenient and efficient way.

Keywords: decision tree, nomogram, hepatitis B virus, liver fibrosis, prediction

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major global health problem and affects approximately 360 million persons in the world (1). Liver fibrosis is a critical indicator of anti-virus treatment for patients with HBV infection. A precise assessment of the degree of liver fibrosis is of great importance for guiding clinical treatment and predicting prognosis (2). Liver biopsy has

traditionally been considered as a reference standard for assessing and staging fibrosis. But there are several shortcomings such as invasiveness, low compliance, high side-effect, sampling error during the assessment of liver fibrosis (3–6). As a result it is difficult for chronic hepatitis B (CHB) patients to early diagnose or rapid screen liver fibrosis. The non-invasive biomarkers and models have been built to decrease the use of unnecessary liver biopsy. Nowadays, some combined indicators such as index of the relationship of aspartate transaminase to platelete (APRI), fibrosis index based on the four factors (FIB-4), and complex models have been used to predict liver fibrosis as non-invasive methods (7, 8). Although these methods have good diagnostic accuracy, it is pretty hard to get these biomarkers in general hospitals, which always be neglected by researchers. For example, serum microRNA profiles serve as novel biomarkers in a model built by Li et al. (9). Therefore, it is very important to construct the predictive model of liver fibrosis using conventional biomarkers.

In most studies, continuous indicators are directly used to construct predictors or models (5, 10–12). But, as we know, small changes in continuous data have little effect on the prediction and classification. The predictors or models based on the continuous values could will be inefficient in classification or discrimination. The reasonable and effective transformation of the continuous indicator is more beneficial to improve prediction accuracy. For example, the risk of disease changes less with each year of age in a cohort study, and it may be not significant. But when the age increases by 5 years, the risk becomes apparent. Therefore, continuous data were often transformed into ordinal or discrete data in medical and epidemiological research according to the mean, median, percentiles, or reputed clinical threshold (13–16). However, the real impact and characteristics of indicators were not accounted on this condition. Decision trees are simple and effective classification algorithms, which provide human-readable rules of classification (17). In this study, continuous indicators were transformed into ordinal predictors according to the optimal candidate value which was produced by the decision tree. Additionally, a more detailed classification in liver fibrosis is the crucial factor to determine whether to suffer a biopsy. And it is a necessary part for constructing a more reasonable and effective prediction model, which can be more suitable for clinical decision (18).

In order to improve the visualization of results and facilitate the extension of applications, a nomogram is used to build and present predictive models. It can conclude statistical predictive models into a single numerical estimate of the probability of a special event, such as death or recurrence, which is tailored to the profile of an individual patient. Currently, nomograms have been developed rapidly in many fields (19–21). In this study, we aimed to construct a multi-logistic prediction model using routine indicators which could be reasonably grouped by the decision tree, then an intuitive nomogram was determined to clearly and concisely predict the severity of liver fibrosis in CHB patients. It is helpful for clinicians to take reasonable treatment and decision according to the actual situation of patients.

MATERIALS AND METHODS

Study Population

This study was conducted in 2017 in the Shengjing Hospital of China Medical University. We collected the data of 1,224 patients according to the records in the histology laboratory database. The enrolled subjects were selected according to the following criteria: (1) Hepatitis B surface antigen (HBsAg) was positive at least 6 months, and virus was carried more than 2 years; (2) No co-infection with human immunodeficiency virus (HIV), the hepatitis C or hepatitis D and other liver diseases including chronic ethanol consumption, liver tumors and hepatocellular carcinoma; (3) Before liver biopsy, there is no antiviral therapy in patients; (4) No liver transplantation; (5) Within a week of liver function tests, percutaneous liver biopsy, and serum markers; (6) patients' age $\geq$ 18. The exclusion criteria were: (1) insufficient liver tissue for the staging of fibrosis; (2) insufficient data on complete blood count or serum markers; (3) There were no serum markers before treatments. If more than one set of laboratory results were available, the results closest to the time of biopsy were used. Among the 1,224 patients collected in the present data, 964 patients were recruited in the final analysis. Two hundred sixty patients were exclude because of incomplete data, co-infection with hepatitis C and other liver disease (**Figure 1**).

Patient and Public Involvement

All procedures performed in studies involving patients were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Liver biopsy, as an invasive test, was used mainly based on the patient's clinical symptoms, and the patient must sign a consent form. Privacy implications were not involved, and the

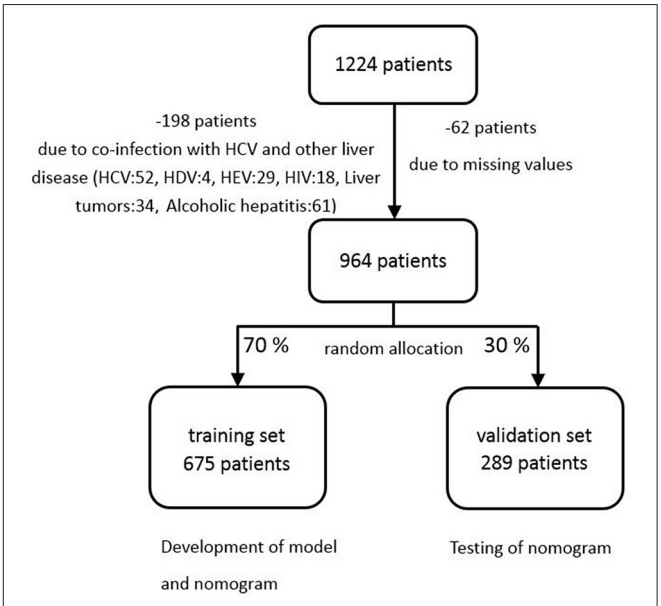

FIGURE 1 | Flow chart of subjects selection.

patients agreed to participate in the study. The study protocol was in accordance with the ethical standards and was approved by the Ethics Committee of China Medical University (CMU6206-1004).

Recruitment of participant into the study was done by health workers based on the inclusion criteria. District and regional health service workers and managers also supported it.

Laboratory Tests and Clinical Characteristics

All patients were evaluated on standard laboratory parameters. The complete blood count was measured on Hematology Analyzer (Beckman Coulter 5 diff, Miami FL) and clinical chemistry tests were performed using 7150 Analyzer (Hitachi, Japan). All recorded indicators were from blood routine examination, coagulation function, liver and kidney function, serum lipid, myocardial enzyme and demographic characteristics. Thirty-nine variables were excluded in this study, because of literature and medical background (28 predictors such as Chlorine, Urea, Uric acid), as well as over-missing values (11 predictors such as C-reactive protein, Hepatitis b E antigen, HBeAb, HBV-DNA).

The variable "sign" is primarily considered as an indicator of clinical feature, which represents the status and symptoms of patients. If a patient had both liver palms and spider nevus, the "sign" was assigned to 2. If a patient had either liver palms or spider nevus, the "sign" was assigned to 1. If a patient has neither liver palms nor spider nevus, the "sign" was assigned to 0.

Liver Histological Examination

Patients received percutaneous liver biopsy with automatic fare cut biopsy needle after signing the informed consent. All the samples were at least 10 mm in length and 1 mm in width. Two pathologists who had no clinical information of patients evaluated all biopsy specimens. The level of fibrosis was evaluated semi-quantitatively according to the METAVIR scoring system, which had previously been applied in other reports on CHB (22). Fibrosis was classified from F0 to F4 stages: F0 for no fibrosis, F1 for portal fibrosis without septa, F2 for few septa, F3 for numerous septa without cirrhosis and F4 for cirrhosis.

Statistical Analysis

The recruited patients were randomly divided into two sets, training set and validation set, by a ratio of 7:3. The training set was used to generate a plausible model, and the validation set was used to accomplish the validation and assess the performance of the model (**Figure 1**). Categorical variables were demonstrated with percentage, and were compared with the chi-squared test. Quantitative variables were shown as median with interquartile range (IQR), which were compared with Mann-Whitney tests. All *P*-values reported were 2-sided, and $P < 0.05$ was considered to be statistical significance. The analysis was carried out by SAS 9.4 and R.3.6.0 software (http://www.R-project.org).

Decision Tree

All recruited patients were included in a decision tree, and the result of individual biopsy was used as the classification of the decision tree. Then the optimal threshold value was calculated for every single covariate. Based on the analysis of the decision tree, all of the predictors are divided into two or more sections. This analysis was carried out using SPSS 20.

Nomogram

Nomogram is a graphic calculating tool helping clinicians quickly evaluate patients with specific models in a visual way, which does not require complex interpretation by computer software. It is based on multivariate regression analysis that integrates multiple indicators and then uses segments with scales to plot on the same plane at a certain scale to express the interrelations between variables in the prediction model.

A multinomial model was developed using categorized predictors and biopsy information. The classification of fibrosis stages (response variable) was divided into three categories: nil-mild fibrosis (F0–F1), moderate fibrosis (F2), and severe fibrosis/cirrhosis (F3–F4). The independent predictors included in the model were basic information and biochemical indicators. When carrying out a multinomial regression model, stepwise forward selection procedures were used to select the predictors in the model.

The established model was translated into a nomogram to display its outcome and corresponding probabilities conveniently. We can get the total point of every patient by accumulating points for each line. Then it is easy to get the corresponding lp (linear predicator) and the exponentiated point by drawing a vertical line from the total point axis straight to Exp(lp.m) or Exp(lp.s) axis, and then calculate the final probabilities of three fibrosis stages through the following formulas:

$$P_{F0-F1} = \frac{1}{1 + Exp(lp{\cdot}m) + Exp(lp{\cdot}s)} \quad (1)$$

$$P_{F2} = \frac{Exp(lp{\cdot}m)}{1 + Exp(lp{\cdot}m) + Exp(lp{\cdot}s)} \quad (2)$$

$$P_{F3-F4} = \frac{Exp(lp{\cdot}s)}{1 + Exp(lp{\cdot}m) + Exp(lp{\cdot}s)} \quad (3)$$

Of course, we can also calculate Exp(lp) without finding it in the plot. The Exp(lp) equal to e^{ip}, and lp is the linear predictor that you can get from nomogram.

Model Evaluation

To get bias-corrected estimates of predicted vs. observed values based on non-parametric smoothers, we established calibration plots using bootstrapping. The receiver operating characteristic curves (ROC) were constructed to analyze the accuracy of the model. Diagnostic accuracy for discriminating the stage of fibrosis was expressed as the area under the receiver operating characteristic curve (AUROC) for each outcome probability, both in the training set and validation set. We can also get the sensitivity, specificity and likelihood ratio from it.

RESULTS

Population Characteristics

The basic characteristics of the 964 study patients are shown in **Table 1**. According to the METAVIR score, 529 (54.88%) patients are in F0 stage, 213 (22.10%) patients are in F1 stage, 145 (15.04%) patients are in F2 stage, 74 (7.68%) in F3 stage and 3 (0.31%) patients are in F4 stage. Three continuous variables, Total bilirubin (TBIL), Hydroxybutyrate dehydrogenase (HBDH), D-Dimer, and one binary variable with no statistical significance were excluded from the next step ($P > 0.05$) and the rest of variables all showed statistical significance within different levels of liver fibrosis ($P < 0.05$). And there is no difference between training set and validation set (**Supplementary Table 1**).

Transformation of Indicators

In this study, continuous indicators were transformed into discrete ones according to the optimal candidate value produced by the analysis of the decision tree. Nine indicators were transformed into dichotomous indicators. Eight indicators were transformed into three-category indicators. Three indicators were transformed into four-category indicators and two indicators were transformed into five-category indicators. The

TABLE 1 | Clinical and laboratory characteristics of HBV patients in different levels.

Variables		F0/F1 ($n = 742$)	F2 ($n = 145$)	F3/F4 ($n = 77$)	*P*-value
Gender	Male	487 (65.63)	101 (69.66)	54 (70.13)	0.5088
[n (%)]	Female	255 (34.37)	44 (30.34)	23 (29.87)	
Smoking	Yes	135 (18.19)	35 (24.14)	24 (31.17)	0.0110
[n (%)]	No	607 (81.81)	110 (75.86)	53 (68.83)	
Drinking	Yes	148 (19.95)	38 (26.21)	23 (29.87)	0.0472
[n (%)]	No	594 (80.05)	107 (73.79)	54 (70.13)	
SIGN	0	598 (80.59)	119 (82.07)	48 (62.34)	0.0002
[n (%)]	1	121 (16.31)	16 (11.03)	22 (28.57)	
	2	23 (3.10)	10 (6.90)	7 (9.09)	
Age (years)		34 (26–41)	36 (27–43)	38 (32–44)	0.0026
A/G		1.56 (1.4–1.7)	1.5 (1.3–1.6)	1.42 (1.25–1.6)	<0.001
ALT (UI/ml)		45 (26–78.55)	65 (34–119)	69 (38–106)	<0.001
AST (UI/ml)		29 (22–47)	45 (28–78)	42 (29–86)	<0.001
ALB (g/L)		42.6 (40.6–45.2)	41.9 (39.7–43.4)	42 (39.1–43)	<0.001
ALP (U/L)		73 (60–84.7)	79.55 (63.8–103)	79.55 (70.5–110)	0.0007
APOB (g/L)		0.85 (0.71–1)	0.77 (0.65–0.92)	0.78 (0.62–0.99)	0.0123
DBIL (μmol/L)		4.2 (3.1–5.2)	4.73 (3.6–6)	4.73 (3.8–6.6)	<0.001
TBIL (μmol/L)		1 1.9 (9.2–14.6)	13.19 (9.5–15.9)	13.19 (10.7–17.6)	0.0553
CHE (U/L)		7927.91 (6,732–9,363)	7,173 (5,877–8,294)	7,137 (5,746–7,927.91)	<0.001
CYSC (mg/L)		0.82 (0.72–0.92)	0.84 (0.71–0.97)	0.9 (0.77–1.03)	0.0312
CHOL (mmol/L)		4.31 (3.85–4.88)	4.11 (3.68–4.72)	4.04 (3.65–4.76)	0.0069
GGT (U/L)		27 (17–44.91)	44 (25–72)	44.91 (29–86)	<0.001
GLU (mmol/L)		5.17 (4.87–5.45)	5.16 (4.79–5.52)	5.13 (4.86–5.7)	0.0331
HBDH (U/L)		141.3 (126.5–156)	144.6 (127.3–160)	145 (130–162)	0.4098
TBA (μmol/L)		4.8 (2.6–8.95)	8.5 (4.45–12.1)	8.8 (4.4–12.1)	0.0101
AFP (μg/L)		2.63 (1.87–4.3)	4.01 (2.6–7.4)	5.11 (3–9.31)	<0.001
APTT (s)		30.55 (28–33)	32 (29–35)	32 (28–36)	<0.001
D-Dimer (μg/L)		97 (59–156)	88 (50–145)	100 (65–160)	0.0589
FIB (g/L)		2.4 (2.1–2.8)	2.3 (2–2.6)	2.4 (2.1–2.6)	0.007
PT (s)		11.3 (10.8–11.9)	11.6 (11.1–12.2)	11.8 (11.2–12.5)	<0.001
TT (s)		15.9 (15.4–17.4)	16.5 (15.9–18.3)	16.5 (15.8–18.6)	<0.001
MPV (fl)		9.2 (8.1–10.24)	9.8 (8.7–11)	9.2 (8.4–10.1)	0.0012
PDW (fl)		16.1 (14.6–16.59)	15.24 (13.4–16.5)	16.3 (15.24–16.7)	0.0109
PLT (10^9/L)		183.5 (153.1–220)	150 (127–184)	149 (119–180)	<0.001

Data are presented as number (%) or median (interquartile range). A/G, Albumin/globulin; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALB, Albumin; ALP, Alkaline phosphatase; APOB, Apolipoprotein-B; DBIL, Direct bilirubin; TBIL, Total bilirubin; CHE, Cholinesterase; CYSC, CystatinC; CHOL, Cholesterol; GGT, γ-glutamyl transpeptidase; GLU, Glucose; HBDH, Hydroxybutyrate dehydrogenase; TBA, Total bile acid; AFP, Alpha fetoprotein; APTT, Activated partial thromboplastin time; FIB, Plasma fibrinogen; PT, Prothrombin time; TT, Thrombin time; MPV, Mean platelet volume; PDW, platelet distribution width; PLT, Platelets count.

TABLE 2 | The levels and optimal candidate values of final indicators.

Factors	Score (actual range)				
	0	1	2	3	4
AGE	≤31	>31			
CHOL	≤4.09	>4.09			
APTT	≤35.7	>35.7			
PT	≤11.2	>11.2			
PDW	≤15.2	>15.2			
TT	≤15.3	15.3–16.4	>16.4		
ALP	≤51.3	51.3–109	>109		
GGT	≤24	24–54	54–85	>85	
PLT	≤137	137–166	166–223	>223	
AFP	≤1.47	1.47–2.87	2.87–3.45	3.45–6.78	>6.78

Every original indicator of patients can be transformed into score (0, 1, 2, 3, or 4) according to this table, and then the scores can be used in the nomogram plot to calculate the risk of liver fibrosis.

TABLE 3 | Multinomial estimates from the final multinomial logistic regression model.

Predictive determinants	Moderate fibrosis vs. Nil-mild fibrosis			Severe fibrosis/cirrhosis vs. Nil-mild fibrosis		
	β	OR (95%CI)	*P-value*	β	OR (95%CI)	*P-value*
AGE	0.203	1.224 (0.752–1.994)	0.416	0.994	2.702 (1.343–5.438)	0.005
ALP	0.128	1.137 (0.653–1.979)	0.651	1.075	2.929 (1.449–5.92)	0.003
CHOL	−0.521	0.594 (0.369–0.956)	0.032	−0.603	0.547 (0.296–1.01)	0.054
GGT	0.323	1.382 (1.069–1.787)	0.014	0.296	1.345 (0.964–1.876)	0.081
AFP	0.391	1.478 (1.195–1.829)	<0.001	0.299	1.348 (1.021–1.78)	0.035
APTT	0.608	1.838 (0.936–3.607)	0.077	1.079	2.941 (1.341–6.451)	0.007
PT	0.531	1.701 (1.028–2.815)	0.039	0.675	1.964 (0.999–3.862)	0.05
TT	0.629	1.875 (1.316–2.673)	0.001	0.501	1.65 (1.052–2.588)	0.029
PDW	−0.522	0.594 (0.352–1.001)	0.051	0.543	1.721 (0.807–3.674)	0.16
PLT	−0.39	0.677 (0.536–0.854)	0.001	−0.578	0.561 (0.411–0.766)	<0.001

ALP, Alkaline phosphatase; CHOL, Cholesterol; GGT, γ-glutamyl transpeptidase; AFP, Alpha fetoprotein; APTT, Activated partial thromboplastin time; PT, Prothrombin time; TT, Thrombin time; PDW, platelet distribution width; PLT, Platelets count.

specific classification and optimal candidate values of final indicators were shown in **Table 2**.

Multinomial Logistic Regression

Based on multinomial logistic regression, we constructed predictive models of the degree of liver fibrosis in the training set. Ten biochemical markers were included in the final model with nil-mild fibrosis as a reference. **Table 3** showed relative factors of liver fibrosis. They are age (AGE), Alkaline phosphatase (ALP), Cholesterol (CHOL), γ-glutamyl transpeptidase (GGT), Alpha fetoprotein (AFP), Activated partial thromboplastin time (APTT), Prothrombin time (PT), Thrombin time (TT), platelet distribution width (PDW), and Platelets count (PLT).

Multinomial Nomogram

The nomogram enabled to calculate the probabilities of moderate (**Figure 2A**) and severe fibrosis/cirrhosis (**Figure 2B**). We can get the total point of every patient by accumulating points for each line, and the corresponding linear predictor (lp). We can also get the Exp(lp) by drawing a vertical line from the linear predictor axis straight to Exp(lp) axis, and then calculate the final probabilities of three fibrosis stages through the above mentioned formulas.

Calibration Plot

The calibration plot only tells us the bias of a classifier and has no connection with the classification quality. The dashed line indicates the ideal model in which predicted and actual probabilities were perfectly identical. The dotted line indicates actual model performance. The solid line presents the bootstrap corrected performance of our model. The bootstrap calibration plot (**Figure 3A**) indicated a good agreement between nomogram-predicted and observed probability of different fibrosis level for mild-moderate fibrosis group. However, it showed a good agreement for severe fibrosis group (**Figure 3B**). But the track of dotted line and solid line is different with ideal line which indicated predictions may slightly differ from reality.

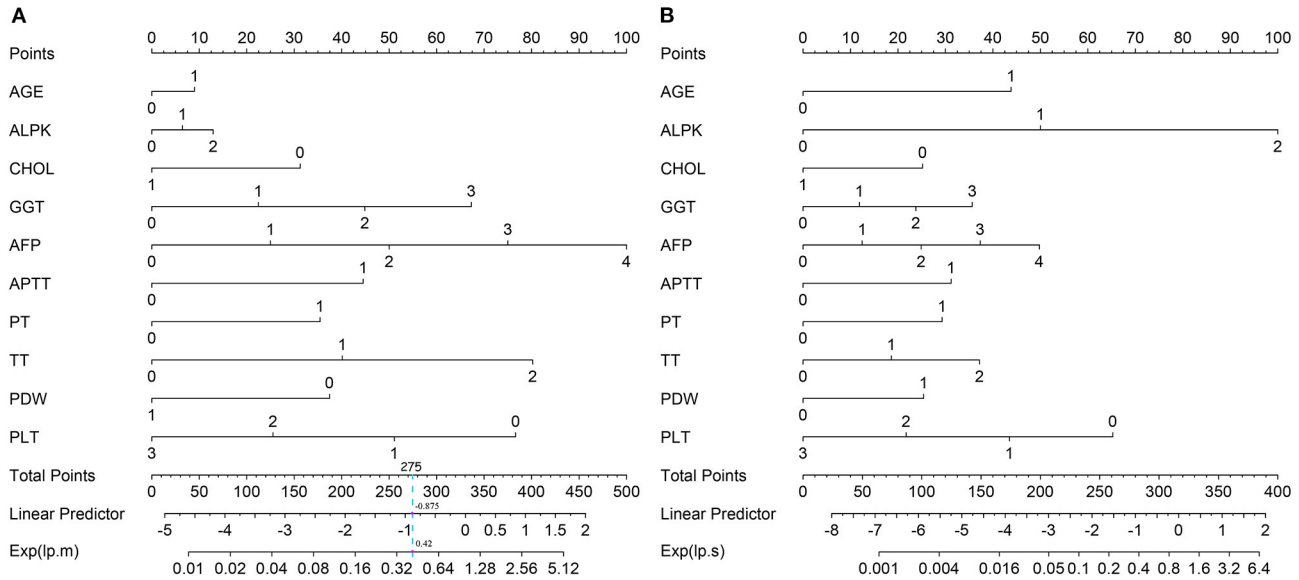

FIGURE 2 | The multinomial nomogram for the prediction of mild-moderate fibrosis **(A)** and severe fibrosis/cirrhosis **(B)**.

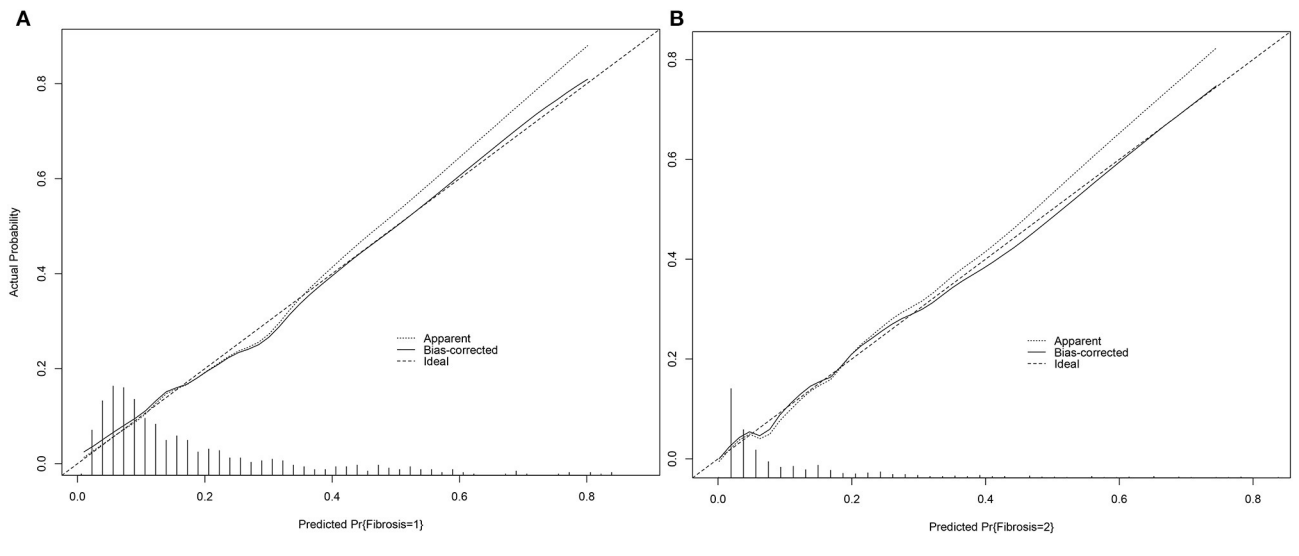

FIGURE 3 | Calibration plot of nomogram for mild-moderate fibrosis **(A)** and severe fibrosis/cirrhosis **(B)**.

Model Validation

For nil-mild fibrosis, we got AUROCs of 0.792 (95% CI 0.760–0.822) for the training set (**Figure 4A**) and 0.843 (95% CI 0.796–0.883) for the validation set (**Figure 4B**). For moderate fibrosis, our model enabled correct identification of patients with AUROCs of 0.750 (95% CI 0.715–0.782) for the training set (**Figure 4C**) and 0.781 (95% CI 0.729–0.827) for the validation set (**Figure 4D**). For severe fibrosis/cirrhosis (F3–F4), the model showed a good discrimination performance with AUROCs of 0.809 (95% CI 0.778–0.838) in the training set (**Figure 4E**) and 0.879 (95% CI 0.836–0.915) maintained in the validation set (**Figure 4F**), which demonstrated an intrinsic robust performance of the predictive model in terms of discrimination.

The detail information of the model in predicting fibrosis was shown in **Table 4**. The model predicted severe fibrosis with a sensitivity of 80.00% and a specificity of 73.66% in the training set at the optimal cutoff. In the validation set, the same cutoff yielded a sensitivity of 94.12% and a specificity of 69.85% accompanied with an LR+ 3.12 and LR− 0.084. Choosing the point on the ROC curve corresponding to the best cutoff, the model predicted moderate fibrosis with a sensitivity of 58.49% and a specificity of

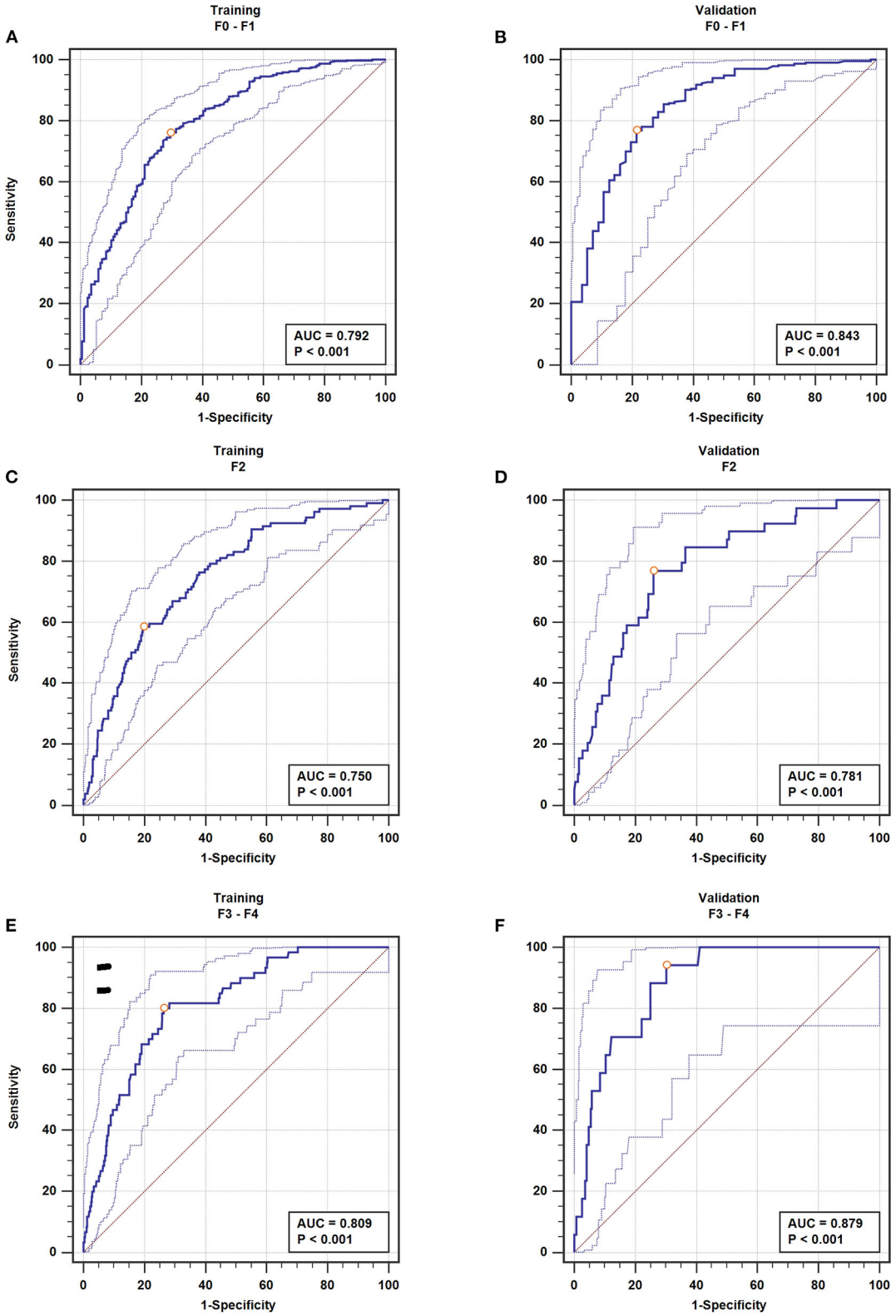

FIGURE 4 | The AUROC of fibrosis. nil-mild fibrosis **(A)**, moderate fibrosis **(C)**, severe fibrosis/cirrhosis **(E)** in the training set; nil-mild fibrosis **(B)**, moderate fibrosis **(D)**, severe fibrosis/cirrhosis **(F)** in the validation set.

TABLE 4 | The detail information of the multinoimal nomogram in predicting of fibrosis.

Fibrosis	Nil-mild fibrosis		Moderate fibrosis		Severe fibrosis/cirrhosis	
Data set	Training	Validation	Training	Validation	Training	Validation
Cut-off	0.7302		0.2135		0.0967	
Sensitivity (%)	76.03 (72.1–79.7)	76.83 (70.9–82.1)	58.49 (48.5–68.0)	76.92 (60.7–88.9)	80.0 (67.7–89.2)	94.12 (71.3–99.9)
Specificity (%)	70.48 (62.9–77.3)	78.57 (65.6–88.4)	80.14 (76.7–83.3)	74.00 (68.1–79.3)	73.66 (70.0–77.1)	69.85 (64.0–75.2)
LR+	2.58 (2.0–3.3)	3.59 (2.2–5.9)	2.95 (2.3–3.7)	2.96 (2.3–3.9)	3.04 (2.5–3.6)	3.12 (2.5–3.9)
LR–	0.34 (0.3–0.4)	0.29 (0.2–0.4)	0.52 (0.4–0.7)	0.31 (0.3–0.6)	0.27 (0.2–0.5)	0.084 (0.01–0.6)
AUC	0.792 (0.760–0.822)	0.843 (0.796–0.883)	0.750 (0.715–0.782)	0.781 (0.729–0.827)	0.809 (0.778–0.838)	0.879 (0.836–0.915)

LR+, positive likelihood ratio; LR–, negative likelihood ratio; AUC, Area under of ROC curve.

80.14% in the training set with an LR+ 2.95, and LR− 0.52. In the validation set, the same cutoff yielded a sensitivity of 76.92% and a specificity of 74.00% accompanied with an LR+ 2.96 and LR− 0.31. In the same way, the model predicted nil-mild fibrosis with a sensitivity of 76.03% and a specificity of 70.48% accompanied with an LR+ 2.58 and LR− 0.34. In the validation set, the same cutoff yielded a sensitivity of 76.83% and a specificity of 78.57% accompanied with an LR+ 3.59 and LR− 0.29.

DISCUSSION

Liver fibrosis is known as the major problem causing morbidity and mortality in chronic HBV patients. The evaluation of liver fibrosis stage in CHB patients is not only conducive to precision treatment by doctors, but also can reduce the burden of patients (23). We investigated HBV patients who had liver biopsies in the same hospital, and over 50% of them were actually in F0 stage. However, they are also at risk from unnecessary biopsies. Therefore, it is necessary to find a non-invasive method to determine whether a patient must further undergo an invasive procedure. Several biomarkers and combining markers are related to liver fibrosis and many non-invasive models have been suggested as good choices for screening liver fibrosis in order to overcome the limitations of liver biopsy (24–27). In our study, routine biomarkers and clinical markers were used to establish noninvasive predictive models for liver fibrosis. The final model included routine biomarkers which can be easily obtained from general hospital and even in local clinics with laboratory, such as AGE, ALPK, CHOL, GGT, AFP, APTT, PT, TT, PDW, PLT, which is conducive to the expansion of clinical applications.

Decision tree classification with a single classifier has been very successful in general classification problems. It provides human-readable rules of classification (28). The optimal separating points and the number of categories are based on the characteristics of every indicator and its influence on the target outcome, and the relationship between the outcome and indicators make each classification more reasonable. But, in several researches, continuous indicators were directly used without considering the fact that the tiny changes in a primitive continuous variable may obscure its role in the final model, which may result in this significant indicator being excluded from the model (29–31). On the other hand, the impact of extreme values could be reduced by transforming variables into categorical variables before the modeling process, although some of the original information may be lost. Classification of continuous variables by decision trees has been applied and the good result had been obtained (28). We used the decision tree to automatically classify 22 meaningful continuous indicators into dichotomous indicators, three-category indicators, four-category indicators or five-category indicators. The classification can better reflect the influence of different levels of indicators on liver fibrosis.

In our study, a multinomial logistic regression was conducted to build a predictive model instead of an ordinal logistic regression in view of the limitations of the application conditions of ordinal logistic regression. In addition, covariates' effects are the same independently of response categories considered in ordinal logistic regression model, but in practice, we suspect that a set of coefficients does not contribute to good predictive performance. So, the multinomial model became our ultimate choice. We put the multinomial logistic regression formula into an obvious nomogram plot to eliminate the tedious calculations. The nomogram accompanied with the formula can be used to calculate each patient's probability of two kinds of fibrosis in CHB patients. As a method to identify the high-risk or low-risk individual, it is easy and fast, and saves public resources. In our study, the nomogram is very effective in predicting the degree of liver fibrosis in more detail, such as nil-mild fibrosis, moderate fibrosis, and severe fibrosis. These showed good discrimination ability for nil-mild fibrosis with AUROCs of 0.792 in the training set and 0.843 in the validation set. For moderate fibrosis, AUROCs were 0.750 and 0.781. Especially for severe fibrosis, the nomogram showed better accuracy with AUROCs of 0.809 and 0.879. Compared with other validated widely non-invasive models (32), such as FIB-4 with AUROC of 0.766, APRI with AUROC of 0.728, Wang I with AUROC of 0.766, PP with AUROC of 0.772, our model got a better

result. Though WHO recommends APRI as the preferred non-invasive test to assess significant fibrosis or cirrhosis and FIB-4 to detect of fibrosis stages ≥F3 (33), the AUROC of our model is bigger than theirs. And even better than Forns Index, another serum non-invasive fibrosis test, has cholesterol more than FIB-4 in the formula (34). Transient elastography performed with FibroScan (Echosens, Paris) has been evaluated widely and has a good performance of predicting cirrhosis, which is corroborated by Guidelines Development Group. But it requires more expensive equipment and professional technicians, so they considered it was the most useful test for the assessment of cirrhosis in middle-income countries. Some researchers also included transient elastography as a variable in logistic regression established the nomogram. And it showed good prediction results (35). Compared with it, the AUROC of our nomogram is close to it, even our regression model has a better predictive effect, but only the routine serum biochemical indicators are used. Our model has more variables than other methods, but these variables are routine blood biochemical indicators, which are easy to implement in general medical examinations. The variables of our model are also available when the variables of the APRI or other model are obtained, so it is not difficult to practice. The final score of each patient accumulated through different variables can be used to estimate the risk of liver fibrosis, which is intuitive and more applicable to the use of primary hospitals. In addition, continuous indicators were transformed into ordinal predictors by the decision tree before multinomial logistic regression in our nomogram. It could improve prediction accuracy and made the AUROC bigger than nomogram by traditional regression model (36).

However, there were some limitations in our study. It was conducted in a specialized department for infectious diseases. All enrolled individuals were inpatients, not a completely random sample of all CHB patients. These inpatients could pay more attention to their own health. They are hospitalized as soon as possible to slow down the development of the disease. However, many CHB patients don't care about their health. They have not been hospitalized in time, and their condition has developed into fibrosis without knowing it. Therefore, our study might potentially underestimate the percentage of mild fibrosis in CHB patients. In addition, owing to the limitation of retrospective investigation, we did not collect some information such as HBV genotypes, virus load, dietary habit, use of health food (37). Therefore, we could not determine whether these variables should be included in the model.

In conclusion, this study presents nomograms covers mild-moderate fibrosis, and severe fibrosis, and it can be effectively used to predict the degree of liver fibrosis in CHB patients. We have confirmed that the nomogram based on decision tree could improve the more accuracy of individualized prediction and clinical benefit.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee Group of China Medical University (CMU6206-1004). The patients/participants provided their written informed consent to participate in this study. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XX conducted the design of study, performed statistical analysis, and wrote the initial manuscript after consultation with the other authors. HL improved the design, revised the manuscript, and approved the final version. WW collected the preliminary data and helped revise the manuscript. QZ participated in the design and acquisition of preliminary data. WC collected and sorted the preliminary data. MW and TQ participated in the collection of the data. All authors have read and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Hypoxia-Inducible Factors as Key Players in the Pathogenesis of Non-alcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis

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Non-alcoholic fatty liver disease (NAFLD) and its more severe form non-alcoholic steatohepatitis (NASH) are a major public health concern with high and increasing global prevalence, and a significant disease burden owing to its progression to more severe forms of liver disease and the associated risk of cardiovascular disease. Treatment options, however, remain scarce, and a better understanding of the pathological and physiological processes involved could enable the development of new therapeutic strategies. One process implicated in the pathology of NAFLD and NASH is cellular oxygen sensing, coordinated largely by the hypoxia-inducible factor (HIF) family of transcription factors. Activation of HIFs has been demonstrated in patients and mouse models of NAFLD and NASH and studies of activation and inhibition of HIFs using pharmacological and genetic tools point toward important roles for these transcription factors in modulating central aspects of the disease. HIFs appear to act in several cell types in the liver to worsen steatosis, inflammation, and fibrosis, but may nevertheless improve insulin sensitivity. Moreover, in liver and other tissues, HIF activation alters mitochondrial respiratory function and metabolism, having an impact on energetic and redox homeostasis. This article aims to provide an overview of current understanding of the roles of HIFs in NAFLD, highlighting areas where further research is needed.

Keywords: non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), hypoxia-inducible factor (HIF), chronic intermittent hypoxia, obstructive sleep apnea, fibrosis, metabolism, inflammation

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a progressive, widespread form of chronic liver disease with a large global burden. Worldwide, around 25% of the population have NAFLD and its prevalence is increasing (1). NAFLD initially presents as relatively benign fatty liver but worsens with time, leading to fibrosis and the inflammatory, more severe non-alcoholic steatohepatitis (NASH). Eventually, even cirrhosis or hepatocellular carcinoma can occur (2). It is also an important independent risk factor for cardiovascular disease (1). Despite this, specific treatment options for NAFLD are lacking. In order to develop such specific treatments, a better understanding of disease mechanisms and the (patho-)physiological signalling systems involved in NAFLD progression are needed.

The hypoxia-signalling system has been implicated in the pathogenesis of NAFLD (3). Central to cellular oxygen-sensing is the hypoxia-inducible factor (HIF) family of transcription factors which regulate the expression of genes underpinning the cellular and systemic response to hypoxia. HIFs are heterodimers, made up of an alpha subunit (of which three are currently known: HIF1α, HIF2α, and HIF3α), and a beta subunit (HIF1β). Current understanding of the regulation and function of HIF1α and HIF2α, is much greater than that of HIF3α, which remains under-investigated (4). The 2019 Nobel Prize in Physiology or Medicine was awarded to William Kaelin Jr., Peter J. Ratcliffe, and Gregg L. Semenza for their work in revealing how HIFs sense oxygen levels and coordinate the cellular response to hypoxia. The sensing mechanism, which has been reviewed elsewhere (5), involves targeted destruction of HIFα subunits in the presence of oxygen (**Figure 1**). Under normoxic conditions, HIF-prolyl hydroxylase domain proteins (PHD1-3) hydroxylate proline residues in cytoplasmic HIFα subunits in an oxygen-dependent manner. This allows recognition by the E3 ubiquitin ligase von-Hippel Lindau protein (VHL), leading to ubiquitination of HIFα and subsequent proteasomal degradation. PHD-mediated hydroxylation does not occur in hypoxia, allowing HIFα stabilisation, translocation to the nucleus and dimerization with HIF1β. Activated HIFs bind to hypoxia response elements in the promoters of target genes, leading to the transcription of genes required for adaptation to hypoxia, such as *Vegfa,* encoding vascular endothelial growth factor, and genes encoding many glycolytic enzymes (6). Owing to their roles in the regulation of diverse processes such as metabolism and angiogenesis, there is great potential for the involvement of HIFs in multiple key aspects of NAFLD, and accumulation of HIFs has been demonstrated to occur in the livers of patients with NAFLD (3). This makes HIF signalling a promising therapeutic target for this disease, especially since pharmacological modulators of the HIF pathway already exist (7–9).

Potential Mechanisms of HIF Activation in NAFLD

The canonical driver of HIF activation is tissue hypoxia. Hypoxia in the liver has been shown to occur in mice fed a high fat diet (HFD) for 8 weeks, though it remains unclear how this local hypoxia develops (10). The liver displays a steep oxygen gradient, with higher partial pressures of oxygen in the periportal regions, but lower oxygenation in perivenous regions (11). In NAFLD, this gradient could become dysregulated, leading to hepatic hypoxia, and this has been observed using pimonidazole staining in mice fed a HFD (12). Pimonidazole is a small molecule that reacts with thiol groups in proteins and peptides specifically under hypoxic conditions allowing for the detection of hypoxia using immunohistochemical techniques (13). Dysregulation of the oxygen gradient in the liver could result from increased size of hepatocytes (which increases the diffusion distance for oxygen), e.g., due to steatosis, or from increased oxygen consumption, which may occur in early stages of NAFLD development as appears to be the case in HFD fed rats (14, 15). This increase in oxygen consumption may be a result of increased fat oxidation to avoid lipid accumulation in a state of high fat intake. In addition to hypoxia, HIF stabilisation also occurs in response to reactive oxygen species (ROS) production (16), which is commonly seen in animals fed a HFD (17), and can be caused by cholesterol accumulation (18). ROS production could also result from reduced levels of the sirtuin SIRT4, which have been observed in patients with NAFLD (19). HIF activation can also result from succinate accumulation (20). SIRT1 has also been shown to be an important component of HIF activation (21). However, it should be noted that SIRT1 is generally downregulated in patients with NAFLD (22), and it is therefore unclear whether this mechanism is involved in regulation of HIFs in this context. While localised hypoxia has been demonstrated in steatotic mouse livers (10), it remains unclear whether this is driven by increased diffusion distance, increased oxygen consumption, or a combination of both. Further, other mechanisms of HIF activation, such as ROS production and importantly, chronic intermittent hypoxia (CIH), remain under-investigated in this context. CIH occurs in humans with obstructive sleep apnoea (OSA), which causes nocturnal bouts of low blood oxygen caused by breathing difficulties (23). It is common in patients with obesity (24), and has been linked to NAFLD severity (25), but it remains unclear to what extent it is required for HIF activation in patients with NAFLD, and whether HIF mediated pathophysiological mechanisms differ between patients of NAFLD with and those without OSA. It should be noted that while rodents do not spontaneously develop OSA (meaning CIH does not occur in rodent models of NAFLD), HIF accumulation has been demonstrated in the livers of rodent models of NAFLD. This supports the view that CIH is not necessarily a requirement for HIF activation in NAFLD. The uncertainty around the mechanism driving HIF activation in NAFLD is of note, as mechanistic into this very common disease remains lacking (26), making it crucial to address such gaps in our understanding of the pathology of NAFLD.

Metabolic Roles of HIFs in NAFLD

Regulation of cellular metabolism is a major canonical function of HIFs. In order to maintain energy charge in hypoxia, HIFs increase the expression of genes encoding glycolytic enzymes such as lactate dehydrogenase (27), while repressing the expression of genes involved in oxidative metabolism,

Abbreviations: *Acox*, Acyl coenzyme A oxidase; *Acta*, α smooth muscle actin (gene); α-SMA, α smooth muscle actin (protein); ATP, Adenosine triphosphate; BDL, Bile duct ligation; CD36, Cluster of differentiation 36; CIH, Chronic intermittent hypoxia; *Col1a1*, Type 1 Collagen; *Cpt1*, Carnitine palmitoyltransferase 1; ECM, Extracellular matrix; *Epas1*, Endothelial PAS domain containing protein (gene); FAO, Fatty acid oxidation; *Fas*, Fatty acid synthase; HFD, High fat diet; HIF, Hypoxia-inducible factor; HRGP, Histidine rich glycoprotein; HSC, Hepatic stellate cell; IL-1b, Interleukin 1b; *Irs2*, Insulin receptor substrate 2; *Lox*, Lysyl oxygenase; MCP1, Macrophage attractant protein 1; NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OSA-Obstructive sleep apnea; PAI-1, Plasminogen activator-inhibitor 1; PDGF, Platelet derived growth factor; *Pepck*, Phosphoenolpyruvate carboxykinase; *Pgc1a*, Peroxisome proliferator-activated receptor gamma coactivator 1α; *Phd*, Prolyl hydroxylase domain protein (gene); PPAR, Peroxisome proliferator-activated receptor; ROS, Reactive oxygen species; *Scd1*, Stearoyl coenzyme A-desaturase 1; *Tnfa*, Tumour necrosis factor 1α; *Vegf*, Vascular endothelial growth factor; *Vhl*, Von Hippel-Lindau protein (gene).

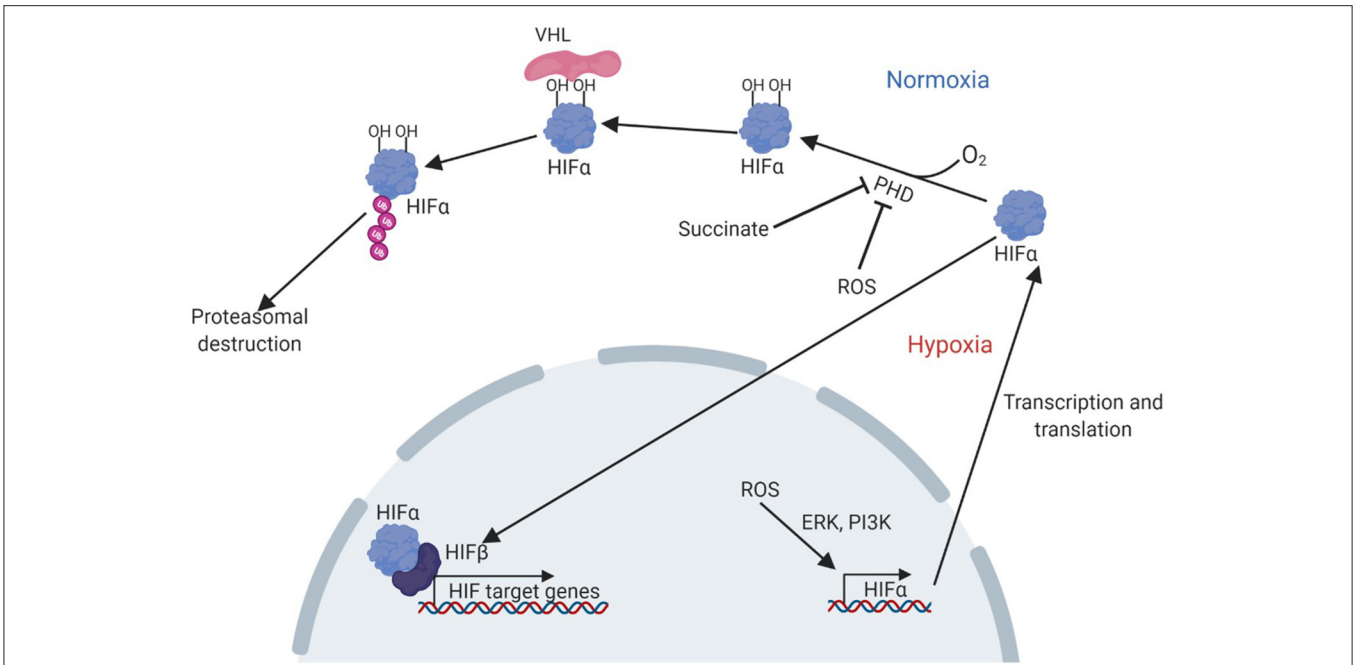

FIGURE 1 | Pathway of HIF activation in hypoxia. Adapted from Lee et al. (7). Under normoxic conditions, PHD enzymes hydroxylate proline residues in the HIFα subunit, in an oxygen-dependent manner. The hydroxylated residues are bound by VHL, which ubiquitinates HIFα allowing recognition and destruction of HIFα by the proteasome. Under hypoxic conditions, hydroxylation cannot occur and HIFα can instead translocate to the nucleus, bind HIFβ and other cofactors to activate transcription of target genes. HIF accumulation can also result from PHD inhibition by succinate or ROS, or by increased transcription and translation due to a ROS induced, ERK and PI3K mediated pathway. ERK, extracellular-signal related kinase; HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase domain proteins; PI3K, phosphoinositide 3-kinase; VHL, Von Hippel Lindau protein.

particularly fatty acid oxidation (FAO) (28). This serves to decrease oxygen requirements for ATP production, and protects against cellular damage in short-term hypoxia. However, chronic activation of HIFs in patients and models of fatty liver disease (3) may inhibit FAO to such an extent that it leads to or worsens hepatic lipid accumulation. HIF activation also appears to worsen steatosis by increasing the expression of genes required for lipogenesis, and the uptake and storage of lipids (9). Under normal circumstances, this may be an adaptive response to acute hypoxia, acting to store energy sources that cannot be utilised due to the general limitation on oxidative metabolism, and to package potentially toxic fatty acids as less harmful triglycerides. Overall, however, the resulting lipid accumulation appears to represent a harmful role for HIFs in steatotic liver diseases, such as NAFLD. It may also explain part of the association between severe OSA severity and incidence of NAFLD (29). Evidence of an insulin-sensitising role of HIFs in metabolic disease (30) complicates the overall effect of HIF activation in fatty liver disease, which is typically associated with insulin resistance (31).

Considerable evidence points toward HIF-mediated downregulation of FAO in hepatic steatosis. In particular, HIF2α activation, which occurs in the livers of patients with NAFLD as well as in mouse models (3), appears to worsen lipid accumulation (see **Figure 2**). Early studies in *Vhl*-deficient mice, showed that HIF2α, but not HIF1α, is responsible for the suppression of FAO in these mice (32–34). *Vhl*-deficient mice had lower expression of peroxisome proliferator-activated receptor α (PPARα)-target genes, such as carnitine-palmitoyl transferase 1 (*Cpt1*) and acyl CoA oxidase (*Acox*), lowering fatty acid-supported oxidative phosphorylation (33). PPARs are a family of transcription factors activated by unsaturated fatty acids, amongst other ligands. They play a key role in the control of fatty acid metabolism, and PPARα in particular is a major regulator of FAO in the liver (35). The reduced expression of PPARα target genes in *Vhl*-deficient mice was prevented by deletion of *Epas1* (*endothelial PAS domain containing protein 1*, the gene encoding HIF2α) but not *Hif1a* deletion (32). Similarly, primary hepatocytes from *Vhl*-deficient mice showed increased lipid accumulation alongside low expression of PPARα target genes (36). Indeed, HIF2α binds the PPARα promoter to repress its expression in HEK293 cells (28). *Hif2a* deletion or knockdown using siRNA prevents hypoxia-associated lipid accumulation in the human hepatocellular carcinoma HepG2 cell line (37, 38). Hypoxia appears to cause lipid accumulation in these cells by stabilising HIF2α, thereby lowering expression of FAO genes such as *Cpt1* and PPARγ coactivator α (*Pgc1α*) (38). Expression of these genes was normalised by *Hif2a* deletion, leading to decreased lipid accumulation. These studies demonstrate a potential role for HIF2α activation in decreasing the capacity for FAO in the liver, which could worsen steatosis in the context of NAFLD, when dietary fat intake is typically high.

While it is clear that HIF, and in particular HIF2α, activation can limit FAO in the liver to worsen steatosis, the studies outlined above did not investigate whether this occurs in NAFLD.

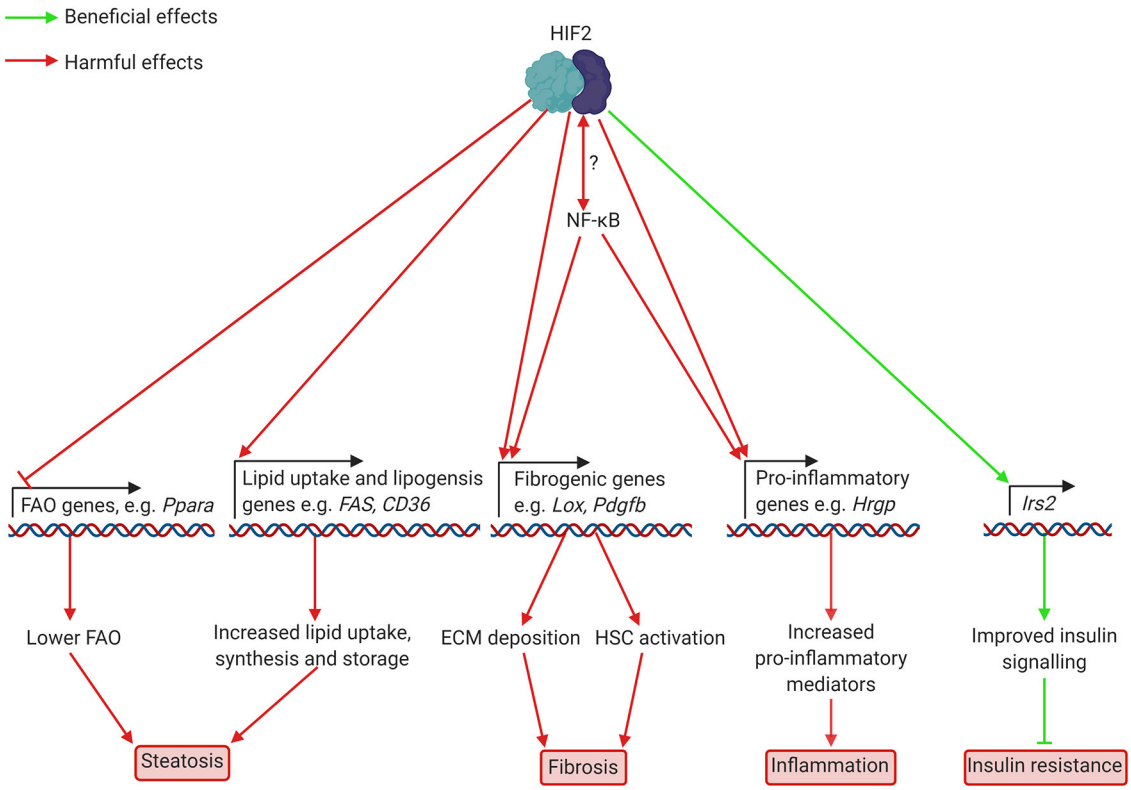

FIGURE 2 | Putatively beneficial and harmful effects of HIF2 activation in hepatocytes in NAFLD and NASH. HIF2 activation leads to lower expression of FAO genes, including *Ppara,* which encodes PPARα. This decreases FAO, leading to increased lipid accumulation. Higher levels of fibrogenic mediators such as LOX and potentially PAI-1, which are involved in ECM deposition, also occur as a result of HIF2 activation. Increased production of HSC activators may also occur but this has not yet been demonstrated in NAFLD/NASH. HIF2 mediated upregulation of the pro-inflammatory cytokine HRGP worsens inflammation. Interplay between HIF2 and NF-κB appears likely, but details of this interaction are unknown. Finally, increased transcription of the insulin signalling component Irs2 appears to improve insulin signalling to prevent insulin resistance. ECM, extracellular matrix; FAO, fatty acid oxidation; HIF, hypoxia-inducible factor; Hrgp, histidine rich glycoprotein; HSC, hepatic stellate cell; Irs2, insulin receptor substrate 2; Lox, lysyl oxidase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Ppara, peroxisome proliferator-activated receptor α; Fas, fatty acid synthase; CD36, cluster of differentiation 36; Pdgfb, platelet derived growth factor b.

Studies in *in vitro* systems and animal models of NAFLD suggest that this is indeed the case. Mice exposed to a HFD to induce hepatosteatosis showed decreased lipid accumulation when treated with a HIF2α antagonist (9), though FAO was not investigated in this study. In L02 human hepatocytes treated with fatty acids to model NAFLD *in vitro*, hypoxia worsened lipid accumulation, and this was associated with increased HIF2α levels, decreased expression of *Ppara* and transcriptional targets of PPARα such as *Cpt1a* and *Acox*, and lower oxidation of oleate (39). Silencing of *Hif2a* or treatment with a PPARα agonist, normalised expression of FAO genes and oleate oxidation, thereby lowering lipid accumulation, while treatment with a PPARα inhibitor prevented the beneficial effect of HIF2α-silencing. The authors also found that exposure of HFD fed mice to CIH, which models OSA, (see **Table 2** for an overview of hypoxia animal and cell culture models) increased lipid accumulation in the liver and decreased the expression of FAO genes including *Ppara*, *Cpt1a*, and *Acox2*. PPARα agonist treatment reversed the effects of hypoxia on steatosis. Chen et al. (39) did not investigate whether HIF2α activation played a role in lipid accumulation in the absence of a hypoxic stimulus, although other studies have demonstrated that HIF2α accumulation occurs in animal models of NAFLD without added hypoxia (40). Hepatic *Hif1α* deletion in a mouse model of NAFLD (mice fed a choline deficient diet), however, led to lower Lipin1 mediated PPARα/PGC1α pathway activation, which worsened steatosis relative to wild type mice (41), suggesting HIF1α is required to maintain FAO in NAFLD. Further work is required to determine whether HIF2α activation in NAFLD leads to lower FAO in animal models and human patients, especially in the absence of imposed hypoxia, though current evidence suggests that HIF2α activation in NAFLD contributes to hepatic steatosis, and that HIF2α activation can limit fatty acid oxidation, whereas HIF1α appears to be required for FAO in NAFLD.

Increased lipogenesis is an important feature of NAFLD in human patients (42–44). Again, studies support a potential HIF-mediated upregulation of this process in the context of NAFLD, although the current evidence for this role of HIF is conflicting. Studies of animal models of NAFLD suggest that HIF2α activation in this disease context may drive increased lipogenesis,

thus worsening lipid accumulation in the liver (see **Figure 2**). Treatment with the HIF2α specific antagonist PT2399 lowered hepatic steatosis in HFD fed mice (9), and this was associated with decreased expression of lipogenic genes in the liver. In L02 human hepatocytes treated with fatty acids, hypoxia (1% oxygen in hypoxic cell culture incubators) increased the expression of lipogenic genes such as *Fas* and stearoyl CoA dehydrogenase 1 (*Scd1*), and this was normalised by HIF2α silencing (39). Similarly, mice fed a HFD and subjected to CIH showed increased expression of *Fas* and *Scd1* relative to HFD fed mice not exposed to CIH (39). Conversely, oxygen therapy, which prevented HIF2α accumulation, lowered hepatic steatosis in HFD fed mice, and lipid accumulation in primary hepatocytes exposed to fatty acids. This also normalised expression of lipogenic genes in both *in vivo* and *in vitro* models of hepatosteatosis (40). Thus, it appears that HIF2α-activation, resulting from hypoxia, worsens diet induced steatosis by activating lipogenic gene expression. However, it should be noted that genetic HIF2α activation *via Vhl* disruption has been associated with decreased expression of lipogenic genes such as fatty acid synthase (*Fas*) (32), or, in other studies using the same mechanism, with only a temporary increase in lipogenic gene expression 3 days after the *Vhl* disruption (34). These conflicting results may be due to the differing mechanisms of HIF2α activation. In addition to increasing lipogenesis, HIF2α upregulation in NAFLD appears to increase lipid uptake by upregulating the fatty acid transporter Cluster of Differentiation 36 (CD36) (45). CD36 expression correlates with HIF2α levels in patients with NAFLD, and hypoxia induces CD36 expression in mouse AML12 hepatocytes exposed to hypoxia (45). Therefore, there is evidence that HIF2α activation (*via* genetic manipulation or hypoxia) can cause steatosis *via* inhibition of FAO and upregulation of lipid uptake, that liver hypoxia and HIF2α activation occur in NAFLD, and that HIF2α upregulates lipogenesis in diet-induced steatosis, which worsens lipid accumulation and can be prevented by treatment with HIF2α antagonists. However, whether HIF2α also impairs FAO in NAFLD remains unclear.

OSA also induces metabolic changes that may be mediated by HIF signalling. Levels of the CD36 are higher in the livers of patients with OSA than in those of healthy controls, and correlate with severity of OSA (46). CIH, which mimics OSA, induces the expression of lipogenic genes, such as S*cd1*, and CD36 in wild type (46) and *ob/ob* mice (47). Moreover, CIH increased HIF2α, but not HIF1α levels in HFD fed mice, while decreasing the expression of FAO genes such C*pt1a* (39). Thus, it appears likely that HIF signalling decreases FAO and increases lipid uptake and lipogenesis to worsen steatosis in the context of OSA and CIH, though the link between CIH/OSA and HIF signalling has not yet been established.

The role that HIF activation plays in the context of obesity associated disease is complicated by evidence of a link between HIF and insulin signalling. Both HIF1α and HIF2α activation have been shown to alter insulin sensitivity and glucose handling, most likely in a beneficial manner (see **Figures 2**, **3**). Owing to its role in upregulating glycolytic enzymes, it seems likely that HIF1α could improve glucose handling in obesity and diabetes. Indeed, HIF1α was upregulated in the livers of mice fed a high-fat, high-sucrose diet (30). Hepatocyte-specific deletion of *Hif1a* was associated with worsened glucose handling and insulin sensitivity. This was associated with lower levels of hepatic glucokinase (30). Treatment of HFD fed mice with HIF1α antisense oligonucleotides, however, decreased blood glucose and insulin levels (48). Unlike the hepatocyte-specific deletion employed by Ochiai et al. (30), this not only interfered with *Hif1a* in the liver, but also in adipose tissue, which may explain the opposing results. Shin et al. (48) found increased energy expenditure and lower body weight in *Hif1a* antisense oligonucleotide-treated animals. *Hif1a* antisense oligonucleotide treatment was also associated with lower liver steatosis, increased hepatic *Ppara* expression, and decreased expression of the lipogenic genes *Scd1* and acetyl-CoA carboxylase (48), though again it is unclear whether this was due to *Hif1a* interference in the liver or secondary to effects in other tissues. Overall, it appears that HIF1α activation can have opposing effects on insulin sensitivity, which may be tissue specific. This could explain why OSA severity is associated with worsened insulin resistance in patients with NAFLD (29) while liver-specific deletion of *Hif1a* worsens HFD induced glucose intolerance in mice (30).

HIF2α also appears to be involved in hepatic insulin signalling, *via* direct modulation of components of the insulin-signalling pathway. Liver-specific HIF2α (but not HIF1α) activation led to improved insulin tolerance and glucose handling (49). HIF2α directly upregulates the insulin-signalling pathway component insulin receptor substrate 2 (IRS2) by binding to HREs in its promoter and *Irs2* was required for the HIF2α-mediated effect on insulin sensitivity. Similarly, hepatic deletion of *Phd3*, which specifically upregulated HIF2α, was associated with increased *Irs2* transcription, improving insulin sensitivity (50). Again, this beneficial effect required both *Hif2a* and *Irs2*. *Phd3* deletion was associated with lower expression of gluconeogenic [e.g., phosphoenolpyruvate carboxykinase (*Pepck*)] and lipogenic (e.g., *Fas*) genes. Interestingly, unlike other models of liver specific HIF2α activation, *Phd3* deletion was not associated with worsened steatosis. The authors observed that deletion of *Phd1-3*, which increased HIF2α stabilisation still further, did worsen steatosis, suggesting that lower level HIF2α activation may be predominantly beneficial *via* improved insulin sensitivity, while higher levels of stabilisation, as occurs with *Phd1-3* and *Vhl* deletion (and potentially in long-term NAFLD) has a detrimental effect due to inhibition of FAO, leading to worsened steatosis.

Overall, significant evidence points toward a steatosis-promoting role for chronic HIF2α activation in liver, likely occurring *via* inhibition of FAO and upregulation of lipogenesis, though studies investigating the effect of *Hif2a* deletion in NAFLD on FAO are needed to confirm this. Meanwhile, low levels of HIF2α activation in metabolic diseases appear to have a beneficial effect on insulin sensitivity and glucose handling. Whether HIF1α activation is protective or harmful in the context of metabolic disease and hepatic steatosis remains less clear. There are conflicting results which may be the result of opposing roles in different cell types and tissues, although in hepatocytes specifically, HIF1α activation in obesity appears to improve insulin

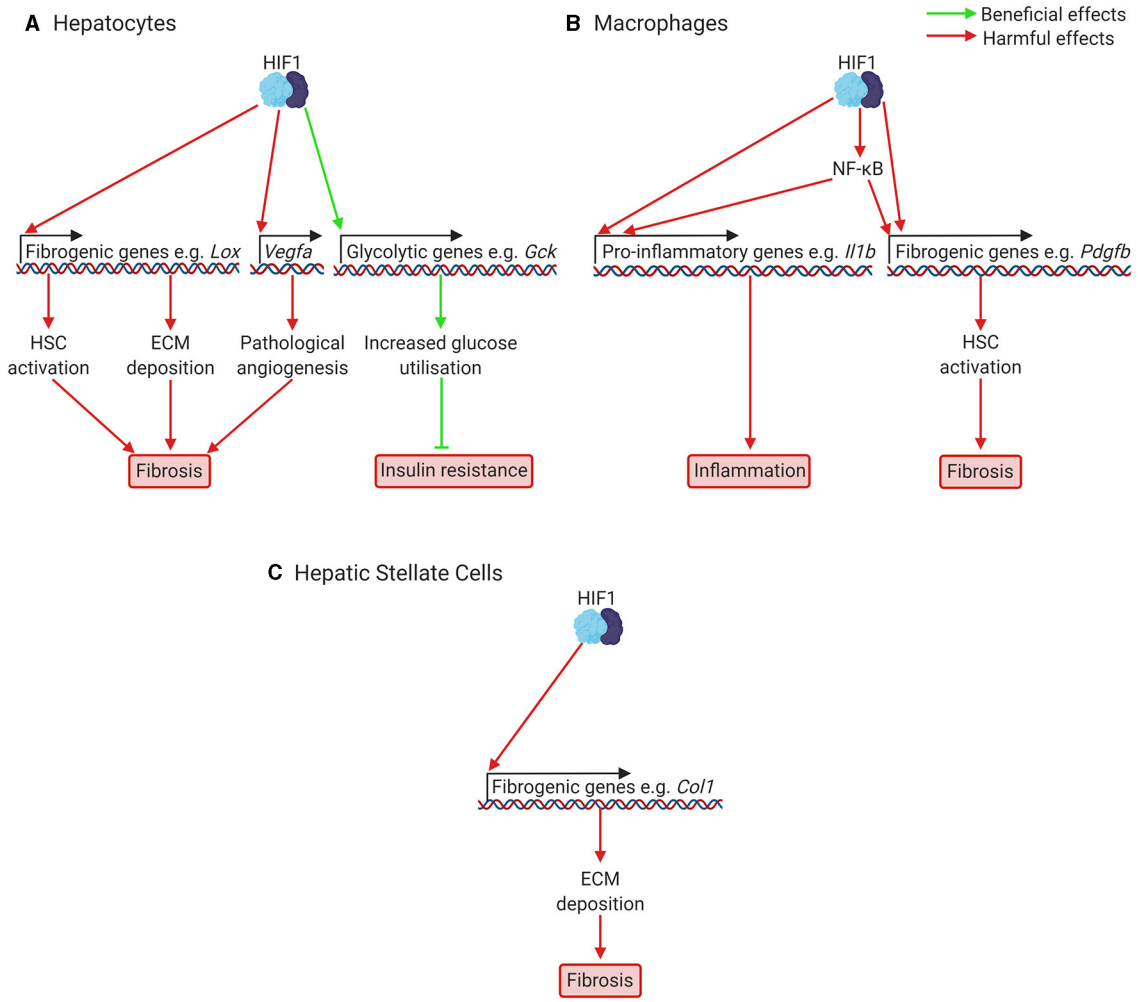

FIGURE 3 | Putatively beneficial and harmful effects of HIF1 activation in NAFLD and NASH. **(A)** In hepatocytes, HIF1 activation leads to increased expression of fibrogenic genes, including genes responsible for HSC activation (e.g., Pdgfb), ECM deposition (e.g., Lox), and pathological angiogenesis (Vegfa). Pathological angiogenesis has only been investigated in fibrosis models, not NAFLD models. HIF activation also increases expression of glycolytic genes such as glucokinase and glucose transporters, which increases glucose consumption and improves glucose handling. This can help prevent insulin resistance. **(B)** In macrophages, including resident macrophages in the liver, Kupffer cells, HIF1 activation increases expression of pro-inflammatory cytokines such as Il1b, leading to inflammation, and of fibrogenic genes, such as the HSC activator Pdgfb, leading to fibrosis. HIF1 also appears to activate NF-κB, a key regulator of fibrosis and inflammation. **(C)** In HSCs, which will be activated at increased levels due to signals from other cells resulting from HIF1 activation, HIF1 activation is also required for transcription of fibrogenic genes, such as Col1, encoding Type 1 Collagen. For many of these effects of HIF1 activation, the precise mechanisms are not yet clear and not all genes mentioned are necessarily under direct HIF1 control. Col1, Type 1 Collagen; Gck, glucokinase; ECM, extracellular matrix; HIF, hypoxia-inducible factor; HSC, hepatic stellate cell; Il1b, interleukin 1b; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Pdgfb, platelet derived growth factor b; Vegfa, vascular endothelial growth factor A.

sensitivity and may be required to maintain FAO and prevent increased lipogenesis.

HIFs and Fibrosis in NAFLD and NASH

Fibrosis is a key component of NAFLD in its most severe forms (51) and can occur both in patients of non-inflammatory non-alcoholic fatty liver and of inflammatory NASH (52). It is associated with worse outcomes and higher mortality rates in patients with NAFLD (53, 54). HIF-signalling likely contributes to fibrosis in NAFLD as shown by studies of fibrosis in general, and of fibrosis in NAFLD in particular. Liver hypoxia has been demonstrated in animal models of fibrotic and cirrhotic liver disease (see **Table 1** for an overview of fibrosis, cirrhosis, and NAFLD models), including in diethylnitrosamine cirrhosis (72), CCl_4 induced fibrosis (73), bile duct ligation (BDL) (74), and high dietary trans-fat induced NAFLD (56), and increased levels of HIF1α have been found in mouse models (75) and patients with fibrotic liver disease (76). Deletion of *Hif1a* protects against liver fibrosis in mouse models of both fibrotic liver disease, such as mice subjected to BDL (74), and models of NAFLD

TABLE 1 | Rodent models for the study of NAFLD.

Disease	Model	Aspects of NAFLD/NASH captured	References
NAFLD	High fat diet with varying fat content	Obesity, hepatic steatosis, often insulin resistance, sometimes liver fibrosis, inflammation	(17, 30)
	ob/ob mouse	Obesity, steatosis, mild fibrosis	(55)
	db/db mouse	Obesity, insulin resistance, steatosis, mild fibrosis	(55)
	High trans-fat diet	Obesity, with steatosis, fibrosis and some inflammation	(56)
NASH	Gubra-Amylin-NASH diet (high fat, high fructose, high cholesterol)	Obesity, severe steatosis, moderate inflammation, moderate fibrosis	(57)
	ob/ob mouse with high calorie feeding	Obesity, steatosis, moderate fibrosis, inflammation, moderate fibrosis	(55)
	db/db mouse with high calorie feeding	Obesity, insulin resistance	(55)
	Choline-deficient, L-amino acid-defined diet	No obesity, steatohepatitis and fibrosis	(3)
	Methionine/choline deficient diet	No obesity, steatohepatitis and fibrosis	(3, 58)
Cholestatic, fibrotic liver disease	Bile duct ligation	Liver fibrosis	(59)
	Repeated CCl_4 injection	Liver fibrosis	(60–62)
Cirrhotic liver disease	Diethylnitrosamine injection/feeding	Severe liver injury and cirrhosis, can induce hepatocarcinoma	(63)

TABLE 2 | Relevant *in vitro* and *in vivo* models of chronic and chronic intermittent hypoxia.

System	Model	Details of model	References
in vitro	Cells cultured in hypoxic chambers	Constant hypoxia achieved using high levels of nitrogen. Range oxygen concentrations can be used, 1% most common. Wide range of timeframes.	(64, 65)
	Cells treated with cobalt chloride	Model of HIF activation similar to constant hypoxia. Cellular response sometimes differs from true hypoxia.	(65, 66)
	Cells cultured in hypoxic chambers with cycling oxygen levels	Models CIH *in vitro*. Wide range of oxygen levels at nadir and cycle patterns in use.	(65)
in vivo	Rodents in hypoxic chambers	Constant hypoxia achieved using high levels of nitrogen. Range of oxygen concentrations in use.	(67, 68)
	Rodents in hypoxic chambers with cycling oxygen levels	Chronic intermittent hypoxia to model OSA. Oxygen cycles often applied only during sleeping hours of rodents. Wide range of oxygen levels at nadir and cycle patterns in use.	(39, 69)
	Rodents injected with sodium nitrite	Chronic intermittent hypoxemia through methemoglobinemia.	(70, 71)

(56, 77). Similarly, hepatocyte-specific deletion of *Vhl*, which increases both HIF1α and HIF2α signalling led to fibrosis which was normalised by *Hif2a* (but not *Hif1a*) deletion (34), and hepatocyte-specific deletion of *Hif2a* protects against fibrosis in mouse models of NAFLD (3). It therefore appears likely that HIF-signalling contributes to liver fibrosis in NAFLD. HIF-signalling may be involved in fibrosis *via* several mechanisms, including regulation of the expression of fibrogenic mediators in hepatocytes, Kupffer cells (resident macrophages in the liver) and hepatic stellate cells (HSCs) (see **Figure 3**), and by contributing to aberrant angiogenesis, a process that occurs in parallel with fibrosis and appears to be mechanistically linked to it (78).

HIF regulated expression of fibrogenic mediators in hepatocytes has been demonstrated in several relevant *in vitro* and *in vivo* models. Isolated mouse hepatocytes exposed to hypoxia show increased expression of plasminogen activator-inhibitor 1 (PAI-1), and this is partially prevented by *Hif1a* deletion and completely prevented by *Hif1b* deletion, suggesting both HIF1α and HIF2α may be involved (79) (**Figures 2**, **3**). PAI-1 contributes to fibrosis by inhibiting the activities of matrix metalloproteinases, leading to excessive collagen and extracellular matrix (ECM) accumulation (80). Similarly, AML12 mouse hepatocyte cells exposed to hypoxia (81), and HepG2 cells treated with the HIF stabiliser cobalt chloride and free fatty acids (66) show increased expression of genes encoding pro-fibrotic proteins, such as Type 1 Collagen α (COL1A1) and α-smooth muscle actin (α-SMA). In NAFLD models (**Table 1**), hepatocyte-specific deletion of *Hif1a* protects against collagen deposition and suppresses collagen crosslinking in the media of isolated hepatocytes exposed to hypoxia (56). This is likely to be due to decreased lysyl oxidase (*Lox*) expression, which requires *Hif1a*

in vitro (56). *Lox* expression has also been shown by chromatin immunoprecipitation to be under the control of HIF2α (34). In another study of NAFLD models, hepatocyte-specific deletion of *Hif1a* decreased collagen deposition and α smooth muscle actin staining (77). HepG2 cells treated with palmitic acid also showed increased HIF1α levels, and increased Type I Collagen and fibronectin expression, which was prevented by treatment with *Hif1a* siRNA (77). Further, hepatocyte-specific deletion of *Hif2a* protected against fibrosis in choline deficient, amino acid defined diet fed mice, a model of lean NAFLD (3). This was associated with lower levels of *Col1* (Collagen I) and *Acta2* (αSMA) mRNA. Collectively, these studies highlight that one role of HIF activation in liver fibrosis is the direct regulation of fibrogenic genes in hepatocytes and that this likely occurs in NAFLD. However, hepatocytes are not considered major sources of ECM deposition *in vivo*, and so it remains unclear how central this mechanism is to the pathology of NAFLD.

HSCs are the main source of myofibroblasts and therefore fibrosis in liver disease. Myofibroblasts form in the injured liver in response to fibrogenic signals and are the major source of ECM deposition in fibrosis. They are not found in the healthy liver (82). Hypoxia and HIF-signalling appear to play an important role in the activation of HSCs and in regulating the expression of fibrogenic mediators in HSCs. Hypoxia increases the expression of Type I collagen in activated HSCs (72) and HIF-signalling is required for the expression of collagen synthesis genes in isolated HSCs (83) and the production of HSC activators (including platelet derived growth factor (PDGF)-B) in livers in BDL (74), which suggests hypoxia signalling in hepatocytes may play an important role in activating HSCs. Further evidence for this comes from *in vitro* studies; HIF signalling is required for the upregulation of HSC activators in isolated mouse hepatocytes exposed to hypoxia (79), and the conditioned medium of AML12 cells exposed to hypoxia induces α-SMA expression in HSC-T6 cells (81). Similarly, extracellular vesicles isolated from HepG2 cells treated with fatty acids and cobalt chloride to stabilise HIFs induced the expression of fibrotic genes such as Collagen-1 and α-SMA in the human HSC LX2 cell line (66). HSCs are also activated by Kupffer cells and isolated Kupffer cells exposed to hypoxia show increased PDGF-B expression (84). This is normalised by *Hif1b* deletion (84) and myeloid specific deletion of *Hif1a* or *b* protects against fibrosis in BDL (85). Overall, evidence suggests that HIF-signalling is involved in HSC activation by acting directly in HSCs to increase expression of fibrogenic mediators, as well as by increasing the expression of signalling factors that activate HSCs in hepatocytes and Kupffer cells, though this has not been investigated in the context of NAFLD *in vivo* and the relative importance of HIF1 and HIF2 remains unclear.

A further important mechanism linking fibrosis and HIF-signalling is pathological angiogenesis; a common feature of fibrosis and cirrhosis that appears to be closely linked to fibrosis (78). Physiologically, angiogenesis is an important feature of the adaptive response to hypoxia, and is especially vital in liver regeneration after injury, to enable blood supply to re-growing liver regions. It is largely driven by HIF1α-mediated expression of VEGF and treatment with the PHD inhibitor DMOG increases the speed of liver regeneration in rats after portal vein ligation and parenchymal transection, and portal vein ligation alone (86). In pathological or aberrant angiogenesis however, immature neovessels form, which are incapable of resolving localised hypoxia in liver disease, and may lead to chronic HIF activation. Aberrant angiogenesis is likely mediated by increased VEGF expression in fibrosis due to activated HIF signalling (72). Anti-angiogenic treatment with VEGF neutralising antibodies or the VEGF Receptor 2 inhibitor sorafenib can prevent fibrosis in BDL models of liver fibrosis (87, 88), although VEGF may also play a role in fibrosis resolution (88). VEGF expression is increased in hypoxic hepatocytes in a HIF1α-dependent manner (79) and in hypoxic Kupffer cells in a HIF1β-dependent manner (84). VEGF-signalling is highly active in HSCs from areas of active fibrogenesis in patients and animal models, and VEGF stimulates HSC chemotaxis (89). T6-HSCs exposed to hypoxia have reduced levels of VHL, resulting in increased HIF1α and VEGF expression, which is normalised by cyclooxygenase 2 inhibition (90). Thus, chronic HIF-activation may contribute to fibrosis by upregulating VEGF, which contributes to HSC activation and leads to aberrant angiogenesis. However, this has only been investigated in models of fibrotic liver disease, rather than non-alcoholic or metabolic associated fatty liver disease, and further work is required to determine whether pathological angiogenesis is also involved in these conditions.

Hypoxia signalling may also be linked to fibrosis *via* interaction with nuclear factor (NF)-κB signalling. NF-κB is thought to be an important driver of fibrosis and inflammation in NAFLD (91) and inactivation of NF-κB, in particular in Kupffer cells, protects against fibrosis in mice injected with CCl_4 (60). NF-κB signalling is also activated in HSCs and myofibroblasts in the livers of CCl_4 and BDL rodent models, and human patients of fibrotic liver disease (92). There is considerable evidence of crosstalk between NF-κB and HIF signalling (93, 94), particularly in immune cells (95). However, the specific link between HIF and NF-κB signalling in the context of NAFLD remains less clear. While it has been demonstrated that both HIF2α and NF-κB accumulate in the livers of patients with NASH and mice exposed to hypoxia (96), it is unclear whether their respective signalling pathways interact and whether modulation of either can affect the other, and thereby improve fibrosis.

A link between OSA and liver fibrosis in NAFLD also appears likely. In patients with OSA and obesity, more severe OSA was associated with worsened fibrosis (29), and circulating levels of LOX (which is regulated by HIFs) are higher in patients with obesity and more severe OSA (64). In mice fed a high trans-fat diet and exposed to CIH (97), and in rats fed a HFD to induce NASH and injected with sodium nitrite to mimic CIH (70) fibrosis worsened. It is not clear what mechanisms contributed to this. In the NASH rat model (70), sodium nitrite injection was associated with increased HIF1α, VEGFA and VEGF receptor 2 levels. Silencing of HIF1α, however, normalised VEGFA and VEGF receptor 2 levels and improved fibrosis, suggesting pathological angiogenesis driven by HIF1α signalling may play a role. VEGF receptor neutralising antibodies attenuated the

development of fibrosis in CCl_4 induced fibrosis, and VEGF stimulated HSC proliferation *in vitro* (98), further supporting a role for pathological angiogenesis. However, the link between CIH and fibrosis may not always be HIF1α mediated, as, while HIF1α deletion improved liver fibrosis and inflammation in trans-fat diet fed mice with or without CIH, it did so without significant interaction with CIH effects (97). Further research is needed to understand the mechanisms involved in the link between CIH/OSA and liver fibrosis.

HIFs and Inflammation in NAFLD and NASH

Hypoxia is a common feature of chronically-inflamed tissue, and, as highlighted by a number of recent reviews (99–102), HIFs play important roles in inflammation and immunity, including *via* the activation of macrophages and certain types of T cells, and regulation of inflammatory cytokine expression, partly mediated *via* crosstalk with NF-κB signalling (103). Current evidence suggests that both HIF1α and HIF2α play a harmful role in NASH (**Figures 2**, **3**), a more severe form of NAFLD with marked liver inflammation (104). This involves hepatocyte-specific and immune cell-specific roles of HIFs. Hepatocyte-specific normoxic activation of HIF1α and HIF2α *via* deletion of *Vhl* worsens lipid accumulation, fibrosis and inflammation, with global microarray expression analysis showing increased expression of proinflammatory cytokines (34). This pathological phenotype was averted by concomitant deletion of *Hif2a*, but not *Hif1a*, suggesting a greater importance for HIF2α in driving steatohepatitis in hepatocytes. Similarly, in patients with NAFLD, hepatic levels of HIF2α and HIF1α are increased in early, non-inflamed stages of NAFLD, but only HIF2α levels are further increased in the livers of patients with NASH vs. patients with non-inflamed NAFLD (40), though studies in animal models do suggest a possible role for HIF1α as well (97).

In NASH, treatment studies and genetic interference with the HIF pathway point toward HIF activation contributing to inflammation. Treatment with the cardiac glycoside digoxin suppressed HIF1α pathway activation and decreased neutrophil and monocyte infiltration, as well as liver damage, in a mouse model of NASH (105). Further, HIF1α was increased in macrophages from patients and a mouse model of NASH (106). Myeloid specific HIF1α stabilisation worsened steatosis and inflammation, with increased macrophage infiltration in the liver, higher expression of the proinflammatory cytokines macrophage chemoattractant protein 1 (MCP1) and interleukin (IL)-1b in liver macrophages, and higher hepatic levels of *Mcp1* and tumour necrosis factor α (*Tnfa*) mRNA. Palmitic acid treatment also induced HIF1α in macrophages *in vitro*, and silencing of *Hif1a* suppressed the activation of NF-κB (106). HIF2α, which is also increased in patients and mouse models of NASH, appears to influence liver inflammation *via* control of hepatocyte production of the cytokine histidine rich glycoprotein (HRGP) (3). HRGP induces a proinflammatory M1 phenotype in macrophages, and deletion protects against NASH in methionine-choline deficient diet fed mice (107). Choline-deficient, amino acid-defined diet feeding, another model of NASH, increased levels of HRGP and other proinflammatory cytokines (including TNFα) in mouse livers. This was prevented by *Hif2a* deletion, whilst overexpression of *Hif2α* increased HRGP levels in HepG2 cells (3). It therefore appears that both HIF1α and HIF2α contribute to inflammation in NASH, and that this involves HIF-mediated mechanisms in several cell types, especially hepatocytes and macrophages. How these mechanisms function is not entirely clear, however.

HIF-signalling may also be involved in the link between OSA and NAFLD progression generally, and regarding inflammation in particular. Severity of nocturnal hypoxia in OSA correlates with NAFLD/NASH severity, including liver inflammation, independent of other risk factors in patients (25), and subjecting mice to CIH in order to mimic OSA leads to increased liver HIF1α, TNFα, and NF-κB (108). OSA induced inflammation may be mediated in part by changes in the balance between anti-inflammatory regulatory T cells and pro-inflammatory Th17 helper T cells (109). In mice fed a HFD, this ratio was shifted toward the pro-inflammatory Th17 cells, and this shift was even greater when CIH was superimposed through injection of sodium nitrite. Interference of HIF1α partially normalised this shift in the CIH and HFD exposed mice, and in hypoxic T-cells *in vitro*. This suggests HIF signalling in patients with NAFLD/NASH and OSA may induce or worsen inflammation, though more studies are needed to confirm this.

OPEN QUESTIONS

The evidence currently available highlights potential mechanisms by which HIF signalling may be involved in several key aspects of NAFLD, namely steatosis, inflammation and fibrosis. Further work is required to confirm many of these mechanisms and provide a more detailed understanding, and to determine whether targeting HIF signalling is a viable treatment strategy to improve these aspects of the pathology. It also remains uncertain what drives liver hypoxia and HIF activation in NAFLD in the first place.

While high fat feeding has been shown to induce liver hypoxia even in a relatively short time frame (10), it has not yet been determined what processes lead to this. It also remains unclear whether ROS production plays a role in HIF induction in NAFLD. Short-term feeding of NAFLD inducing diets combined with measurement of oxidative metabolism [e.g., using mitochondrial respirometry (110) or metabolomics, especially with isotope tracing (111, 112)] in the liver could elucidate whether development of liver hypoxia is preceded by increased oxygen consumption. Concomitantly, measurement of ROS markers [such as thiol (113) or lipid peroxidation (114)] could show whether ROS production is likely to play a role in HIF activation, which could be followed up with *in vitro* studies using ROS scavengers to investigate whether this prevents HIF activation in *in vitro* models of NAFLD. Investigation of SIRT4 in this context could also be valuable as reduced levels of this sirtuin have been demonstrated in patients with NAFLD (19) and it has been proposed that this may be a driver of increased ROS production in this disease.

It has been demonstrated that HIF2α activation in normoxia can limit FAO in hepatocytes (32). However, as demonstrated by the observation that lipogenic gene expression is decreased in mice with HIF2α activation due to *Vhl* disruption (32), while it is increased in NAFLD rodent models (9) [which also show HIF2α activation (3)], this does not necessarily mean that HIF2α in NAFLD also reduces FAO. Studies that investigate the function and expression of enzymes involved in FAO, and of the key cellular organelle in oxidative metabolism, the mitochondrion, in NAFLD models (with HIF2α deletion or pharmacological inhibition) and patients would help elucidate this. More detailed metabolomic studies in these settings may also provide further insight into how HIF2α activation affects metabolism in NAFLD.

In many cases, details of the signalling pathways by which HIF activation contributes to NAFLD and NASH remain unclear. This includes the pathways leading to increased expression of lipogenic, fibrogenic, and pro-inflammatory genes. Biochemical and molecular biology techniques such as chromatin immunoprecipitation and co-immunoprecipitation may provide targets for further investigation. *In vitro* studies may prove useful to probe these targets due to the greater ease of deletion and overexpression of genes. However, the current lack of consistent *in vitro* NAFLD models may make this more challenging.

Currently, it is unclear to what extent OSA is required for HIF activation in patients with NAFLD, and whether HIF activation resulting from OSA differs in its effects on NAFLD from HIF activation without OSA. This is likely to be the case due to the hypoxia-reoxygenation cycles inherent to OSA, which may affect activation of HIFs (e.g., by favouring HIF1α over HIF2α activation) and other co-activated pathways. Further investigations into how closely linked HIF activation and OSA are in patients with NAFLD would be useful, and studies in animal models of NAFLD exposed to CIH—a way of mimicking OSA in rodents, which do not develop OSA spontaneously—could provide insight into whether and how these pathophysiological mechanisms differ.

The ultimate goal of understanding the involvement of HIF signalling in NAFLD would be to attempt to treat the disease by targeting this pathway. Current evidence supports the use of animal studies to investigate this, and both HIF1α and HIF2α antagonists have been developed, largely with a view to treating cancers (8). Early studies, looking for example at the effect of HIF2α antagonism in HFD induced hepatosteatosis in mice have shown promising results (9), but studies in more severe models of NAFLD and NASH do still need be conducted.

Finally, while this review has focussed on the role of HIF signalling in the liver, some studies point toward roles of HIFs in other tissues and organs that are likely to impact on NAFLD and outcomes in NAFLD. For example, HIF activation in adipocytes (115) and adipose tissue macrophages (116) has been shown to affect insulin resistance, which is likely to affect NAFLD development. Further, the close links between gut and liver are likely to be involved in NAFLD, as shown by the association between inflammatory bowel disease and NAFLD (117). HIFs are known to play an important role in inflammation in the intestine (118) and may be an important part of this inter-organ link. Indeed, HIF activation in the intestine can affect NAFLD directly (119). The role of HIFs in other organs in the broader context of metabolic disease has recently been reviewed elsewhere (120), and better understanding of this, and how it may affect interactions between other organs and the liver is likely to aid in the development of therapeutic strategies for NAFLD.

CONCLUSION

In conclusion, considerable evidence points toward HIF activation occurring in NAFLD and NASH, and having widespread, predominantly harmful effects. Both HIF1α and HIF2α activation appear to worsen inflammation, though the mechanisms involved in this require further study. Further, evidence from studies of fibrosis shows important HIF mediated mechanisms, including control of profibrotic gene expression in hepatocytes and HSCs, regulation of HSC activation and HIF mediated pathological angiogenesis, though only control of profibrotic gene expression has been demonstrated to occur in animal models of NAFLD and NASH. Evidence also highlights a role for HIFs, in particular HIF2α, in driving steatosis. Studies of HIF activation under normoxic conditions suggest that HIF2α can inhibit FAO, while studies that interfere with HIF2α activation in NAFLD *via* oxygen therapy or antagonism suggest that HIF2α drives lipogenesis. These mechanisms could explain the protective effect that *Hif2α* deletion has against steatosis in NAFLD. While some beneficial effects of HIF activation have been noted, such as a potential role in improving insulin sensitivity, on balance, HIF activation appears to be harmful in NAFLD, and may therefore be a useful therapeutic target. Further research is required to fully elucidate the mechanisms by which HIF activation contributes to NAFLD and NASH, in particular the effect on FAO, the signalling pathways involved in regulating the expression of lipogenic, fibrogenic, and pro-inflammatory genes, and the link between HIF signalling and OSA in NAFLD and NASH.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Liver Fibrosis and MAFLD: From Molecular Aspects to Novel Pharmacological Strategies

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Metabolic-associated fatty liver disease (MAFLD) is a new disease definition, and this nomenclature MAFLD was proposed to renovate its former name, non-alcoholic fatty liver disease (NAFLD). MAFLD/NAFLD have shared and predominate causes from nutrition overload to persistent liver damage and eventually lead to the development of liver fibrosis and cirrhosis. Unfortunately, there is an absence of effective treatments to reverse MAFLD/NAFLD-associated fibrosis. Due to the significant burden of MAFLD/NAFLD and its complications, there are active investigations on the development of novel targets and pharmacotherapeutics for treating this disease. In this review, we cover recent discoveries in new targets and molecules for antifibrotic treatment, which target pathways intertwined with the fibrogenesis process, including lipid metabolism, inflammation, cell apoptosis, oxidative stress, and extracellular matrix formation. Although marked advances have been made in the development of antifibrotic therapeutics, none of the treatments have achieved the endpoints evaluated by liver biopsy or without significant side effects in a large-scale trial. In addition to the discovery of new druggable targets and pharmacotherapeutics, personalized medication, and combinatorial therapies targeting multiple profibrotic pathways could be promising in achieving successful antifibrotic interventions in patients with MAFLD/NAFLD.

Keywords: liver fibrosis, metabolic associated fatty liver disease, drug target, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, cirrhosis

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INTRODUCTION

Due to the close association with metabolic disorders and the previous exclusionary diagnostic strategy facing many challenges, a new disease nomenclature, metabolic-associated fatty liver disease (MAFLD), was proposed by expert panels to renovate its former name, non-alcoholic fatty liver disease (NAFLD) (1, 2). However, there are committees and experts who believe that the molecular basis of the disease behind this new definition lacks sufficient understanding, which may lead to uncertainty and negative effects in this field (3). Although many aspects of MAFLD are not well-understood, the similarities of the prevalence, risk factors, and pathological and metabolic traits between MAFLD and NAFLD suggest that evidence from NAFLD over the past decades would provide valuable clues for the discovery of druggable targets for the treatment of MAFLD and its subsequent fibrosis (4–7).

Non-alcoholic fatty liver disease is the most common chronic liver disease globally and affects approximately a quarter of the world population (5, 8, 9). It progresses from simple liver steatosis to nonalcoholic steatohepatitis (NASH) and, in more severe cases, to liver fibrosis and cirrhosis (10). By 2030, the overall number of cases of this disease is projected to increase by 18.3%, and the number of cases of its related advanced liver disease and liver-related mortality will be doubled (11). Facing such a severe public health burden, MAFLD as a new concept still lacks direct and strong evidence from pharmaceutical investigations. Even for NAFLD with sufficient research data to endorse, there are no specific drugs approved by the United States Food and Drug Administration (US-FDA) or the European Medicines Agency (12, 13).

Fibrosis is a consequence of advanced liver injury that is closely associated with cirrhosis and liver carcinoma (14). Therefore, the improvement of liver fibrosis has become an important indicator for evaluating the efficacy of drugs for the treatment of NAFLD. However, the effectiveness of current drugs for hepatic fibrosis is limited (15). The identification of druggable targets and the development of novel reagents for the prevention and reversal of fibrosis will be an important mission of NAFLD/MAFLD research (16). This review summarizes the key endogenous molecules involved in the pathogenesis of NAFLD/MAFLD fibrosis and discusses the compounds or antibodies derived from these druggable targets that could potentially lead to successful treatments for NAFLD/MAFLD (**Supplementary Table 1**).

PATHOGENETIC MECHANISMS UNDERLYING FIBROSIS IN MAFLD

Fibrosis is the primary histological feature of the advanced form of NAFLD/MAFLD (17). Therefore, it is critical to elucidate the mechanism mediating liver fibrosis in NAFLD/MAFLD. Although the majority of the mechanistic discoveries were based on NAFLD, the new terminology MAFLD shares similar driving factors as NAFLD and knowledge from NAFLD provides important implication in the understanding of the pathogenesis of MAFLD. The pathophysiology of NAFLD progression is summarized as the "multiple hits" theory. That is, the "first hit" begins with hepatic triglyceride accumulation, and the responses of insulin resistance (IR)-related lipotoxic substances, and the increased *de novo* hepatic lipogenesis, thereafter oxidative stress, metabolic inflammation, endoplasmic reticulum stress, and autophagy together with the intestinal microbial signals and other links all involved, finally facilitating "parallel, multiple hits" to the liver (18). In the liver injury-repair process, dysregulated hepatocytes or inflammatory cells elicit paracrine signaling that promotes the hepatic stellate cells (HSCs) activation. Meanwhile, circulating factors (e.g., adipokine and fatty acids) from extrahepatic tissues (e.g., visceral adipose tissue or intestine) could activate HSCs directly or mediately (17). In addition, gene polymorphisms, such as PNPLA3, TM6SF2, and HSD17B13, may increase an individual's susceptibility to liver fibrosis during metabolic dysregulation (17, 19, 20). Upon the stimulation of the abovementioned profibrotic factors, HSCs turn into an active form and accelerate the production of fibroblasts, portal vein fibroblasts, and myofibroblasts, which ultimately result in exacerbated extracellular matrix formation (21). Overall, HSCs activation is a dominant manifestation during fibrosis in NAFLD (17, 21) (**Figure 1**).

As the main target of liver injury, hepatocytes first face the imbalance of fatty acid and carbohydrate metabolism caused by metabolic overload in the early stage of NAFLD (22). With the further development of hepatic steatosis, excess, or not timely disposed fatty acids could be metabolized into toxic lipids (such as oxidized phospholipids), causing hepatocyte metabolic stress and damage or death (22). This lipotoxic response leads to hepatocytes apoptosis, which liberates reactive oxygen species (ROS) and free cholesterol (17). Damaged hepatocytes serve as a major driver for HSCs activation via paracrine signaling. For example, lipotoxic associated ROS production from mitochondria, endoplasmic reticulum, and NADPH oxidase (NOX) in hepatocytes have profound and direct impacts on HSCs activation (17, 23, 24). In addition, the receptors for advanced glycation end-products (RAGEs), which is pattern recognition receptor, are highly expressed in HSCs. ROSs are also generated in AGE formation, and oxidized RAGE stimulates NOX1, which contributes to ROS production in HSCs (24). Other signals from hepatocytes, such as leptin and osteopontin, are also involved in mediating the transformation of HSCs into a profibrotic and inflammatory phenotype (17). It should be noted that the innate immune response mainly regulates aseptic inflammation triggered by metabolic stress (25, 26). The innate immune system activates the release of inflammatory cytokines and chemokines by sensing metabolic stress and many of them have shown to be important in the pathogenesis of fibrosis, such as IL-1β and IL-18, C-C chemokine ligand types 2 and types 5 (CCL2 and CCL5), together with C-C chemokine receptor type 2 and types 5 (CCR2 and CCR5), etc. (27–29).

Hepatic macrophages can also polarize toward a proinflammatory phenotype, and their TLR4 signaling facilitates the production of transforming growth factor-beta 1 (TGF-β1) in response to metabolic insults. Transforming growth factor-beta 1 coordinates with HSCs to accelerates liver fibrosis (30). Hepatic T cell population is also essential in NASH-associated inflammation or stellate cell activation. Maintaining a good amount of $CD8^+$ tissue-resident memory T cells protected mice from fibrosis progression by predisposing activated HSCs to FasL-Fas-mediated apoptosis in a CCR5-dependent manner (31, 32). There is a large number of B cells in the liver, immune regulatory properties of HSCs promote the profibrogenic activity of B cells (33). Platelets are an essential cellular source of PDGFβ and TGFβ that activate HSCs and promote fibrosis in NASH. Extracellular signals from resident and inflammatory cells collectively modulate HSCs activation by stimulating autophagy, oxidative stress, endoplasmic reticulum stress, and retinol metabolism, thereby further modulating liver fibrosis (17). Since NAFLD is a component of metabolic syndrome that affects multiple organs, circulating factors, and signals from extrahepatic tissues and organs, such as the intestinal microbiome (34), adipose tissue, and skeletal muscle,

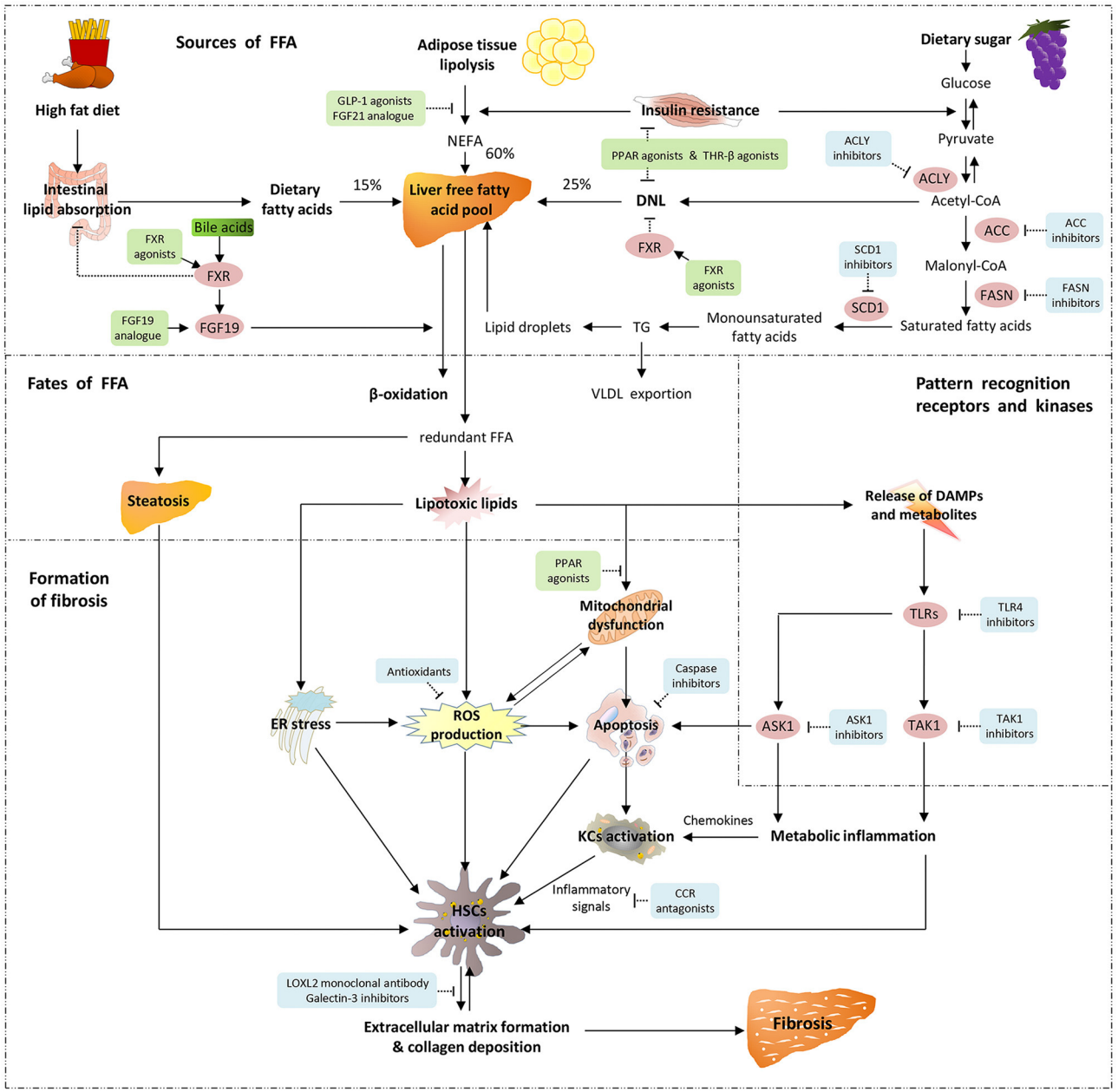

FIGURE 1 | Pathogenetic mechanisms underlying fibrosis in MAFLD/NAFLD and molecular target of drug therapy. There are three sources of hepatic free fatty acids (FFA): 60% from the adipose tissue lipolysis or non-esterified fatty acid (NEFA) pool, 25% from *de novo* lipogenesis (DNL), and the remaining 15% from the intestinal absorption of diet. The two main metabolic pathways of hepatic FFA are mitochondria-mediated β-oxidation and esterification to form triglyceride (TG). Triglyceride is able to be exported into the circulation in the form of very-low-density lipoprotein (VLDL), and the excessive TG are stored in lipid droplets. When FFA are overaccumulated or their disposal is not timely, the redundant FFA act as substrates to produce lipotoxic lipids, which lead to endoplasmic reticulum (ER) stress, production of reactive oxygen species (ROS), impaired mitochondrial function and release of danger-associated molecular patterns (DAMPs). Pattern recognition receptors such as toll-like receptors (TLRs) sense the continuous production of DAMPs and metabolites, thereby triggering downstream signaling pathways. Apoptosis signal-regulating kinase 1 (ASK1) and TGF-β-activated kinase 1 (TAK1) are crucial intracellular signal transduction components that are activated by post-transcriptional modification, and further activate their downstream pivotal kinases and transcription factors, leading to the occurrence of metabolic inflammation. As metabolic stress leads to the expression and release of inflammatory chemokines, Kupffer cells (KCs) polarize into pro-inflammatory phenotypes and participate in the activation of hepatic stellate cells (HSCs) in coordination with ROS, apoptotic signals and ER stress. These exacerbate extracellular matrix formation and collagen deposition, and result in liver fibrosis. Emerging therapeutic agents and their molecular targets for fibrosis in MAFLD/NAFLD are also indicated. Agonists and analogs are marked in green, while antagonists, inhibitors and antibodies are marked in blue.

it also influences liver fibrosis (17, 35). Especially, intestinal flora-derived pathogen-associated molecular patterns and danger-associated molecular patterns, as well as endotoxins, could directly promote fibrosis by signaling through innate immune receptors like TLR4 on HSCs (17). Different types of epigenetic modifications, including DNA methylation, histone covalent modifications, and the expression of some non-coding RNAs (such as miR-29a and miR-590-5p), were found to play essential roles in regulating HSCs activation during the progression of NAFLD to NASH (36, 37).

Given the development of liver fibrosis as a result of interactions between various hepatic cell types under metabolic stress and the mediation of intercellular communication by secreted mediators or circulating mediators, which regulate lipid toxicity, inflammation, apoptosis, extracellular matrix formation, and fibrosis, the discovery of molecular targets and the development of novel pharmacological strategies covers all these aspects.

LIVER FIBROSIS DRIVEN BY NON-ESTERIFIED FATTY ACID-DERIVED LIPID SYNTHESIS

Glucagon-Like Peptide-1 and GLP-1 Agonists

Glucagon-like peptide-1 (GLP-1) is a pleiotropic peptide hormone excreted by intestinal L cells that enhances insulin secretion and improves glucose homeostasis, thereby reducing liver non-esterified fatty acid (NEFA) overload caused by triglyceride decomposition. In addition to its metabolic benefits, GLP-1 has been shown to delay gastric emptying and limit body weight, as well as inhibit inflammation and cell apoptosis (38). These features make GLP-1 receptor agonists well-suited for the treatment of MAFLD, which is characterized by metabolic disorders, and allow them to help reduce multiple upstream links of HSCs activation, such as IR, lipid toxicity, and metabolic inflammation. Animal studies have shown that GLP-1 receptor agonists alleviate hepatic steatosis and inflammation, and play an antifibrotic role by improving HSCs phenotypes (12).

Liraglutide is a long-acting GLP-1 receptor agonist that reduces body weight in NAFLD patients. In a phase 2 study (NCT01237119), NAFLD patients who received subcutaneous injections of Liraglutide achieved histological improvement with attenuated fibrosis progression (39). Adverse effects of Liraglutide mainly consist of mild to moderate gastrointestinal reactions. Another GLP-1 receptor agonist in clinical application is Exenatide, which yields better improvement in the noninvasive Fibrosis 4 index and greater benefit in terms of body weight and liver enzymes than insulin Glargine in NAFLD patients with type 2 diabetes (NCT02303730) (40). A new-generation GLP-1 receptor agonist, Semaglutide, also improved steatohepatitis in a phase 2 trial (NCT02970942). It should be noted that although the administration of Semaglutide improved the fibrosis stage in nearly half of the patients, the excessive fibrosis regression rate in the placebo group made the difference between the groups non-significant (43 vs. 33%, $P = 0.48$) (41). Cotadutide is a dual receptor agonist of GLP-1 and Glucagon. Preclinical evidence has demonstrated that it is more effective than Liraglutide in improving liver fibrosis in NASH models (42). The current phase 2 study of NAFLD with compensatory fibrosis in patients with obesity (NCT04019561) completed the data collection of primary outcomes. Tirzepatide, a dual receptor agonist of GLP-1 and glucose-dependent insulinotropic polypeptide, also reported an improvement in fibrosis biomarkers in NAFLD patients with type 2 diabetes (NCT03131687) (43). Although it has only been approved by the US-FDA for the treatment of type 2 diabetes at present, a wide variety of GLP-1 receptor agonists have become attractive candidate drugs for the treatment of MAFLD due to their metabolic benefits.

LIVER FIBROSIS DRIVEN BY *DE NOVO* LIPOGENESIS-DERIVED LIPID SYNTHESIS

Acetyl-CoA Carboxylase and ACC Inhibitors

De Novo lipogenesis (DNL) is a pivotal step in liver fatty acid metabolism and plays a major role in triglyceride accumulation in hepatocytes (44). Acetyl-CoA carboxylase (ACC) is a crucial enzyme in DNL regulation that catalyzes the rate-limiting step of acetyl-CoA to malonyl-CoA conversion and modulates mitochondrial fatty acid oxidation. Therefore, ACC is an attractive therapeutic target for restoring the balance of hepatic fatty acid metabolism (44). An animal study confirmed that inhibiting ACC reduced lipid toxicity in hepatocytes by lessening DNL. This mechanism resulted in the direct suppression of HSCs activation and impairment of HSCs profibrogenic activity, thereby reducing liver fibrosis in a rat model (45).

Firsocostat (also known as GS-0976 and NDI-010976) is a small-molecule allosteric inhibitor of ACC in the liver that significantly inhibited hepatic DNL in a metabolically overburdened population in a dose-dependent manner (46). Treatment with 20 mg of Firsocostat daily for 12 weeks reduced hepatic steatosis and fibrosis marker levels in patients with NASH (NCT02856555) (47). In order to explore more suitable treatment regimens for NAFLD patients with advanced liver fibrosis, the ATLAS phase 2b study (NCT03449446) focused on the improvement of F3–F4 fibrosis after 48 weeks of treatment with three agents alone or in combination (48). The combination of Firsocostat and either of the other two drugs allowed more patients to achieve the primary endpoint of fibrosis improvement $\geq$1 stage than the monotherapy regimen, although this benefit did not have a significant advantage over placebo. The combination of 20 mg of Firsocostat and 30 mg of Cilofexor transformed the fibrosis pattern in the biopsy area into $\leq$F2 and led to significant improvements in NASH activity, the enhanced liver fibrosis score and the liver stiffness as determined by transient elastography, revealing a potential antifibrotic effect (48). Most patients treated with Firsocostat experienced a remarkable increase in serum triglyceride levels. This asymptomatic hypertriglyceridemia subsided spontaneously in some patients and could partially be resolved by treatment with fish oil or fibrates (47, 48). Hypertriglyceridemia may be

the result of decreased polyunsaturated fatty acids produced by malonyl-CoA, which makes the expression of sterol regulatory element-binding protein 1 increase in a compensatory manner, resulting in increased very-low-density lipoprotein secretion in the liver and peripheral triglyceride accumulation (44). In addition, Firsocostat in combination with Cilofexor increased the total cholesterol level and decreased the high-density lipoprotein cholesterol level in the ATLAS study (48). In consequence, further research on the long-term effects of Firsocostat on the cardiovascular system is needed.

PF-05221304 is another oral liver-directed ACC inhibitor that significantly improved the hepatic steatosis, fibrosis, and inflammation induced by metabolic stress in both human-derived *in vitro* systems and rodent models (49). An ongoing phase 2 study (NCT04321031) is evaluating whether it has a beneficial effect on fibrosis as assessed by liver biopsy in NASH patients.

ATP-Citrate Lyase and ACLY Inhibitors

ATP-citrate lyase (ACLY) is a pivotal lipogenic enzyme positioned at the coupling between glycocatabolism and lipid anabolism, and serves as a metabolic checkpoint for detecting excessive nutrients (12, 50). ATP-citrate lyase catalyzes mitochondrial-derived citrate into cytoplasmic oxaloacetate and acetyl-CoA, which in turn promotes the synthesis of fatty acids and cholesterol, and the acetylation of proteins (50). Studies have shown that the expression of ACLY is increased in liver samples from NAFLD patients (51). The increase of ACLY in hepatocytes may be due to the impairment of an E3 ligase-drived ubiquitination-dependent degradation of ACLY during metabolic stress (52). ATP-citrate lyase is involved in inflammatory and IL-4-induced macrophage polarization and activates the transcription factor Stat6 to induce coordinated fibrosis and tissue remodeling (50, 53). ATP-citrate lyase activity inhibition and gene silencing help prevent hepatic steatosis, reduce oxidative stress and prostaglandin E2 inflammatory mediators, thereby indirectly contribute to the improvement of fibrosis in metabolism-induced liver disease (12, 54, 55). Bempedoic acid (ETC-1002) is a first-class, prodrug-based direct competitive inhibitor of ACLY, which reduces ACLY activity and hepatic lipids in rodents (55, 56). Analysis of its phase 2 and 3 clinical studies demonstrated that bempedoic acid has good safety and favorable effects on lipid profiles and inflammation represented by high-sensitivity C-reactive protein (57). This evidence suggests that ACLY may serve as a new therapeutic target for regulating metabolism and MAFLD-related fibrosis. Further clinical trials are needed to evaluate the safety and efficacy of bempedoic acid in improving the primary outcomes of metabolic overload-induced liver disease and its fibrosis.

Fatty Acid Synthase and FASN Inhibitors

As a member of the lipogenic enzymatic cascade, fatty acid synthase (FASN) is a core modulator of hepatic DNL that catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA (58, 59). The powerful rate-limiting capacity of FASN for lipogenesis makes it a promising target for the treatment of liver disease caused by metabolic stress. The inhibition of FASN reduced hepatic DNL and improved liver steatosis in animal models and in patients with NAFLD (58–60). TVB-2640 is a novel FASN inhibitor that significantly reduces liver DNL and hepatic fat in obese subjects after 10 days' treatment (NCT02948569) (61). Given that the inhibition of hepatic DNL decreases intrahepatic fat accumulation, inflammation and fibrosis, a FASCINATE-1 (NCT03938246) phase 2a study focused on the efficacy of 25 mg or 50 mg of TVB-2640 in NASH patients (62). After 12 weeks of treatment, TVB-2640 reduced the liver fat and inflammatory biomarkers levels in a dose-dependent manner. More encouragingly, TVB-2640 produced significant improvements for several serum markers of fibrosis in NASH patients after such a short-term treatment (62). The benefit of TVB-2640 on fibrosis may be a result of the reduction in the DNL-triggered activation of HSCs or from inhibiting the indirect effect of lipotoxicity-mediated fibrosis (62). Encouraged by the success of the pilot study, a 52-week long-term study FASCINATE-2 (NCT04906421) was initiated with a higher drug dose (50 or 75 mg) and is recruiting NASH patients with F2–F3 liver fibrosis.

Stearoyl-CoA Desaturase 1 and SCD1 Inhibitors

Stearoyl-CoA desaturase 1 (SCD1) is also a key enzyme for hepatic lipid anabolism that catalyzes the rate-limiting step of converting saturated fatty acids into monounsaturated fatty acids (63). Studies have indicated that SCD1 is overexpressed in activated HSCs, and is involved in diet-induced steatohepatitis and fibrosis by regulating Wnt signaling (64, 65). Decreasing SCD1 expression through genetic disruption or pharmacological inhibition reduced HSCs activation and alleviated liver fibrosis and steatohepatitis in murine models (66, 67). Arachidyl-amido cholanoic acid (Aramchol) downregulated SCD1 to inhibit DNL in the liver, reduce steatosis and inflammation, and reverse fibrosis in mice (66). In a phase 2a clinical trial (NCT01094158), 300 mg of Aramchol daily for 3 months dramatically reduced the liver fat content in NAFLD patients without significant adverse effects (67). Because the parameters in the above study, such as liver enzymes and insulin sensitivity did not improve, and the efficacy of the 300 mg was better than that of 100 mg, the ARREST phase 2b trial (NCT02279524) carried out a further study with doses of 400 and 600 mg. At the end of the 1-year treatment, Aramchol demonstrated dose-dependent benefits in reducing hepatic steatosis and fibrosis with good tolerability (63). Therefore, the ARMOR phase 3 trial (NCT04104321) was launched to evaluate the efficacy and safety of 300 mg of Aramchol twice a day in NASH patients with stage 2–3 fibrosis and metabolic disorders. The primary endpoint is fibrosis improvement for stage 1 or above and NASH regression, and the trial is currently in the recruitment stage (68).

LIVER FIBROSIS DRIVEN BY DIETARY INTAKE-DERIVED LIPID SYNTHESIS

Fanitol X Receptor and FXR Agonists

Fanitol X receptor (FXR) is a nuclear receptor widely expressed in the liver and small intestinal mucosa that plays an essential role in the sensation of bile acid signals. Fanitol X receptor

senses bile acid signals and regulates their secretion by negative feedback, resulting in decreased intestinal lipid absorption, downregulation of the expression of key lipogenic genes in the liver, and reduced hepatic lipid levels in the end (69). The beneficial effects of the ligation of FXR with bile acids on metabolism also include promoting fatty acid oxidation in the liver, regulating glycogenolysis and gluconeogenesis, as well as restoring insulin sensitivity in muscle and adipose tissue (12). These processes help to reduce toxic lipid production and suppress HSCs activation. The activation of FXR in HSCs has been confirmed to reduce extracellular matrix production and weaken the response of HSCs to profibrotic signals such as TGF-β, thus playing a protective role in fibrosis (70). In patients with fatty liver disease, the expression of hepatic FXR was negatively correlated with disease severity. In a rodent model, the deletion of FXR in the liver acerbates metabolic stress-induced liver steatosis, inflammation and fibrosis (12). Therefore, agonists of FXR are emerging as promising therapeutic agents for treating metabolic stress-induced liver fibrosis.

Obeticholic acid (OCA) is a semisynthetic FXR agonist that is more than 100-fold more potent than the endogenous ligand chenodeoxycholic acid (12). There is evidence from a number of clinical trials and animal studies showing that OCA has a promising effect on improving fibrosis due to NASH (71–73). Animal studies have indicated that OCA reduces metabolic stress-induced liver fibrosis and lipid infiltration and effectively improves systemic IR in obese and diabetic mice (12, 74). In the FLINT phase 2 clinical trial (NCT01265498), liver fibrosis and disease histological features were significantly improved in NASH patients who received 25 mg of OCA daily compared to those who received placebo (72). The benefits of OCA on NASH-induced liver fibrosis were also identified in interim analysis results published in the ongoing REGENERATE phase 3 trial (NCT02548351). Taking 25 mg of OCA daily improved liver fibrosis in nearly a quarter of NASH patients with stage 2–3 fibrosis, which is approximately twice the proportion observed in the control group (73). Twenty-five milligrams of OCA also outperformed placebo in reducing the NAFLD activity score (NAS). Regrettably, the expected endpoint of NASH regression was not achieved after 18 months of OCA treatment. The limitations of OCA treatment include not only mild to moderate dose-dependent pruritus but also more worrisome dyslipidemia, characterized by elevated low-density lipoprotein cholesterol (LDL-c) levels during treatment, which may pose an additional risk of atherosclerosis in NASH patients who are already overweight or suffering from type 2 diabetes (73). Overall, the treatment benefits on mid-term histological endpoint of the REGENERATE study are still uncertain, and the benefits do not significantly outweigh the potential risks. Therefore, in June 2020, the US-FDA rejected the pharmaceutical company Intercept's application for approval of OCA in treating fibrosis due to NASH, recommending that Intercept provide more interim data from REGENERATE and maintain long-term studies on the benefit-risk ratio of OCA. Currently, the efficacy and safety of OCA therapy in patients with compensated cirrhosis due to NASH are being evaluated in a phase 3 clinical trial (NCT03439254). Considering that FXR activation influences plasma lipoprotein concentrations, the combination of OCA and statins was considered and tested in the CONTROL phase 2 study (NCT02633956). After 16 weeks of treatment, OCA-induced increases in LDL-c in patients with NASH were mitigated with atorvastatin. This combination was generally safe and well-tolerated (75).

In addition to OCA, several FXR agonists have been examined in clinical trials or proven to be effective in animal studies. Cilofexor (formerly GS-9674), a small-molecule non-steroidal agonist of FXR, significantly reduced hepatic steatosis, liver biochemical marker, and serum bile acid levels, but did not improve liver fibrosis or stiffness after 24 weeks of treatment (NCT02854605) (76). However, as previously mentioned, it has preferable antifibrotic potential in combination with the ACC inhibitor Firsocostat (NCT03449446). A novel FXR agonist, EDP-305, also demonstrated a potent effect in terms of reducing liver fibrosis triggered by metabolic stress, bile duct ligation, and methionine-choline deficient (MCD) diet in rodent models (77, 78). Similar compounds, e.g., Tropifexor (LJN452) (NCT03517540 and NCT04065841), Nidufexor (LMB763), PX-104, EYP001, and TERN-101, are under investigation (79). In summary, FXR signaling decreases in patients with metabolic overload-induced fatty liver disease, especially in the fibrotic stages. Restoring FXR expression using a FXR agonist demonstrates promising therapeutic potential for treating fatty liver disease and fibrosis. Currently, there are side effects that limit the application of this strategy in clinical practice. Thus, the development of more precise drug targets or combination therapies is warranted.

Fibroblast growth factor 19 (FGF19), secreted by the intestines during feeding, is a signaling hormone downstream of FXR activation and is currently under clinical investigation for the potential treatment of NASH and its associated fibrosis (80). Fibroblast growth factor 19 (mouse ortholog FGF15) promotes NEFA oxidation in mitochondrial and glycogen synthesis via its interaction with FGF receptors (FGFR) in hepatocytes and thus suppresses metabolic stress-induced signaling activation in HSCs and liver fibrosis (80, 81). In addition, FGF19 plays a crucial role in the regulation of systemic glucose and lipid metabolism, as well as maintaining energy homeostasis (81). However, efforts involving the FGF19-FGFR pathway have encountered setbacks. Carcinogenicity were observed in mice treated with FGF19 (81, 82). To overcome these barriers, alternative approaches and molecules need to be developed. Aldafermin (alias NGM282) is an engineered non-carcinogenic FGF19 analog that maintains a key region of the protein involved in receptor interactions and signaling modulation but does not activate the FGF19-STAT3 carcinogenic pathway (82, 83). A 5-aminoacid deletion (P24–S28) coupled with the substitution of three amino acids at crucial positions (Ala30Ser, Gly31Ser, and His33Leu) within the amino terminus of Aldafermin enables biased FGFR4 signaling; thus, Aldafermin retains the ability to potently repress CYP7A1 expression (82). The first phase 2 trial (NCT02443116) assessing the safety and efficacy of Aldafermin for the treatment of NASH revealed that 12 weeks of Aldafermin treatment rapidly decreased liver fat content measured by MRI-proton density fat fraction (MRI-PDFF), and non-invasive markers of inflammation and

fibrosis (84). Because the histological features of NASH were not assessed at the end of this study, an open-label trial of Aldafermin with histological endpoints in patients with NASH was conducted (NCT02443116). Consistently, this study confirmed that Aldafermin improved the NAS and fibrosis score of NASH patients after 12 weeks of treatment (85). Due to the encouraging success in trials of 12 weeks of Aldafermin treatment, the efficacy and safety of 24 weeks of treatment were further evaluated in patients with biopsy-proven NASH (NCT02443116). In this trial, Aldafermin reduced liver fat and produced a trend toward fibrosis improvement, and few adverse events were reported (86). To further evaluate the benefits of Aldafermin on liver fibrosis, two multicenter randomized controlled trials (RCTs) of Aldafermin in subjects with F2/F3 fibrosis (NCT03912532) or compensated cirrhosis (NCT04210245) are underway. It is worth mentioning that Aldafermin modulates CYP7A1-mediated bile acid homeostasis and may lead to an increase in serum cholesterol. An appropriate combination with statins may counteract Aldafermin-induced side effects on the lipid profile (NCT02443116) (87). Although no liver tumors were observed in multiple animal models after prolonged exposure to Aldafermin (82), trials of longer duration are still warranted for further safety evaluation.

Fibroblast growth factor 21 (FGF21) demonstrates large functional overlap with FGF19 in terms of regulating energy homeostasis and metabolism (80, 81). Unlike FGF19, FGF21 is produced in the liver during fasting and in response to elevated NEFA levels. The increased level of FGF21 in plasma has a negative-feedback effect on lipolysis in peripheral tissue (81). Moreover, FGF21 facilitates glucose and lipid uptake and adipogenesis in adipose and muscle tissue, which prevent ectopic lipid accumulation in the liver (80). Fibroblast growth factor 21 has shown great therapeutic potential as a treatment for NASH, but it has poor pharmacokinetic and biophysical properties (88). Numerous FGF21 analogs have been synthesized and developed for the treatment of metabolic diseases. Pegbelfermin (BMS-986036), a PEGylated human FGF21 analog, was tested in patients with NASH in a phase 2a clinical trial (NCT02413372). The administration of Pegbelfermin for 16 weeks significantly reduced the hepatic fat fraction as measured by MRI-PDFF by over 10%, and deceased liver fibrosis biomarkers, e.g., N-terminal type III collagen propeptide (pro-C3) (89). Further research using liver biopsies to assess the effects of 24 weeks of Pegbelfermin treatment on patients with histologically confirmed NASH with stage 3 liver fibrosis (FALCON 1; NCT03486899) or compensated cirrhosis (FALCON 2; NCT03486912) was initiated in 2018 (90). Efruxifermin is an engineered fusion protein formed by linking human IgG1 Fc to modified FGF21, and it has a balanced and long-lasting agonistic effect on FGFR1c, 2c, and 3c (91, 92). In the 16-week phase 2a BALANCED study (NCT03976401), Efruxifermin resulted in a significant reduction in the liver fat fraction as measured by MRI (92). This improvement in hepatic steatosis was accompanied by a reduction in biomarkers of fibrosis and the enhanced liver fibrosis scores. More importantly, 55% of patients achieved stage 1 or greater fibrosis improvement, and half of these patients even met all the exploratory endpoints (92). The major adverse effects of FGF21 analogs is mild gastrointestinal reactions. The promising efficacy and mild side effects of FGF21 analogs demonstrate their potential as treatments for NASH.

LIVER FIBROSIS DRIVEN BY MULTIPLE METABOLIC PATHWAY-DERIVED LIPID SYNTHESIS

Peroxisome Proliferator-Activated Receptors and PPAR Agonists

Peroxisome proliferator-activated receptors (PPAR), a superfamily of nuclear hormone receptors, are extensively involved in the regulation of metabolic homeostasis and inflammatory response in the liver (63). The distribution and functions of the three PPAR hypotypes, α, δ, and γ, are not identical. PPARα is mainly located on liver cells. After activation, it promotes the oxidation of fatty acids in the liver and enhances the expression of superoxide dismutase and catalase to protect liver cells from oxidative stress-induced damage (12). The expression of PPARδ is more extensive and serves various functions, such as inhibiting inflammation, enhancing NEFA oxidation, suppressing adipogenesis, and regulating the immune system (12). PPARα and PPARδ play an important role in suppressing liver fibrosis by inhibiting liver steatosis and inflammation (93). PPARγ is mainly expressed in adipocytes and pancreatic β cells and is able to accelerate the differentiation and storage capacity of adipocytes and regulate glucose metabolism (12, 94). Importantly, PPARγ can be activated by various ligands, such as fatty acids and thiazolidinedione, to inhibit HSCs proliferation and improve liver fibrosis (93). Research-based evidence suggests that PPARγ mediates the effect of liver-protective docosahexaenoic acid in ameliorating liver fibrosis by inducing cell cycle arrest and apoptosis in HSCs (95). In summary, some agonists targeting PPAR may have promise for the treatment of liver fibrosis.

Pirfenidone is an oral PPARα agonist with antisteatogenic and antifibrotic effects that is currently approved for the treatment of idiopathic pulmonary fibrosis (96). To evaluate its value for application in treating advanced liver fibrosis, the PROMETEO phase 2 study (NCT04099407) applied a sustained-release formulation with less potential toxicity to liver metabolism and a longer-lasting plasma concentration (97). The ratio of stage 3 to stage 4 fibrosis in the study population was approximately 1:3, and nearly half of the patients had advanced fibrosis due to NAFLD. Taking 600 mg of Pirfenidone twice a day improved fibrosis in 35% of patients after 12 months and reduced liver enzyme levels in nearly half of the patients. Moreover, the serum TGF-β1 level was lower, and the quality of life appraised by the Euro-QoL scale was better after Pirfenidone treatment. As the serum Pirfenidone concentration was higher in patients with fibrosis regression than in patients with fibrosis progression, Pirfenidone was associated with a better antifibrotic effect (97). The PPARγ agonist Pioglitazone, a first-generation thiazolidinedione agent, has been shown to improve the fibrosis score in NASH patients without diabetes (NCT00994682) (98). However, for patients with diabetes, the

combination of Pioglitazone and vitamin E did not improve liver fibrosis, although this regimen was superior to vitamin E alone or placebo in terms of steatohepatitis resolution (NCT01002547) (99). Due to side effects, such as fluid retention, osteoporotic fracture or hypoglycemia, Pioglitazone may increase the overall risk of patients with metabolic disorders, and second-generation PPARγ agonists have been developed and tested in clinical trials. Novel agents, such as MSDC-0602K, limited the common side effects of Pioglitazone. However, MSDC-0602K failed to improve liver histological features in NASH patients with stage 1–3 fibrosis in a 52-week phase 2b trial (NCT02784444) (100).

Agonists that act on multiple PPAR hypotypes at the same time seem to be more effective than those that only act on one PPAR hypotype. Elafibranor (formerly GFT505) is a dual-pathway agonist that acts on both PPARα and PPARδ. It has been researched in a phase 2b study of NAFLD patients without cirrhosis (NCT01694849). In a *post-hoc* analysis aimed at the degree of steatohepatitis resolution, the researchers found that 120 mg of Elafibranor daily had a better effect on disease activity in the population with a NAS $\geq$4. More importantly, patients who achieved the primary outcome are often accompanied by a reduction in the degree of liver fibrosis (101). Another dual-path agonist, Saroglitazar, targets PPARα and PPARγ and has shown beneficial effects on serum lipid levels and liver biochemical parameters in patients with NAFLD (NCT03061721) (102, 103). It leads to improvement in non-invasive-assessed liver fibrosis parameters in NAFLD patients with diabetic dyslipidemia (104). It may produce antifibrotic effects by reducing oxidative stress and the production of lipotoxic substances, as well as inhibiting leptin signaling. The efficacy and safety of this drug in NAFLD patients with advanced fibrosis are still under evaluation (NCT04469920). Lanifibranor (IVA337), a pan-PPAR agonist that acts on all three receptor subtypes, causes fibrosis regression and ameliorates HSCs-related phenotypes in preclinical models of advanced chronic liver disease (105). In the phase 2b study NCT03008070 completed just a few months ago, Lanifibranor reached the steatosis active fibrosis score endpoint and demonstrated histological fibrosis improvement, with good tolerability. In the forthcoming multicenter phase 3 study NCT04849728, Lanifibranor will be further evaluated in adult patients with non-cirrhotic NAFLD and stage 2/3 liver fibrosis. In general, the benefits of poly/pan-PPAR agonists for liver fibrosis appear to be better than those of single-subtype agonists. We still need a lot of clinical evidence to highlight the direction for the application of such drugs.

Thyroid Hormone Receptor-β and THR-β Agonists

As with the PPAR family, thyroid hormones widely participate in the regulation of lipid and glucose metabolism. Thyroid hormone receptor (THR)-β is mainly expressed in the liver and specifically enhances the oxidative utilization of hepatic fat and cholesterol metabolism (12). Preclinical studies revealed that the specific activation of THR-β reduces hepatic steatosis and fibrosis and improves insulin sensitivity and hepatocyte injury (106). There are two selective THR-β agonists presently under clinical development that can optimize the liver benefits while avoiding the adverse cardiac and skeletal effects of activating THR-α.

Resmetirom (MGL-3196) is a liver-directed THR-β agonist. In a phase 2 study (NCT02912260), the oral administration of 80 mg of Resmetirom daily for 36 weeks improved fibrosis activity markers in NAFLD patients with stage 1–3 fibrosis and caused the resolution of steatohepatitis in nearly 30% of patients, who also showed an improvement in the liver fibrosis stage compared with those treated with placebo (107). To obtain more data on the safety and efficacy of Resmetirom in non-invasive assessments, an open-label extension study (NCT02912260) was conducted in 31 patients from the aforementioned study with sustained mild to significantly increased liver enzyme levels. A reduction in fibrosis markers such as pro-C3 and liver stiffness assessed by transient elastography was observed after 36 weeks of Resmetirom treatment (108). Unlike the aforementioned OCA or Aldafermin, Resmetirom resulted in reduced levels of multiple lipids that carry the risk of atherosclerosis, such as LDL-c and triglycerides (108). In addition, Resmetirom was well-tolerated, and the main adverse effects were transient mild diarrhea or nausea (107, 108). The considerable efficacy and safety support the ongoing phase 3 clinical study (NCT03900429) to explore the efficacy for NAFLD patients with F2–F3 fibrosis. Another selective THR-β agonist is VK2809 (formerly called MB07344), which significantly improved liver fat content in NAFLD patients with hyperlipidemia (NCT02927184); the publication of the study result is expected. A 52-week phase 2b study, VOYAGE, is currently being conducted in NAFLD patients with stage F1–F3 fibrosis to investigate the benefits of 1.0, 2.5, 5.0, and 10 mg of VK2809 compared with placebo on liver fat content, fibrosis, and histopathology (NCT04173065). Compared with VK2809, Resmetirom has more clinical evidence to support its therapeutic potential in NASH.

LIVER FIBROSIS DRIVEN BY CELLULAR STRESS AND APOPTOSIS

Vitamin E

Excessive fatty acids and subsequent mitochondrial dysfunction lead to ROS production, which plays a crucial role in NASH and advanced fibrosis (17, 24, 109, 110). Vitamin E, as a major fat-soluble chain-scission antioxidant, prevents plasma lipid and low-density lipoprotein peroxidation and protects the structural integrity of cells from damage caused by lipid peroxidation and oxygen-free radicals (110, 111). In addition to its powerful antioxidant effects, vitamin E also induces adiponectin expression, reduces inflammatory signaling, and regulates macrophage polarization, making it a potential treatment option for suppressing oxidative stress and metabolism-related liver diseases (111, 112). In the metabolic stress-induced NASH model in mice, vitamin E reduces oxidative stress, improves hepatic fibrosis and HSCs activation, and alleviates hyperinsulinemia. The antifibrotic properties may be due to the inhibition of TGF-β expression after the downregulation of ROS production, thereby reducing the activation of HSCs (112). A clinical study indicated that using 800 IU of vitamin E per day significantly

improved transplant-free survival and liver decompensation in NASH patients with stage F3–F4 fibrosis (113). However, there is still a lack of direct and conclusive evidence of the beneficial effect of vitamin E on NASH-induced liver fibrosis. Vitamin E did not cause significant regression of liver biopsy-proven fibrosis in RCTs (NCT00063622, NCT00063635, and NCT01002547), although it induced varying degrees of improvement in NASH histology (99, 114, 115). In general, the liver benefits of vitamin E alone were more embodied in the reduction of oxidative stress marker levels and improvement of liver function and the NAS (99, 111, 116). It must be emphasized that there is evidence suggesting that dietary vitamin E supplementation might increase cancer risk (NCT00006392) and mortality in the healthy population (117, 118). However, meta-analyses from recent years have shown that the adverse effects of vitamin E supplementation on all-cause mortality or cancer risk are not significant, supporting dietary intake of this natural antioxidant (119, 120). Therefore, hepatologists may be inclined to use vitamin E in combination with other reagents in the study of NAFLD/MAFLD.

Caspase Inhibitors

Chronic liver injury induced by excessive toxic lipid leads to increased hepatocyte apoptosis, which is an important feature of NASH (121, 122). Apoptotic hepatocytes activate immune cells and HSCs, thereby promoting liver fibrosis and cirrhosis (122, 123). Caspases, a family of cysteine proteases, play a central role in the progression of NAFLD/NASH due to their role in the regulation of liver apoptosis and inflammation (124). Caspase inhibitors have been studied and tested as therapeutic agents for NASH (122). Emricasan (IDN-6556) is an irreversible pan-caspase inhibitor that ameliorates apoptosis and liver fibrosis in NASH mouse models (125). In a short-term clinical study (NCT02077374), Emricasan suppressed caspase activation and liver enzyme levels in patients with NASH after 4 weeks of treatment, with decent safety and tolerance (126). Moreover, 3 months of treatment with Emricasan improved liver function more in patients with cirrhosis caused by NASH than in those with cirrhosis caused by viral hepatitis or alcoholic liver disease and showed a potential beneficial effect on portal hypertension (NCT02230670) (127). However, Emricasan did not show a significant beneficial effect on fibrosis regression in RCTs with a larger sample of NASH patients. In NASH patients with stage F1–F3 fibrosis (NCT02686762), usage of Emricasan at 10 or 100 mg daily for 72 weeks failed to improve liver fibrosis or lead to NASH resolution. Moreover, fibrosis and hepatocyte ballooning were aggravated in the Emricasan group (128). In another study conducted in NASH patients with compensatory cirrhosis (NCT02960204), Emricasan at 10, 50, or 100 mg daily did not cause an improvement in clinical outcomes (129). In NASH patients with decompensated cirrhosis (NCT03205345), administering 10 or 50 mg of Emricasan daily also did not reduce the amount of decompensation events or improve liver function after 48 weeks (130). These three studies indicated a robust effect of Emricasan on caspase activity inhibition and a good safety, but none demonstrated a significant therapeutic benefit. This may be due to excessive inhibition of apoptosis activating alternative forms of cell death, such as necroptosis and pyroptosis (124, 128). It is also possible that cirrhosis leads to many pathological changes, such as reduced number of functional hepatocytes, decreased hepatic blood flow and transporter protein expression, resulting in unsatisfactory drug bioavailability. Therefore, a daily dose of 50–100 mg may be insufficient for patients with cirrhosis (129, 130). Although none of these studies achieved the primary endpoint, they provided valuable reference data and ideas for design optimization in future clinical research on NASH-associated fibrosis. In a recent study in an HCV patient treated with liver transplantation, 24 months of Emricasan therapy showed a beneficial effect on moderate liver fibrosis. Although the pathogenesis of HCV-related fibrosis differs from that in NASH, this positive result inspires the initiation of treatment in NASH patients with moderate fibrosis (131).

LIVER FIBROSIS DRIVEN BY THE INNATE IMMUNE SYSTEM AND INFLAMMATION

The liver consists of a network of innate immune cells, which collectively form the first line of defense against invading organisms and toxins (132). Under excessive metabolic stress, the hepatic innate immune system is over-activated to further trigger hepatic cell injury and liver fibrosis (22, 25, 133, 134). There are a number of molecular targets that function as hubs controlling the inflammatory signaling flow in the progression of fatty liver disease, and they have been emerging as targets in the development of drugs for the treatment of MAFLD/NAFLD and fibrosis (48, 135–140).

Apoptosis Signal-Regulating Kinase 1 and ASK1 Inhibitor

Studies have revealed that apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, is hyperactivated in the liver of NASH patients. Upon receiving metabolic stress signals, ASK1 activates the downstream c-Jun N-terminal kinase (JNK) 1/2-mitogen-activated protein kinase 14 (p38) signaling cascade to trigger hepatic inflammation and fibrosis during the development of MAFLD (135, 136). Apoptosis signal-regulating kinase 1 functions as a molecular hub controlling cellular signal transduction in NASH. It has been considered an essential target for the development of drugs for NASH.

A number of clinical trials evaluating the efficacy of ASK1 inhibitors against NASH have been performed. There have been two large phase 3 studies in patients with NASH and advanced fibrosis. They compared the effect of the ASK-1 inhibitor Selonsertib (GS-4997) with that of placebo in ∼1,700 patients with NASH and bridging fibrosis (F3, STELLAR-3) or compensated cirrhosis (F4, STELLAR-4) (NCT03053050 and NCT03053063). Although Selonsertib successfully suppressed the expression of hepatic phospho-p38, it did not significantly improve liver fibrosis on liver biopsy (137, 138). There are several explanations for the failure of these large trials. First, there are a number of signaling pathways involved in the pathogenesis of NASH, particularly in the advanced stages, which suggests

that combination therapy may be required in the treatment of NASH. In a phase 2 clinical trial, 72 patients with NASH and stage F2–F3 fibrosis were treated with either 6 or 18 mg of GS-4997 orally once daily alone or in combination with a once-weekly injection of 125 mg of Simtuzumab (a humanized monoclonal antibody directed against lysyl oxidase-like molecule 2) for 24 weeks (NCT02466516). Reduced liver hardness on MRI elastography, reduced collagen content and lobular inflammation on liver biopsy, and improved serum markers of apoptosis and necrosis all suggested improvement in liver fibrosis. The assessed results showed that the proportion of patients with a reduction of fibrosis of at least one stage at week 24 was 20% in the Simtuzumab -alone group (2 of 10; 95% confidence interval (CI), 3–56), 30% in the 6-mg Selonsertib group (8 of 27; 95% CI, 14–50), and 43% in the 18-mg Selonsertib group (13 of 30; 95% CI, 26–63). The changes in fibrosis stage were correlated with the changes in hepatic collagen content ($r = 0.54$, $P < 0.001$). The median percent change in the morphometric collagen content of patients who were treated with Simtuzumab alone was 2.1%, while that of patients treated with 6 and 18 mg of Selonsertib was −8.2 and −8.7%, respectively. In summary, compared with patients treated with Simtuzumab alone, patients treated with Selonsertib showed a higher rate of fibrosis improvement and a lower rate of fibrosis progression. These findings suggest that Selonsertib combined with Simtuzumab may reduce liver fibrosis in patients with NASH and stage 2–3 fibrosis (139).

Another study was performed to evaluate the safety and efficacy of Selonsertib, Firsocostat, Cilofexor, and combinations in participants with bridging fibrosis or compensated cirrhosis due to NASH (NCT03449446). In this study, 392 patients with bridging fibrosis or compensated cirrhosis due to NASH were randomized to receive 18 mg of Selonsertib, 20 mg of Firsocostat, or 30 mg of Cilofexor, alone or in two-drug combinations, once daily for 48 weeks. Histological parameter analysis showed that for the primary endpoint of an improvement in fibrosis of ≥1 stage without the worsening of NASH, the proportion of patients was 12% (4 of 33, $P = 0.94$) in the Firsocostat group, 12% (4 of 34, $P = 0.96$) in the Cilofexor group, 15% (11 of 71, $P = 0.62$) in the Firsocostat/Selonsertib group, 19% (13 of 68, $P = 0.26$) in the Cilofexor/Selonsertib group, and 21% (14 of 67, $P = 0.17$) in the Cilofexor/Firsocostat group. A higher response rate was observed in the combination groups than in the monotherapy groups, but the differences between the treatment and placebo arms did not reach statistical significance. However, patients treated with Cilofexor/Selonsertib (8%; $P = 0.018$ vs. placebo) were significantly less likely to progress to cirrhosis than those treated with placebo (41%). These results suggest that combination therapy with Selonsertib offers the possibility of fibrosis reversal in the long-term treatment of patients with advanced NASH and fibrosis (48). Additional studies are warranted to confirm the potential therapeutic effects of ASK1 inhibitors on liver fibrosis in NASH.

Since ASK1 plays essential roles in physiological function, modulating its activity via posttranslational modification could be a more appropriate strategy in the treatment of disease. Many ASK1-negative regulators have been reported to significantly inhibit the development of NASH-associated fibrosis in rodents and preclinical models (140–142). For instance, the disassociation of milk fat globule-epidermal growth factor-factor 8 from ASK1 accelerates its dimerization and phosphorylation in hepatocytes under metabolic stress, thus leading to liver steatosis and fibrosis (140, 141). The deubiquitinating enzyme tumor necrosis factor-alpha-induced protein 3 (TNFAIP3) directly interacts with and deubiquitinates ASK1 in hepatocytes and ameliorates metabolic stress-induced hepatic inflammation and fibrosis (144). Tumor necrosis factor receptor-associated factor 6 promotes the polyubiquitination of Lys6 connections and the activation of ASK1, in turn exacerbating inflammatory and fibrotic responses in the liver (143). A high-fat diet also induces the overexpression of hepatic E3 ligase Skp1-Cul1-F-box protein F-box/WD repeat-containing protein 5 (FBXW5), which is a key endogenous activator of ASK1 ubiquitination and activation, and small molecules that mimic FBXW5 (S1) and FBXW5 (S3) can block the ubiquitination of ASK1 in MAFLD (144). Future clinical trials could aim to these molecules that regulate the activity of ASK1 in the posttranslational modification process, which may lead to better therapeutic effects in the treatment of NASH.

TGF-β-Activated Kinase 1 and TAK1 Inhibitors

TGF-β-activated kinase 1 (TAK1) is a member of the MAP3K family and is known as a central signalosome in the regulation of the inflammatory response (145). Conventionally, TAK1 is activated by proinflammatory cytokines and agonists of toll-like receptors to activate MAPK and NF-κB signaling pathways (146). There is accumulating evidence showed that metabolic stress also promotes TAK1 signalosome formation and activity in hepatocytes, which leads to the development of NAFLD and NASH (147). However, previous studies showed that the complete deletion of TAK1 expression also accelerates NASH progression, suggesting that maintenance of the normal enzymatic activity of TAK1 is also critical for sustaining homeostasis in metabolism and inflammation (148). Therefore, posttranslational modifications are essential in fine-tuning the activity of the TAK1 signalosome under such conditions. Recent studies have revealed endogenous molecules that are important in the regulation of TAK1 ubiquitination or phosphorylation without suppressing its physiological activity, which may serve as potential targets in the development of treatments for NASH. Evidences from mouse or preclinical non-human primate models showed that the deubiquitinating enzyme cylindromatosis, TNFAIP3-interacting protein 3, ubiquitin-specific protease (USP) 4, and USP18 mitigate liver steatosis, inflammation, and fibrosis by deubiquitinating metabolic stress-induced TAK1 ubiquitination and activation (149–153), while dual-specificity phosphatase 14 and regulator of G protein signaling 5 dephosphorylate TAK1, resulting in the reduced activation of TAK1 and its downstream signaling pathways (154, 155). Although these molecules show strong potency, their safety and efficacy required to be tested in prospective studies.

Toll-Like Receptors and TLR4 Inhibitors

Due to the unique anatomical association of the liver with the intestine, the blood supply of the liver is enriched in microbial-associated molecular patterns (PAMPs) and nutrients. Thus, Toll-like receptors (TLRs) play an essential role in liver physiology and pathophysiology (156). Previous evidence has shown that TLRs are involved in the pathogenesis of NASH and liver fibrosis (157–159). Among these TLRs, the role of TLR4 has been the most extensively studied due to its importance in recognizing gut-derived endotoxin (160). The genetic deletion or pharmaceutical inhibition of TLR4 improved liver steatosis, inflammation, and fibrosis in response to a high-fat diet in mice and nonhuman primates (158). A small long-acting molecule, JKB-121, inhibits TLR4, which inhibits liver fibrosis by repressing the redox status and stellate cell activation in the liver (NCT02442687). Recently, a novel TLR4 antagonist, JKB-122, was developed and shown to be effective in reducing autoimmune hepatitis-associated liver necrosis and inflammation in animal models (161). A phase 2 study testing the efficacy of JKB-122 for 52 weeks in subjects with NASH with fibrosis was initiated in 2020 (NCT04255069).

Vascular Adhesion Protein 1 and VAP-1 Inhibitor

Vascular adhesion protein 1 (VAP-1) is continuously expressed as a membrane-bound amine oxidase along the sinusoidal endothelium, which facilitates the accumulation of inflammatory cells into the inflamed environment in concert with other leukocyte adhesion molecules (162). The soluble form of VAP-1 (sVAP-1) is also found in the serum of healthy adults, and its expression is increased under inflammatory conditions and in metabolic disorders (163). Vascular adhesion protein 1 can modulate leukocyte migration in both its transmembranous and soluble forms. Studies have shown that hepatic VAP-1 and serum sVAP-1 expression is increased in patients with NAFLD compared with control individuals (164). In addition, VAP-1 plays an essential role in hepatic fibrosis due to a number of etiologies, such as NAFLD, HBV, and HCV (163–165). Mechanistically, VAP-1 directly affects stellate cells by enhancing the expression of profibrotic genes and promoting liver fibrosis (163). The VAP-1 mutant strain showed significant attenuations of liver inflammation and fibrosis in the MCD diet model (163). PXS-4728A is a selective and orally active VAP-1 inhibitor with potent efficacy observed in animal trials (166). In 2015, the data from a phase 1 clinical trial showed that PXS-4728A administered for 14 days at doses between 3 and 10 mg was safe and well-tolerated. The data suggest that low doses are effective in inducing persistent enzyme inhibition, but further clinical trials are needed to verify the effectiveness of the drug in treating NASH.

C-C Chemokine Receptor and Ligand and CCR Antagonists

During NASH progression, C-C chemokine receptor type 2 (CCR2) and C-C chemokine receptor type 5 (CCR5), together with their respective ligands, C-C chemokine ligand types 2 (CCL2) and C-C chemokine ligand types 5 (CCL5), promote liver fibrosis by increasing immune cell aggregation and infiltration and amplifying the inflammatory response (167–170). Cenicriviroc is a dual CCR2 and CCR5 antagonist with significant antifibrotic and anti-inflammatory activity in models of fibrosis, such as the mouse peritonitis model, mouse diet-induced NASH model, and rat thioacetamide-induced liver fibrosis model (171). Recently, it has been explored in the treatment of liver fibrosis associated with NASH (172). The 2-year phase 2b CENTAUR study showed that Cenicriviroc treatment resulted in liver fibrosis improvement compared to placebo, with a greater effect on advanced fibrosis (172). Due to the success in the phase 2 trial, a phase 3 study examining the efficacy and safety of Cenicriviroc in the treatment of liver fibrosis in adults with NASH was initiated in 2017. Unfortunately, there was a lack of efficacy at the 12-month follow-up in terms of achieving an improvement in fibrosis of at least 1 stage with no worsening of steatohepatitis (NCT03028740). There are emerging studies investigating whether combination therapy provides superior clinical effectiveness in the treatment of NASH and fibrosis. A phase 2 clinical trial in NASH patients exploring the efficacy of a combination of Tropifexor (LJN452, an FXR agonist) and Cenicriviroc has been completed, and the results are pending publication (NCT03517540).

LIVER FIBROSIS DRIVEN BY OTHER MECHANISMS

Lysyl Oxidase-Like 2 and LOXL2 Monoclonal Antibody

Lysyl oxidase-like 2 (LOXL2) is an extracellular copper-dependent enzyme that catalyzes the cross-linking of structural extracellular matrix components in fibrous organs, including the liver (173). The serum LOXL2 level was associated with the severity of liver fibrosis (174). In a preclinical model characterized by advanced fibrosis and portal hypertension, an anti-LOXL2 antibody decreased the portal pressure in Mdr2-knockout mice (175). However, the LOXL2 monoclonal antibody Simtuzumab failed to reduce the liver collagen content and fibrosis in NASH patients with advanced fibrosis and cirrhosis (NCT01672866 and NCT01672879) (176). These findings were also observed in a phase 2 trial of the combination of the ASK 1 inhibitor Selonsertib and Simtuzumab. The coadministration of Selonsertib and Simtuzumab did not provide additional benefits over Selonsertib therapy alone in patients with NASH and moderate to severe fibrosis (139). There are several potential factors explaining the failure of the Simtuzumab trial. First, LOXL2 may be the driver for NASH, and fibrosis or redundancy in other pathways may mediate collagen formation. Second, although Simtuzumab effectively binds LOXL2, the dose and frequency at which it was applied in the study might be insufficient to neutralize its activity.

Galectin-3 and Galectin-3 Inhibitors

Galectin-3 is a β-galactoside-binding animal lectin in the nucleus and cytoplasm and on the cell surface that has been

implicated in a variety of biological processes, including cell proliferation, survival and inflammation. Galectin-3 expression is upregulated in human fibrotic liver disease, and the level is associated with the induction and resolution of hepatic fibrosis in animal models (177). A mechanistic study showed that galectin-3 in HSC is required for TGF-β-mediated myofibroblast activation and matrix production during disease progression (177). Preclinical results showed that the galectin-3 inhibitor Belapectin (GR-MD-02) was effective in mouse models of NASH with advanced fibrosis or cirrhosis (178). Although Belapectin was safe and well-tolerated in a phase 1 trial (179), in this 16-week phase 2 clinical study, Belapectin treatment failed to alleviate liver fibrosis in patients with NASH with advanced fibrosis, as measured by multiparametric MRI corrected T1 mapping (NCT02421094). Similarly, another phase 2b trial of the safety and efficacy of Belapectin in patients with NASH, cirrhosis, and portal hypertension further showed that Belapectin was not associated with a significant reduction in the hepatic venous pressure gradient or fibrosis (NCT02462967) (180). However, treatment with Belapectin reduced the venous pressure gradient in a subset of patients without esophageal varices (180). To confirm this discovery, a phase 2b/3 trial evaluating the efficacy of Belapectin for the prevention of esophageal varices in NASH-associated cirrhosis was initiated and is expected to be completed in 2023 (NCT04365868).

TGF-β AND TGF-β MONOCLONAL ANTIBODY

TGF-β is an important pleiotropic cytokine involved in many biological processes such as cell survival, proliferation, differentiation, angiogenesis, and wound healing (181). In advanced MAFLD, TGF-β is activated by HSCs, triggering a series of responses including tissue repair, extracellular matrix production, growth regulation, and apoptosis, ultimately leading to liver fibrosis (182). Studies in NASH model of wild-type and hepatocellular specific TGF-β receptor type II deficiency mice demonstrated that TGF-β signaling in hepatocytes promotes lipid accumulation by regulating lipid metabolism and enhancing cell death in hepatocytes that accumulate lipid, leading to the development of hepatic steatosis, inflammation, and fibrosis (183). Previous studies have shown a significant increase in TGF-β expression in the liver of patients with NASH fibrosis (184). Preclinical results showed that TGF-β inhibitor Galunisertib affected parenchymal cell fate by regulating the biochemical composition of deposited extracellular matrix and inhibited the progression of liver fibrosis, but did not significantly improve the pathological grading of fibrosis in Abcb4ko mice (185). At present, there have been clinical studies on TGF-β inhibitors restraining fibrosis in other organs (idiopathic pulmonary fibrosis and myelofibrosis), but there are no clinical studies related to liver fibrosis. It is believed that researchers will conduct many clinical studies on TGF-β inhibitors in the fibrosis process of MAFLD in the future.

CONCLUSION AND PERSPECTIVES

The burden of MAFLD/NAFLD is increasing rapidly with the ongoing metabolic disease epidemic (6, 7). MAFLD/NAFLD have shared and predominate causes from nutrition overload to persistent liver damage and eventually lead to the development of liver fibrosis and cirrhosis (10, 186). Discoveries have revealed that the pathogenesis of fibrosis in NAFLD involves multiple mechanisms and factors, such as lipid metabolism, inflammation, cell apoptosis, oxidative stress, extracellular matrix formation, and intestinal flora, as well as genetic and epigenetic regulation (17, 134). Reagents specifically targeting these pathways and receptor/ligand interactions have been developed, including agents acting on lipid synthesis, i.e., GLP-1 agonists, ACC inhibitors, FXR agonists, PPAR-α/δ agonists, and THR-β agonists, agents acting on cell stress and apoptosis, i.e., vitamin E and caspase inhibitors, agents acting on the innate immune system and inflammation, i.e., ASK1 inhibitors, TLR4 inhibitors, VAP-1 inhibitors, and CCR2/5 antagonists, and agents acting on other mechanisms, i.e., LOXL2 monoclonal antibodies and galectin-3 inhibitors (**Supplementary Table 1**). Although substantial advances have been made in the development of novel antifibrotic targets and therapeutic compounds, very few have reached clinical primary endpoints without significant side effects in large clinical studies. Therefore, it is important to recognize the boundaries and drawbacks of the traditional paths of drug discovery. First, since the majority of mechanistic investigations are based on rodent models, the application of models in large animals, such as non-human primates, with a closer resemblance to humans in the preclinical phase would likely allow for a higher chance of translating basic discoveries to clinical practice. Second, as the pathways involved in fibrosis are complex and targeting one mechanism may trigger alternative compensatory mechanisms, combination therapies targeting multiple profibrotic pathways could be promising in achieving successful antifibrotic interventions in patients with MAFLD/NAFLD. Third, the inconsistent results in previous trials have indicated that there may be large variations in the genetic predisposition and mechanisms involved in the pathogenesis of MAFLD/NAFLD fibrosis among individuals. A one-size-fits-all strategy would not be applicable for the treatment of MAFLD/NAFLD. Fourth, although liver biopsy is the gold standard for diagnosing NASH and assessing the stage of fibrosis in patients with NAFLD, this methodology misses the systemic evaluation of liver pathology and involves interobserver variations and biopsy bias. Liver biopsy cannot be performed for screening and follow-up in large populations due to its well-known limitations. There is an urgent need to improve the methodology in the evaluation of liver fibrosis.

In summary, the high prevalence of MAFLD/NAFLD paired with end-stage complications emphasizes the need for the discovery of effective and safe pharmaceutical treatments. In the current situation, one should keep in mind that appropriate lifestyle interventions with improvements in metabolic risk factors can potentially impede

the development of MAFLD/NAFLD (13). In addition to accelerating the discovery of new pharmacotherapeutics, personalized medicine, combination therapies targeting multiple profibrotic pathways, and different methodologies for evaluating fibrosis would be beneficial for the development of new treatment strategies with good tolerability and efficacy.

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WQ and TM collected the data and drafted the first edition of the paper. All authors listed have made a substantial, intellectual and direct contributions to this review, and authorized the publication of it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.761538/full#supplementary-material

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Longitudinal Associations Between Hand Grip Strength and Non-Alcoholic Fatty Liver Disease in Adults: A Prospective Cohort Study

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Purpose: This study aimed to determine the longitudinal association between hand grip strength (HGS) and the development of non-alcoholic fatty liver disease (NAFLD) in adults.

Design: A cohort study.

Methods: This study was conducted in a general Chinese population ($n = 14,154$) from 2013–2018. NAFLD was diagnosed by liver ultrasonography during evaluating alcohol consumption. The associations between the HGS and NAFLD were assessed using a multivariable Cox proportional hazards regression model.

Results: During the study period with a mean follow-up duration of 3.20 years, 2,452 participants developed NAFLD. The risk of NAFLD decreased progressively with increasing HGS in both men and women (P for trend < 0.0001). The multivariate-adjusted hazard ratios (95% *CI*) for NAFLD incidence across the quartiles of HGS were 1 (reference), 0.90 (0.79, 1.02), 0.69 (0.60, 0.79), and 0.44 (0.37, 0.52) for men and 1 (reference), 0.82 (0.69, 0.96), 0.54 (0.45, 0.66), and 0.41 (0.33, 0.52) for women, respectively. The interaction terms for body mass index (BMI)-HGS and waist-HGS were significant in men and women (all $P < 0.0001$). The participants with normal BMIs and waist circumferences had the lowest hazard ratios on the subgroup analyses. The sensitivity analysis that defined NAFLD using the hepatic steatosis and fatty liver indices revealed results that were similar to the main analyses.

Conclusion: The present study indicates that the HGS is inversely associated with the incidence of NAFLD.

Keywords: hand grip strength, non-alcoholic fatty liver disease, cohort, China, general adults

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INTRODUCTION

The non-alcoholic fatty liver disease (NAFLD) represents a spectrum of liver diseases not attributable to alcohol consumption, such as simple fatty infiltration, inflammation, and cirrhosis. Globally, the NAFLD is one of the most important causes of liver disease (1) and the previous studies have demonstrated that it is associated with metabolic syndrome (2), diabetes (3), and hypertension (4). As reported in a meta-analysis conducted in 2016, 25% of the global adult population were afflicted with NAFLD (5). Moreover, in China, the prevalence of NAFLD among adults in the general population is >20% and has paralleled the increase in obesity (6). In addition, the obesity prevalence rose from 3.1% (2.5–3.7) in 2004 to 8.1% (7.6–8.7) in 2018 (7). In parallel with increasing prevalence, the economic burden of NAFLD is enormous, especially at the time of diagnosis (8). Therefore, it is important to identify the modifiable risk factors and develop preventive strategies.

Insulin resistance is shown to be an important factor in NAFLD progression (9). A muscle is a target organ for insulin (10), and the previous studies suggested that the skeletal muscles secrete a variety of metabolically bioactive factors, such as myostatin, interleukin-6, and irisin (11, 12) that are subsequently involved in the regulation of insulin resistance and lipid metabolism. Thus, it is plausible that the muscles play an important role in the development of NAFLD. Indeed, several cross-sectional studies have shown that muscle strength is associated with NAFLD (13–18). For example, a cross-sectional study involving 5,132 adults in China showed that the low muscle strength was positively and independently associated with NAFLD [odds ratio (*OR*), 1.47; 95% *CI*, 1.21, 1.80] (13). Another nationwide population-based cross-sectional study demonstrated that the high hand grip strength (HGS) was negatively associated with the hepatic steatosis index (HSI) in 4,764 participants of Koreas (14). Because of the cross-sectional design of these studies, however, a causal relationship could not be identified.

To our knowledge, there has been no cohort study conducted to investigate the associations between muscle strength and the incidence of NAFLD. Thus, we conducted this prospective study to better understand the association between the HGS and NAFLD using data from a large population-based cohort study in adults in China.

METHODS

Participants

This prospective study was based on a large prospective dynamic cohort study conducted in Tianjin, China (19). Between 2013 and 2018, a total of 29,551 participants had at least two health examinations with adequate data related to the NAFLD diagnosis, lifestyle (*via* questionnaire), and physical performance tests. After exclusions (**Figure 1**), the cohort consisted of 15,773 participants at baseline. As 1,619 participants did not complete the follow-up health examinations, the final study population comprised 14,154 participants (follow-up rate of 89.74%). The mean duration of follow-up was 3.20 years (range, 0.50–5 years). The protocol of this study was approved by the Institutional Review Board of the Tianjin Medical University. The subjects provided the written informed consent to participate in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

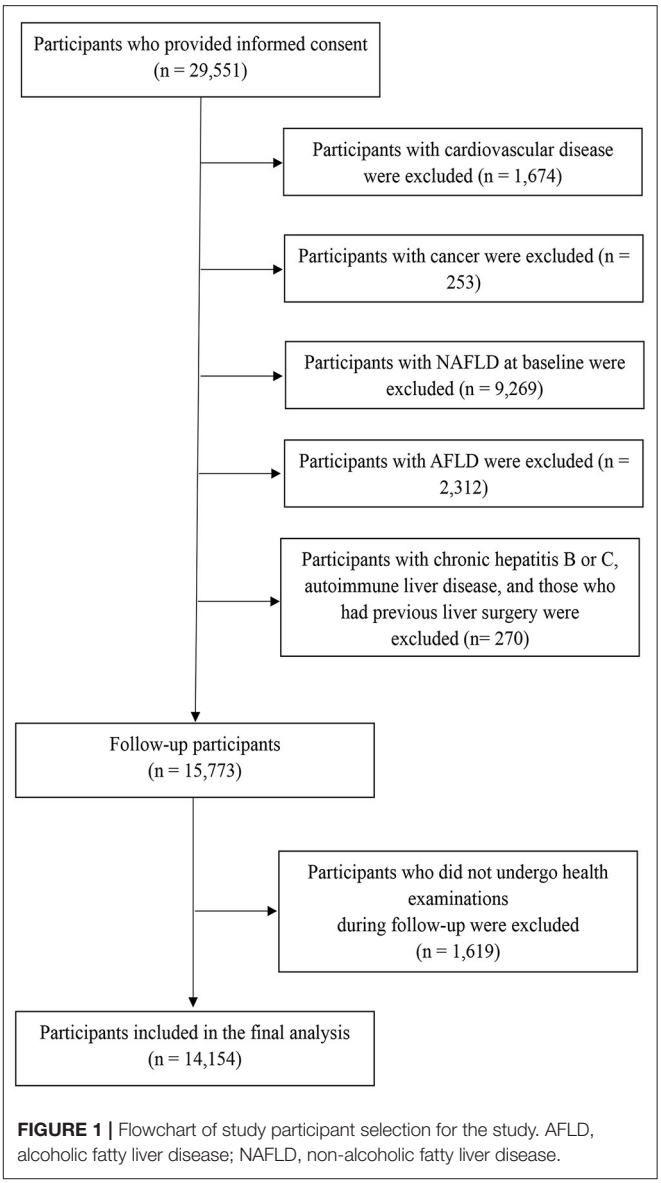

FIGURE 1 | Flowchart of study participant selection for the study. AFLD, alcoholic fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

Assessment of NAFLD

Liver ultrasonography was performed by the trained sonographers using a Toshiba SSA-660A instrument (Toshiba, Tokyo, Japan) with a 2–5 MHz curved array probe. According

Abbreviations: AFLD, alcoholic fatty liver disease; AUC, area under the curve; BMI, body mass index; CI, confidence interval; FFQ, food frequency questionnaire; FLI, fatty liver index; HGS, hand grip strength; HIS, hepatic steatosis index; HRs, hazard ratios; MET, metabolic equivalent; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; Q, quartile; ROC, receiver operating characteristics; SDS, self-rating depression scale.

to the revised 2018 NAFLD definition and treatment guidelines promulgated by the Chinese National Workshop on Fatty Liver and Alcoholic Liver Disease (20), the participants were diagnosed with NAFLD based on the detection of brightness in the liver and a diffusely echogenic change in the liver parenchyma on abdominal ultrasonography despite no history of heavy alcohol consumption (defined as >210 g of alcohol intake per week in men and >140 g per week in women).

In the sensitivity analysis, the participants were diagnosed with NAFLD using the HSI and fatty liver index (FLI), which were developed to identify the presence of suspected NAFLD. The HSI was calculated using the following algorithm: HSI = 8 × the alanine aminotransferase-to-aspartate transaminase ratio + the body mass index [BMI] (+ 2, if type 2 diabetes was present and +2 if female). An HSI value >36.0 predicted the NAFLD with a specificity of 92.4% (95% *CI*, 91.3–93.4) (21). The FLI was calculated using the following algorithm: FLI = $(e^{0.953*\log e\ (\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e\ (\text{ggt}) + 0.053*\text{waist circumference} - 15.745})/ (1 + e^{0.953*\log e\ (\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e\ (\text{ggt}) + 0.053*\text{waist circumference} - 15.745})*100$ (22). According to a previous study that explored the validation of the FLI for NAFLD in the Chinese, an FLI value ≥ of 30 was used as the cut-off point for NAFLD with a sensitivity of 79.89% and a specificity of 71.51% (23).

Measurement of Muscle Strength

The muscle strength was assessed using the HGS, which is a feasible and convenient indicator of the overall muscle strength with good test-retest reliability and responsiveness (24). The participants were tested by the trained technicians using a handheld dynamometer (EH101; Camry, Guangdong, China). The participants were asked to stand upright with the dynamometer beside but not against their bodies and to perform two maximum force trials for each hand; the greatest force was used as the final score. Furthermore, HGS was normalized to the bodyweight to account for the proportion of HGS relative to the body weight [HGS (kg)/body weight (kg)] (25).

Assessments and Definitions of Other Variables

The sociodemographic variables, such as sex, age, education, employment, and household income, were assessed *via* questionnaire, as were cigarette smoking and alcohol consumption. The BMI was calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2). The physical activity in the most recent week was assessed using the short form of the International Physical Activity Questionnaire (26). High depressive symptoms were assessed using the Chinese version of the Zung Self-Rating Depression Scale (SDS), a useful and well-validated questionnaire commonly used by the Chinese psychiatrists; (27) the participants were defined as high depressive symptoms when the SDS score was ≥45 (28). Hypertension was defined as an average systolic blood pressure ≥140 mm Hg or average diastolic blood pressure ≥90 mmHg or as the use of antihypertension medication (29). Hyperlipidemia was defined as a total cholesterol level ≥5.20 mmol/L, a triglycerides level ≥1.70 mmol/L, or as a self-reported clinical diagnosis of hyperlipidemia according to the 2016 Chinese guidelines for the management of dyslipidemia in adults (30). Dietary intake was assessed using a modified version of the Food Frequency Questionnaire (FFQ) that included 100 food items with specified serving sizes; detailed information about this FFQ has been described elsewhere (31, 32). The Chinese Food Composition tables were used as the nutrient database to calculate the total energy intake per day (33). The factor analysis was applied to generate the major dietary patterns and food loading for all the 100 food items and beverages in grams. The factors were named descriptively according to the food items showing high loading (absolute value > 0.3) with respect to each dietary pattern as follows: sweet foods pattern, vegetable pattern, and animal foods pattern. The dietary patterns scores were used for further analyses as confounding factors.

Statistical Analysis

The characteristics of participants at the baseline are described according to sex and NAFLD status. The continuous variables are presented as least-square means and 95% *CI*, in which the categorical variables are presented as percentages. The quartiles were categorized across HGS based on the distribution of the scores and used for further analyses. The Cox proportional hazards regression model was used to estimate the hazard ratios (HRs) and 95% *CI*s for NAFLD incidence in relation to the HGS. The linear trend across increasing the quartiles of HGS was tested using the median value of each quartile as a continuous variable based on the Cox proportional hazards regression analysis. The crude model was used to calculate the crude HR without any adjustment. Model 1 adjusted for age and BMI. Model 2 additionally adjusted for cigarette smoking status, alcohol consumption status, educational level, employment status, household income, physical activity, energy intake, type 2 diabetes, hypertension, hyperlipidemia, depressive symptoms, and intake of sweet foods pattern, vegetable pattern, and animal foods pattern. The receiver operating characteristics (ROCs) curves were performed to quantify the area under the curve (AUC) and an optimal cut-off value of HGS associated with the incidence of NAFLD.

To study the BMI–HGS and waist–HGS interactions, the analyses according to different subgroups of BMI and waist circumferences were performed. The subgroups of BMI and waist circumferences were defined according to the Working Group on Obesity in China (normal BMI, <24 kg/m^2; high BMI, 24–28 kg/m^2; and obesity, ≥28 kg/m^2 and normal waist, < 80 cm for women and <85 cm for men; and high waist, ≥ 80 cm for women and ≥ 85 cm for men) (34). The *P*-values for the interaction were also calculated by testing the multiplicative term of HGS and BMI or HGS and waist circumference. The sensitivity analyses were performed by defining the NAFLD using the HSI and FLI (21, 22). We then repeated the primary analyses by adjusting Model 2 in men and women. All the analyses were performed using the Statistical Analysis System (version 9.3 for Windows; SAS Institute, Inc., Cary, NC, USA). All the *P*-values were two-tailed, and the differences with *P*-values < 0.05 were considered statistically significant.

TABLE 1 | The characteristics of participants by sex at baseline[a].

Characteristics	All (n = 14,154)	Men (n = 5,931)	Women (n = 8,223)
No. of NAFLD* in follow-up	2,452	1,534	918
Sex (male %)	41.8	-	-
Age (years)	39.6 (11.5)[b]	40.9 (12.5)	38.7 (10.7)
BMI	22.9 (3.0)	23.9 (2.9)	22.1 (2.8)
Waist circumference (cm)	78.4 (9.1)	84.2 (7.7)	74.3 (7.7)
Depressive symptoms score[c]	36.8 (7.8)	36.4 (7.8)	37.1 (7.7)
Physical activity (METs × hours/week)	19.6 (32.7)	23.1 (35.5)	17.1 (30.4)
Energy intake (kcal/d)	1,984.8 (837.6)	2,057.4 (885.2)	1,932.7 (797.7)
Education (≥College graduate, %)	69.3	69.2	69.5
Household income (≥10,000 Yuan, %)[d]	31.7	31.0	32.2
Dietary pattern scores (Multiplied by 10)			
Sweet pattern	0 (10)	−0.3 (10.6)	0.2 (9.5)
Vegetable pattern	0 (10)	0.8 (10.7)	−0.6 (9.4)
Animal foods pattern	0 (10)	1.3 (10.9)	−0.9 (9.2)
Smoking status (%)			
Smoker	14.4	33.4	0.9
Ex-smoker	3.7	8.1	0.5
Non-smoker	81.9	58.5	98.6
Drinker (%)			
Everyday	3.1	6.6	0.7
Sometime	53.1	72.0	39.7
Ex-drinker	9.0	9.5	8.6
Non-drinker	34.8	11.9	51.0
Employment status (%)			
Managers	46.6	46.7	46.4
Professionals	15.7	19.7	12.9
Other	37.7	33.6	40.7
Hypertension (%)	13.3	20.8	8.0
Hyperlipidemia (%)	32.5	36.2	29.8
Diabetes (%)	1.7	2.7	1.0

**Non-alcoholic fatty liver disease (NAFLD) was diagnosed by ultrasonography and alcohol intake. [a]NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; MET, metabolic equivalent. [b]Mean (SD) (all such values). [c]Assessed using Zung Self-rating Depression Scale. [d]1 Yuan = 0.1555 dollar (2021-09-16 08:47).*

RESULTS

Participant Characteristics

The baseline characteristics of the participants according to sex are presented in **Table 1**. A total of 14,154 participants were enrolled. During the study period (mean follow-up, 3.20 years; range, 0.50–5 years), 2,452 participants (17.32%) developed NAFLD (incidence = 72.29 per 1,000 person-years). The proportion of men was 41.8%. The mean (SD) ages were 39.6 (11.5), 40.9 (12.5), and 38.7 (10.7) for all the participants, men, and women, respectively.

HGS and Incidence of NAFLD

As shown in **Table 2**, the baseline HGS [HGS/weight (kg/kg)] was negatively associated with the incidence of NAFLD in men ($P <$ 0.0001) and women ($P < 0.0001$) before and after adjusting for the confounding factors. The multivariate-adjusted HRs (*CI*s) for NAFLD incidence across the quartiles of HGS were 1 (reference), 0.90 (0.79, 1.02), 0.69 (0.60, 0.79), and 0.44 (0.37, 0.52) for men and 1 (reference), 0.82 (0.69, 0.96), 0.54 (0.45, 0.66), and 0.41 (0.33, 0.52) for women. The optimal cut-off values of HGS (HGS/weight [kg/kg]) were 0.61 and 0.43 for men and women, respectively. The AUC (95% *CI*) values of HGS were 0.65 (0.63, 0.66) and 0.67 (0.66, 0.69) for men and women, respectively.

BMI–HGS and Waist–HGS Interactions as Related to NAFLD Incidence

The interaction terms of BMI–HGS and waist–HGS were significant in both men and women after adjusting for the confounding factors (all $P < 0.0001$). The associations among the different subgroups according to BMI and waist circumference are presented in **Figures 2**, **3**, respectively. Compared with the participants in the lowest HGS quartiles, the lowest HRs (*CI*s) were observed among the participants who were in the normal BMI group (<24 kg/m^2) in both men (HR: 0.32, 95% *CI*: 0.24, 0.43) and women (HR: 0.31; 95% *CI*: 0.23, 0.43), respectively. Similarly, in the subgroup analyses according to waist circumference, the associations between HGS and NAFLD were stronger in the participants who were in the normal waist group than those who were in the high waist group. Compared with the participants in the lowest HGS quartiles, the HRs (*CI*s) of NAFLD in the highest quartiles were 0.33 (0.25, 0.44) and 0.43 (0.31, 0.59) in men and women who had normal waist circumferences, respectively.

Sensitivity Analysis

Figure 4 shows the association between the HGS and NAFLD incidence defined using the HSI and FLI in men and women. These associations were similar to those derived from the main analyses in which NAFLD was identified using liver ultrasonography and history of drinking. HGS was negatively associated with the incidence of NAFLD in both men and women (all P values < 0.0001). Compared with the participants in the lowest quartiles of HGS, the HRs (*CI*s) of NAFLD which defined using HSI in the highest quartiles were 0.53 (0.36, 0.78) and 0.33 (0.20, 0.53) in men and women, respectively. Compared with the participants in the lowest quartiles of HGS, the HRs (*CI*s) of NAFLD which defined using FLI in the highest quartiles were 0.40 (0.24, 0.67) and 0.29 (0.15, 0.58) in men and women, respectively.

DISCUSSION

Our large population-based cohort study in which we prospectively determined the association between HGS and NAFLD in the Chinese adults suggested that a higher HGS was associated with a lower risk of NAFLD. These associations were independent of the socio-demographic, behavioral,

TABLE 2 | Association between hand grip strength (HGS) (HGS/weight, kg/kg) and NAFLD* by sex[a].

	Categories of hand grip strength (n = 14,154)				P for trend[b]
Men	**Level 1**	**Level 2**	**Level 3**	**Level 4**	
HGS (kg/kg)	0.17, 0.53	0.53, 0.60	0.60, 0.66	0.66, 1.23	
No. of participants	1,483	1,483	1,482	1,483	
No. of participants with NAFLD	560	449	331	194	
Crude model	Reference	0.81 (0.71, 0.92)[c]	0.58 (0.51, 0.67)	0.34 (0.28, 0.39)	<0.0001
Adjusted model 1[d]	Reference	0.90 (0.79, 102)	0.69 (0.60, 0.79)	0.44 (0.37, 0.52)	<0.0001
Adjusted model 2[e]	Reference	0.90 (0.79, 1.02)	0.69 (0.60, 0.79)	0.44 (0.37, 0.52)	<0.0001
Women	Level 1	Level 2	Level 3	Level 4	
HGS (kg/kg)	0.18, 0.39	0.39, 0.44	0.44, 0.50	0.50, 1.63	
No. of participants	2,057	2,055	2,055	2,056	
No. of participants with NAFLD	414	253	153	98	
Crude model	Reference	0.60 (0.52, 0.70)	0.36 (0.30, 0.43)	0.23 (0.19, 0.29)	<0.0001
Adjusted model 1[d]	Reference	0.80 (0.68, 0.94)	0.53 (0.44, 0.65)	0.40 (0.31, 0.50)	<0.0001
Adjusted model 2[e]	Reference	0.82 (0.69, 0.96)	0.54 (0.45, 0.66)	0.41 (0.33, 0.52)	<0.0001

**NAFLD was diagnosed by ultrasonography and alcohol intake. [a]HGS, hand grip strength; NAFLD, non-alcoholic fatty liver disease; BMI, body mass index. [b]Multiple Cox regression analysis. [c]Hazard ratios (95% CI) (all such values). [d] Adjusted for age and BMI. [e]Adjusted for age, BMI, smoking status, drinking status, educational level, employment status, household income, physical activity, energy intake, type 2 diabetes, hypertension, hyperlipidemia, depressive symptoms, and intake of sweet foods pattern, vegetable pattern, and animal foods pattern.*

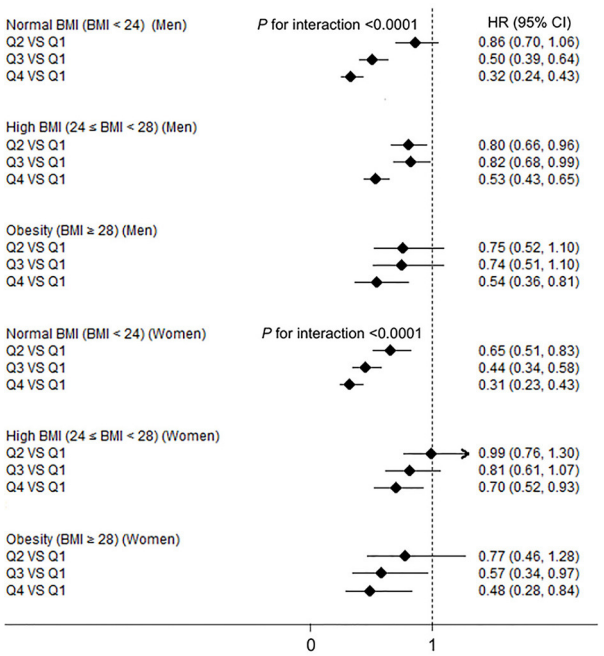

FIGURE 2 | The associations between hand grip strength (HGS) and NAFLD according to body mass index (BMI) in men and women. NAFLD, non-alcoholic fatty liver disease; HGS, hand grip strength; BMI, body mass index; HR, hazard ratio; Q, quartile. Adjusted for age, smoking status, drinking status, educational level, employment status, household income, physical activity, energy intake, type 2 diabetes, hypertension, hyperlipidemia, depressive symptoms, and intake of sweet foods pattern, vegetable pattern, and animal foods pattern.

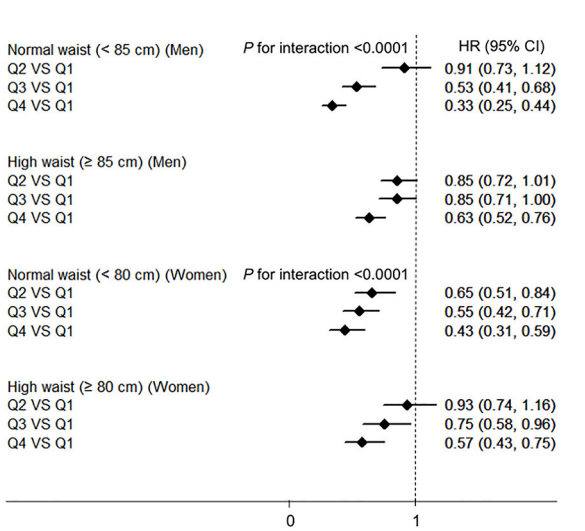

FIGURE 3 | The associations between HGS and NAFLD according to the waist circumference in men and women. NAFLD, non-alcoholic fatty liver disease; HGS, hand grip strength; BMI, body mass index; HR, hazard ratio; Q, quartile. Adjusted for age, BMI, smoking status, drinking status, educational level, employment status, household income, physical activity, energy intake, type 2 diabetes, hypertension, hyperlipidemia, depressive symptoms, and intake of sweet foods pattern, vegetable pattern, and animal foods pattern.

psychological, dietary, and health status factors. Furthermore, there were significant interaction effects between HGS and both BMI and waist size on the incidence of NAFLD. The results of our sensitivity analysis, which defined NAFLD using the HSI and FLI, were similar to those of the main analysis.

The muscles have been shown to play an important role in the development of NAFLD (35). The previous studies found that sarcopenia, which is a progressive and generalized skeletal muscle disorder that involves the accelerated loss of muscle mass and function (36), was shown to be associated with NAFLD

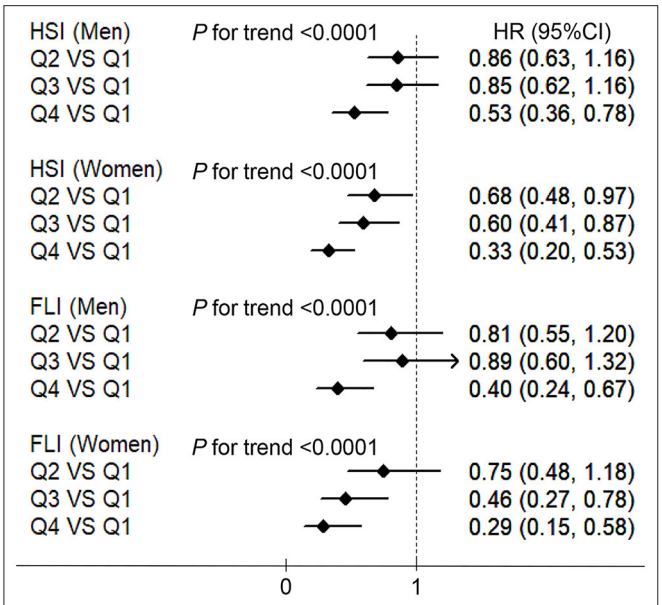

FIGURE 4 | The associations between HGS and NAFLD which defined using the HSI and fatty liver index (FLI) in men and women. HSI, hepatic steatosis index; FLI, fatty liver index; NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; HGS, hand grip strength; HR, hazard ratio; Q, quartile. Adjusted for age, BMI, smoking status, drinking status, educational level, employment status, household income, physical activity, energy intake, type 2 diabetes, hypertension, hyperlipidemia, depressive symptoms, and intake of sweet foods pattern, vegetable pattern, and animal foods pattern.

(37, 38). Compared with measuring muscle mass, measuring muscle strength (an indicator of muscle function) is easy to do in both the clinical and community settings (39). Thus, muscle strength could be a valuable predictor of NAFLD. In recent years, several cross-sectional studies have determined the association between HGS and the prevalence of NAFLD (13–17). For example, a cross-sectional study of 20,957 Chinese adults reported that increased HGS was independently associated with a lower prevalence of NAFLD (17). Compared with the participants who had the lowest HGS, the *OR* (95% *CI*) for the highest HGS was 0.67 (0.57, 0.79) (17). Another cross-sectional study also suggested that the low muscle strength was positively associated with NAFLD (*OR*, 1.47; 95% *CI*, 1.21, 1.80) in 5,132 Chinese adults (13). The Korea National Health and Nutrition Examination Survey also found that the lower BMI-adjusted HGS was associated with NAFLD in the Korean adults (n = 8,001) (16) as well as in elderly participants (n = 4,764) (14). Moreover, another study revealed a linear decrease in the NAFLD index that was commensurate with the incremental HGS level changes among the 538 elderly Korean participants (15). No cohort study has investigated the associations between HGS and NAFLD prospectively; considering the cross-sectional designs of previous studies, it was not possible to draw conclusions with respect to causality.

Consistent with previous cross-sectional studies, the present cohort study suggested that HGS was negatively associated with the incidence of NAFLD after adjusting for the confounding factors. The mechanisms that have been proposed to explain this association are chiefly related to insulin resistance and inflammation. First, the skeletal muscle is a major site of insulin-stimulated whole-body glucose disposal, and muscle metabolism can influence whole-body glucose homeostasis and insulin sensitivity (40). Thus, low muscle mass may lead to insulin resistance and explain the propagation of NAFLD (35). In addition, the muscle secretes irisin, which is a hormone that reduces obesity and insulin resistance (12), and is shown to be inversely associated with hepatic steatosis (41). Second, sarcopenia is associated with inflammatory indicators, such as the C-reactive protein level (38) and the neutrophil-to-lymphocyte ratio (42). Inflammation is also well-documented as a central component of NAFLD pathogenesis (43). Moreover, a previous study suggested that interleukin-6, which is a myokine secreted by muscle, was shown to have a protective effect on the development of NAFLD in an inflammation-prone animal model (44).

The results of the subgroup analyses suggested that the strongest associations between HGS and NAFLD were found in the participants with normal BMI and waist circumference. Two reasons were possible to explain it. First, BMI (45) and waist circumference (46) are positively associated with NAFLD. Thus, the associations between the HGS and NAFLD could be covered by BMI and waist circumference in the participants with high BMI and waist circumference. Second, the small sample sizes of high BMI and waist circumference subgroups could result in wide *CI*s of the HRs for NAFLD. We further performed the sensitivity analyses by defining NAFLD using the HSI and FLI. The associations revealed using this method were similar to those found when NAFLD was detected using liver ultrasonography and alcohol consumption history, indicating the robustness of the results.

The main strengths of our study were the large sample size and prospective cohort design. The former strength allowed for sufficient statistical power to detect the associations between HGS and NAFLD, while the latter strength helped ensure that the reverse causation would be minimized as much as possible. Moreover, the previous studies did not include dietary confounding factors in the adjustment models, even though the factors are strongly associated with muscle strength (47) and NAFLD (48). In the present study, we adjusted for sociodemographic, behavioral, psychological, dietary, and health status factors as much as possible to ascertain the independence from the association between HGS and NAFLD.

Some limitations are notable in our study. First, owing to its observational study design, the mechanism underlying the associations could not be determined. Second, even though we adjusted for the potential confounding factors, we could not rule out the possibility that other unmeasured factors might contribute to the associations observed. Third, we used hepatic ultrasonography scanning instead of the liver biopsies to detect fatty liver given that liver biopsy was not available

during the health examinations of the target population. Even though a previous study found that ultrasonography had a sensitivity of 89% and a specificity of 93% for NAFLD and was widely used in population-based studies because of its non-invasiveness and accessibility (49), ultrasound has limited sensitivity and does not reliably detect steatosis when the amount of fat was low or in individuals with an elevated BMI. Thus, the patients from the NAFLD group (future NAFLD patients) may have NAFLD at the baseline but are diagnosed without NAFLD when using liver ultrasound. Future studies which use more accurate methods, such as liver biopsy and controlled attenuation parameter, are needed to confirm the observed associations in the present study. Fourth, we excluded participants with choric hepatitis B or C, autoimmune liver disease, and those who have previous liver surgery. Nevertheless, the participants with other causes of NAFLD (such as a drug), celiac disease, or thyroid disease were not excluded. Therefore, the observed associations may be affected. Finally, the mechanisms that underline the associations between HGS and NAFLD may be explained by the metabolic factors (e.g., insulin resistance). Otherwise, various factors play important roles in the development of NAFLD. For example, lean NAFLD was developed without obesity (50), and there is also a substantial proportion of patients with normal BMI NAFLD without insulin resistance. Moreover, the susceptible polygenic host background also contributes to the development of NAFLD (51). Therefore, despite metabolic factors, further studies should also focus on the effect of aforenoted factors on the development of NAFLD.

CONCLUSION

Despite the aforementioned limitations, ours is the first cohort study to demonstrate that HGS is inversely associated with the incidence of NAFLD. The data suggested that a high HGS predicts a lower risk of NAFLD; hence, measuring HGS may serve as a possible strategy for detecting NAFLD at an early stage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved the protocol of this study was approved by the Institutional Review Board of the Tianjin Medical University; the subjects provided written informed consent to participate in the study. The study protocol conformed to the Ethical guidelines of the 1975 Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YX and KN contributed to the study conception and design. YX, LC, YL, XuW, SZ, GM, QZ, LL, HW, YG, YW, TZ, XiW, SS, MZ, QJ, and KS contributed to data collection, assembly, analysis, and interpretation of the data. YX, YZ, and KN contributed to the revising of the manuscript. YX, LC, and YZ contributed to the manuscript drafting and approval of the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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OPINION
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Targeting T Cell Subtypes for NAFLD and NAFLD-Related HCC Treatment: An Opinion

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Keywords: T cell subpopulation, NAFLD, HCC, treatment, cytokines, chemokines

INTRODUCTION

The increasing prevalence of non-alcoholic fatty liver disease (NAFLD), as well as its advanced stage non-alcoholic steatohepatitis (NASH) with the progression of liver inflammation and cell death with or without hepatic fibrosis, brings a heavy burden to public health (1). Non-alcoholic fatty liver disease is commonly associated with the incidence of obesity and diabetes (2, 3). In the United States, the prevalence of obesity raised from 30.5 to 42.4% from years 1999–2000 to years 2017–2018 as the Centers for Disease Control and Prevention (CDC) reported, and the prevalence of severe obesity also increased from 4.7 to 9.2% at this period. Recently, a new nomenclature of NAFLD, metabolic associated fatty liver disease (MAFLD), was recommended, which is thought to be more accurate to reflect the clinical pathogenesis of this disease with metabolic dysfunction (4, 5). There is no appropriate treatment for NAFLD up to date, except for early prevention via change of lifestyle (2, 6). Understanding the cellular and molecular pathogenesis of NAFLD and its relative advanced liver disease is helpful to define new potential targets for treatment.

Hepatic immunity plays a critical role in the pathogenesis of liver diseases (7, 8), including NAFLD, NASH, and end-stage of liver disease. Both hepatic innate and adaptive immune cells, as well as their interaction, orchestrate the progression of NAFLD and NASH (9). For example, the accumulation of activated hepatic B cells driven by gut microbiota impacted liver inflammation and fibrosis via modulating both intrahepatic innate and adaptive immunity during the progression of NASH (10). New functions of special types of T cells are reported to be associated with the progression of NAFLD and hepatocellular carcinoma (HCC) defined by the single-cell RNA sequencing (sRNA-seq) technology (11, 12). Here, we mainly focus on the latest investigation of the function of special types of T cells in NAFLD and NAFLD-related primary liver cancer.

FACTORS CAUSING NAFLD AND NAFLD-RELATED HCC PROGRESSION

Non-alcoholic fatty liver disease is an increasing factor that induces the development of HCC (13). The pathogenesis of NAFLD-related HCC progression remains to be clarified. The causing factors such as genetic factor (e.g., the genetic variant I148M of rs738409 in patatin-like phospholipase domain containing 3, PNPLA3) and epigenetic factors (e.g., histone deacetylase) for NAFLD and NASH may result in liver fibrosis and cirrhosis, and finally leading to the development of HCC (14–17). In addition, several other factors including environmental factors have been identified to be associated with NAFLD-related HCC progression (18), such as lipid metabolism (19),

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and dysregulation of gut microbiota (20). For example, dysregulation of lipid metabolism in NAFLD induced hepatic accumulation of linoleic acid and subsequent loss of $CD4^+$ T cells due to an increase of reactive oxygen species (ROS) (21), resulting in an increased incidence of HCC. Clinical trial studies also showed that anti-programmed death-1 (PD-1) or anti-programmed death-ligand 1 (PD-L1) treatment decreased the overall survival (OS) of human patients with NASH-induced HCC compared to non-NASH-induced HCC patients (22). Cellular mechanism study demonstrated that stimulation with gut microbial extracts from NAFLD-related HCC subjects can increase the frequency of regulatory T cells (Tregs) and decrease the frequency of $CD8^+$ T cells in human peripheral blood mononuclear cells (PBMCs), compared to treatment with bacterial extracts from non-NAFLD subjects (20), which indicates an important role of gut microbiota in modulating immunity in HCC microenvironment. In addition, peripheral PBMCs showed an immunosuppressive phenotype in human patients with NAFLD-related HCC compared to non-NAFLD and NAFLD-cirrhosis patients (20). Independent of these discussed causing factors for liver disease, T cells play an important role in the progression of NAFLD and NAFLD-related HCC. Thus, it is critically important to delicate the function of each subtype of T cells in NAFLD-HCC progression.

FUNCTION OF T CELLS IN NAFLD AND NAFLD-RELATED HCC

Function of $CD8^+$ T Cells in NAFLD

LIGHT (tumor necrosis factor superfamily member 14, TNFSF14) expression in activated $CD8^+$ T cells induced by feeding a choline-deficient high-fat diet (CD-HFD) promoted NASH and HCC progression in mice via interacting with lymphotoxin-β receptor (LTβR) in hepatocytes (23). $CD8^+$ T cells were also increased in the livers of obese human patients with NASH and cirrhosis, which was positively correlated with hepatic stellate cell (HSC) activation, evidenced by the increased expression of α-smooth muscle actin (α-SMA) (24). In contrast, depletion of $CD8^+$ T cells significantly reduced liver inflammation and HSC activation. A 3.5-fold increase of $CD8^+$ T cells with high expression of cytotoxic interleukin (IL)-10 can also be found in obese mice while feeding a western diet (WD) compared to the chow diet (24). High expression of IL-10 may promote the progression of HCC (25). Another study also showed that impairing $CD8^+$ T cell activation in mineralocorticoid receptor (MR)-deficient mice decreased liver steatosis in a methionine-choline deficient diet (MCD)-induced NASH model (26). Tumor development altered fatty acid partitioning in the fatty liver via inhibiting prolyl hydroxylase domain (PHD)3 expression, which results in function loss of cytotoxic $CD8^+$ T cells and impaired anti-tumor function (27). Therefore, enhancing or reversing the role of $CD8^+$ T cells in NAFLD may inhibit NAFLD-HCC progression. Here, we summarize some specific subpopulations of $CD8^+$ T cells in NAFLD-related HCC.

Function of $CD8^+$ T Cells in NAFLD-Related HCC

$PD1^+CD8^+$ T Cells

Preclinical study showed that immunotherapy with anti-PD1 treatment increased the prevalence of exhausted $PD1^+CD8^+$ T cells with high mRNA expression of C-X-C motif chemokine receptor 6 (CXCR6) and tumor necrosis factor-alpha (TNF-α) in the liver of NASH mice, which was associated with impaired immune surveillance and increased incidence of NASH to HCC progression (22). Similar phenotypic and functional $PD1^+CD8^+$ T cells were found in livers from humans with NAFLD/NASH in this report. In addition, both anti-CD8 or anti-TNF plus anti-PD1 antibody treatments can ameliorate liver damage and inflammation and reduce HCC incidence compared to anti-PD1 treatment alone.

$CXCR6^+CD8^+$ T Cells

Liver-resident $CXCR6^+CD8^+$ T cells were increased in NASH mice fed a CD-HFD, and those $CD8^+$ T cells expressed low activity of the Forkhead box protein O1 (FOXO1) transcription factor caused by high expression of IL-15 (28). In addition, the level of hepatic acetate was increased in NASH mice, which can cause auto-aggressive liver $CXCR6^+$ $CD8^+$ T cells to damage hepatocytes, resulting in liver injury. Furthermore, $CXCR6^+CD8^+$ T cells were also shown to increase in human NAFLD/NASH livers, as well as hepatic expression of CXCR6 (28).

$Prf1^{null}CD8^+$ T Cells

Perforin (Prf)-deficient mice on an MCD showed an increased accumulation and activation of $CD8^+$ T cells expressing proinflammatory cytokines (e.g., interferon-gamma, or IFN-γ) compared to wild-type (WT) mice, but not $CD4^+$ T cells (29). The increased IFN-γ levels are closely associated with liver dysfunction in human patients, including liver fibrosis, cirrhosis, and HCC (30). In contrast, an increase of cell proliferation antigen $Ki67^+CD8^+$ T cells producing IFN-γ in response to sorafenib treatment was associated with improved OS and progression-free survival (31).

Function of $CD4^+$ T Cells in NAFLD

Dysregulation of hepatic lipid metabolism in human NAFLD patients and mouse models induced a reduction of liver $CD4^+$ T cells (21, 32). Fatty liver impairs the immunotherapeutic effects (33), such as RNA vaccine (e.g., M30-RNA vaccine) and antibody-mediated therapy [e.g., anti-OX40 (CD134) antibody]. Feeding a high-fat and high-calorie diet caused the proliferation of human $CD4^+$ central and effector memory T cells in immunodeficient mice engrafted with human immune cells (HIL mice) compared to that in mice fed with a chow diet, which was associated with a significant increase of pro-inflammatory cytokines, such as IL-17A and IFN-γ (34). In addition, *in vivo* depletion of human $CD4^+$ T cells in those mice can attenuate hepatic inflammation and fibrosis. In summary, these results show that $CD4^+$ T cells play diverse roles in the development of NAFLD, liver fibrosis, and HCC. Thus, clarifying the function of each type of $CD4^+$ T cell is necessary.

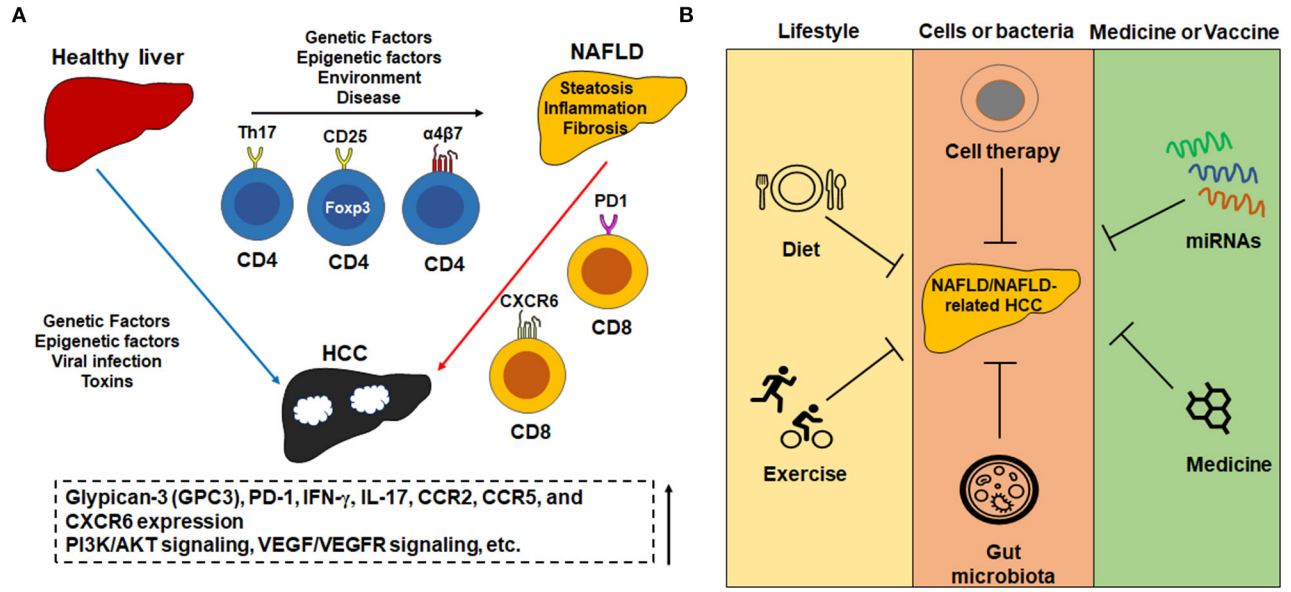

FIGURE 1 | The important role of T cells in the pathogenesis of NAFLD and NAFLD-related HCC and potential treatment options. **(A)** Some important subtypes of T cells and molecules in the pathogenesis of NAFLD and NAFLD-related HCC. For example, $Th17^+CD4^+$, $CD25^+Foxp3^+CD4^+$, and α4β7 integrin-positive $CD4^+$ T cells increase in the progression of NAFLD, while $PD1^+CD8^+$ and $CXCR6^+CD8^+$ increase in NAFLD-HCC progression. The change of T cell population is associated with an increase of cytokines such as IL-17a and IFN-γ which accompany the progression of NAFLD. **(B)** The potential treatment options for NAFLD and NAFLD-related HCC include change of lifestyle, cells or bacteria-mediated therapy such as adoptive transfer of T cells, medicines or vaccines such as microRNA-mediated therapy.

α4β7$^+$ CD4$^+$ T Cells

Recruitment of integrin α4β7$^+$ CD4$^+$ T cells to the liver was associated with NASH progression in $F11r^{-/-}$ mice fed with WD, which was correlated with higher expression of its ligand mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (35). Blocking integrin α4β7 prevented migration of CD4$^+$ T cells, resulting in a significant decrease in liver inflammation and fibrosis. In addition, ablating β7 integrin or MAdCAM-1, as well as β7 integrin deficiency, can reduce concanavalin A (ConA)-induced hepatitis in mice, indicating the role of β7 integrin in liver injury (36).

Th17$^+$ Cells

In the progression of NAFLD to NASH, hepatic IL-17$^+$CD4$^+$ T (Th17) cells were significantly increased, and the ratio of Th17 or Th2 to $CD4^+CD45RA^+CD25^{++}$ resting Tregs (rTregs) was elevated in peripheral blood (37). Imbalance of hepatic Th17/Treg cells was also shown in NAFLD mice fed a HFD (38). The increased frequency of IL-17$^+$ cells in total CD4$^+$ T cells in NASH patients was positively correlated with a higher level of serum concentration of blood endotoxin (LPS) compared to either healthy subjects or non-alcohol fatty liver (NAFL) patients (39).

Treg Cells

The interaction of $Foxp3^+CD25^+CD4^+$ T cells (Tregs) with other immune cells and hepatocytes plays a critical role in liver homeostasis and pathogenesis. Hepatocytes can engulf CD4$^+$ T cells, preferable for Tregs, during liver inflammation to control T cell population, known as enclysis (40). The frequency of $CD25^+CD45^+CD4^+$ T cells was increased in PBMCs of human NAFLD patients with advanced liver fibrosis, while the $PD1^+CD4^+$ T cells were decreased (41), which were significantly and negatively correlated with the ratio of serum fatty acid composition (44, 45).

Moreover, there are other subtypes of T cells that were found to be associated with the progression of NAFLD, such as Vδ2 T cells (42) and γδ T cells (43).

Function of CD4$^+$ T Cells in NAFLD-Related HCC

Treg Cells

Transcription factor Foxp3 can suppress glycolysis and induce oxidative phosphorylation to change metabolic profiles of Tregs to survive in low-glucose and high lactate environments (44). The proliferation of Tregs can suppress the function of cytotoxic CD8$^+$ T cells against liver tumor cells, resulting in the progression of HCC both in mouse models and in human patients (45). A high ratio of effector CD4$^+$ T cells/Treg showed a good prognostic for human HCC (31).

Th17 Cells

Th17 cells and the expression of IL-17a were positively associated with human fatty liver-associated HCC (46). *In vitro* study showed that macrophages are required to mediate IL-17 expression in naive CD4$^+$ T cells through LPS/Toll-like receptor 4 (TLR4) signaling. Furthermore, intra-tumoral infiltration of Th17 cells promoted tumor growth via promoting

angiogenesis and predicted a poor OS in HCC patients (47). In addition to inducing angiogenic factors (e.g., vascular endothelial growth factor/VEGF and prostaglandin E2/PGE2), Th17 cells can activate oncogenic IL-6/Stat3 signaling to enhance tumor growth (48).

Function of Double-Negative T Cells in NAFLD-Related HCC

Double-negative T cells (DNT) defined by T-cell receptor (TCR)$\alpha\beta^+CD3^+CD4^-CD8^-$ T cells and consisting of 1–3% of peripheral T lymphocytes in mice and humans have been shown to play multiple roles in immune responses (49). Adoptive transfer of $CD4^+$ T cells converted DNT was shown to reduce liver inflammation and fat accumulation inducing factors for NASH, by suppressing the infiltration of Th17 cells and M1 macrophages (8). Double-negative T cells can also inhibit the function of effector $CD4^+$ T cells by impairing glucose metabolism and inhibiting mTOR signaling and the expression of inflammatory cytokines IL-17a and IFN-γ (50). Furthermore, DNT was shown to be higher in non-tumor-infiltrating lymphocytes compared to tumor-infiltrating lymphocytes in human HCC (51).

POTENTIAL TREATMENT OPTIONS FOR NAFLD-RELATED HCC BY TARGETING ON T CELLS

Currently, there are some approved first- and second-line treatment options for HCC, which may be also applied in NAFLD-related HCC treatment. In 2008, sorafenib, a multi-kinase inhibitor against VEGF receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and RAF kinases (serine/threonine protein kinases), is the first approved systemic therapy by the U.S. FDA for patients with unresectable HCC (52). In 2018, lenvatinib, a multiple kinase inhibitor against the VEGFR1, VEGFR2, and VEGFR3 kinases, was approved by FDA for systemic treatment for unresectable advanced HCC (53). In 2020, PD-L1 inhibitor atezolizumab was approved by FDA in combination with bevacizumab (anti-VEGF monoclonal antibody) for adult patients with unresectable locally advanced or metastatic HCC without prior systemic therapy (54). In addition, there are some combined treatments such as nivolumab (anti-PD-1 monoclonal antibody) and ipilimumab (anti-cytotoxic T-lymphocyte-associated protein 4/CTLA4 monoclonal antibody) that may approve the outcomes (55). Here, we also review some treatment options by targeting T cells (**Figure 1**).

T Cell-Mediated Treatment

A clinical trial shows that treatment with sorafenib, a protein kinase inhibitor, can increase $Ki67^+CD8^+$ T cells producing IFN-γ to improve progression-free survival and OS of human HCC patients (31). The VEGF/VEGFR signaling was involved in this effect, evidenced by improved sorafenib in combination with VEGFR antagonism (31).

A decrease of Tregs in visceral adipose tissue (VAT) is positively associated with NASH progression (56). Adoptively transfer (ACT) of Tregs from spleens of healthy mice to mice with diet-induced hepatic steatosis promoted liver steatosis with an increase of Tregs in VAT and a decrease of Th1 cells in various tissues (57). Adoptively transfer of Tregs did not impact other metabolic and histologic changes. Recently, a phase I clinical trial showed the initial safety profile and effect of chimeric antigen receptor (CAR)-glypican-3 (GPC3) T-cell therapy for patients with advanced HCC (58). Those CAR T cells include a humanized anti-GPC3 single-chain variable fragment, CD8α hinge domain, CD8α transmembrane domain, CD28 intracellular domain, and CD3ζ intracellular signaling domain. There are some recruiting clinical trials for investigating GPC3-targeted CAR-T Cell for treating HCC, such as trials NCT03198546 and NCT04121273.

Gut Microbiota-Mediated Therapy

Gut microbiota has been shown to play vital roles in human liver diseases (59), through modulating secondary bile acids (BAs), activating TLRs, and influencing the function of immune checkpoint inhibitors (ICIs). For example, gut microbial extracts from NAFLD-HCC patients dramatically suppressed $CD8^+$ T cells and B cells in PBMCs from non-NAFLD healthy people compared to bacterial extract from non-NAFLD controls, but significantly increased the proliferation of $CD3^+CD4^+CD25^+Foxp3^+$ Tregs, inducing an immunosuppressive phenomenon (20). Fecal microbiota transplantation (FMT) from proper donors can restore gut microbiota disorder and ameliorate D-galactosamine-induced liver injury in BALB/c mice, via downregulating the expression of IL-17a, TNF-α, and transforming growth factor-β (TGF-β) and upregulating the expression of IL-10 and IL-22 (60).

miRNA-Mediated Treatment

Overexpression of microRNA-195 (miR-195) can improve the balance of Th17/Treg via regulating CD40 expression in rat liver tissues, accompanying decrease of serum level of proinflammatory cytokines (e.g., TNF-α), total cholesterol (TC) and triglyceride (TG), liver injury markers aspartate transaminase (AST), and alanine aminotransferase (ALT) (61). In addition, hepatocyte-specific overexpression of miR-34a promoted high cholesterol and fructose (HFCF) fat diet-induced NAFLD in mice, while pharmaceutical suppression of miR-34a can reverse NAFLD progression (62). miR-26a can inhibit hepatic expression of IL-17 and IL-6, as lentiviral vector delivered miR-26a treatment significantly decreased total liver weight, liver deposition of TG, and serum ALT concentration compared lentiviral control-treated mice, accompanying decreased infiltration of γδ T cells, and granulocyte-differentiation antigen-1 $(Gr\text{-}1)^+$ cells and $CD11b^+$ cells (63). In addition, Escutia-Gutiérrez et al. reported that miRNAs such as miR-21a-5p, miR-34a-5p, miR-122-5p, and miR-103-3p were increased expression of in livers of MAFLD/NASH (64), the potential targets for HCC treatment.

Chemokine or Cytokine-Mediated Treatment

Treatment with C-C chemokine receptor (CCR)2 antagonist inhibited tumor-infiltrating macrophage (TAMs)-mediated immunosuppression and increased $CD8^+$ T cells in liver cancer (65). In addition, this antagonist improved the therapeutic effect of sorafenib via enhancing tumor necrosis and apoptosis. CCR5/CCL5 signaling pathway plays a critical in the development of HCC in chronic liver disease both in mice and humans (66–68), as a potential treatment target for HCC. Moreover, injection of WSX1 (IL-27 receptor α) can significantly suppress the HCC growth by suppressing PD-L1 expression on tumor cells via blocking phosphoinositide 3-kinase delta (PI3Kδ)/protein kinase B (AKT)/glycogen synthase kinase-3β (GSK3β) pathway to release the cytotoxic effect of $CD8^+$ T cells (69). Combined therapy with regorafenib and anti-PD-1 increased the filtration and activation of $CXCR3^+CD8^+$ T cells via increasing CXCL10 expression in tumors, resulting in inhibition of HCC growth (70). Therefore, modulating chemokines, chemokine receptors, and cytokines can improve anti-tumor immunity to inhibit tumor progression.

DISCUSSION

Obesity and NAFLD are closely linked with each other. NAFLD patients with medium-high risk obesity with body mass index (BMI) >35 kg/m² showed poor response to hepatitis B virus (HBV) vaccine (71). In addition, hepatitis B surface antigen-specific $CD4^+$ T cells showed significantly less proliferation in PBMCs of high-risk obesity NAFLD patients compared to that in low-risk obesity NAFLD subjects. Fatty liver disease also is a serious issue for obese children. Lipid metabolism is one of the major contributing factors for NAFLD (72). Fat metabolism modulates T cell profiles in the liver of NAFLD subjects to impact NAFLD-HCC progression. New technologies (e.g., siRNA-seq) improve our understanding of the pathogenesis of NAFLD. Each subtype of T cells is shown to play different roles in NAFLD progression, such as $TCR\alpha\beta^+CD3^+CD4^-CD8^-$ cells and $CXCR6^+CD8^+$ or $PD1^+CXCR6^+CD8^+$ T cells. Targeting those T cells by orchestrating gut microbiota, treatment of miRNAs, adoptive transfer of T cells, and modulating the expression of small molecules are potential treatment options against NAFLD and NAFLD-HCC progression. In addition, energy restriction is a method to reduce BMI and ameliorate fatty liver disease, which may bring new health concerns. Supplement of lycopene-rich tomato juice to obese children can improve calorie-restricted regimen-induced impairment of glycolysis and mitochondrial metabolism in T cells to enhance their immune surveillance function (73).

T cell populations vary during the development of NAFLD-related HCC, including the changes in subtype and function. For example, Tregs in the early stage of NAFLD/NASH can suppress liver inflammatory function, but in the HCC stage, they can inhibit effector T cell function to suppress tumor progression. Therefore, manipulation of T cell function or population is dependent on the stage of liver disease and microenvironment. In addition, proteomic analysis of NAFLD-HCC infiltrating T cells is awaited to explore the functional proteins to modify those T cell functions except PD-1 and CXCR6. Overall, T cells play a critical role in metabolic fatty liver diseases to HCC progression, and targeting them may provide a novel treatment.

AUTHOR CONTRIBUTIONS

CZ and MY conceived the opinion and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Association of Cholecystectomy With Liver Fibrosis and Cirrhosis Among Adults in the USA: A Population-Based Propensity Score-Matched Study

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Background and Aims: Cholecystectomy is the "gold standard" for treating diseases of the gallbladder. In addition, non-alcoholic fatty liver disease (NAFLD), liver fibrosis or cirrhosis, are major causes of morbidity and mortality across the world. However, the association between cholecystectomy and these diseases is still unclear. We assessed the association among US adults and examined the possible risk factors.

Methods: This cross-sectional study used data from 2017 to 2018 National Health and Nutrition Examination Survey, a population-based nationally representative sample of US. Liver fibrosis and cirrhosis were defined by median stiffness, which was assessed by transient elastography. Furthermore, patients who had undergone cholecystectomy were identified based on the questionnaire. In addition, Propensity Score Matching (PSM, 1:1) was performed based on gender, age, body mass index (BMI) and diabetes.

Results: Of the 4,497 included participants, cholecystectomy was associated with 60.0% higher risk of liver fibrosis (OR:1.600;95% CI:1.278–2.002), and 73.3% higher risk of liver cirrhosis (OR:1.733, 95% CI:1.076–2.792). After PSM based on age, gender, BMI group and history of diabetes, cholecystectomy was associated with 139.3% higher risk of liver fibrosis (OR: 2.393;95% CI: 1.738–3.297), and 228.7% higher risk of liver cirrhosis (OR: 3.287, 95% CI: 1.496–7.218).

Conclusions: The present study showed that cholecystectomy is positively associated with liver fibrosis and cirrhosis in US adults. The discovery of these risk factors therefore provides new insights on the prevention of NAFLD, liver fibrosis, and cirrhosis.

Keywords: cholecystectomy, non-alcoholic fatty liver disease, liver fibrosis, liver cirrhosis, association

INTRODUCTION

Chronic liver diseases, such as non-alcoholic fatty liver disease (NAFLD), liver fibrosis (LF) or cirrhosis (LC), are major causes of morbidity and mortality across the world (1–3). With the rising prevalence of NAFLD, interest is increasing in LF, which is a reversible condition and can progress to irreversible LC and even hepatocellular carcinoma (HCC), thereby leading to a major social and economic burden (4, 5). Previous researches have reported that LF is correlated with long-term outcomes of NAFLD patients (6). However, liver fibrosis continues to threaten public health despite decades of research, and thus scientists are now focused on prevention strategies. Classically, LF is caused by various risk factors, such as viral hepatitis, alcoholism, obesity, and type 2 diabetes (4). Better understanding of the associated risk factors may contribute to the early prevention of the underlying liver disease.

Gallbladder diseases are also among the most prevalent conditions worldwide, affecting 10 to 20% of the adult population (7). Cholecystectomy is widely used as the "gold standard" for the treatment of gallbladder diseases, such as gallstones, acute cholecystitis and benign tumors of the gallbladder (8). However, few studies have evaluated whether cholecystectomy is associated with an increased risk of developing NAFLD. A previous retrospective, multicenter study in Turkey showed that there is no independent association between the presence of cholecystectomy and advanced LF (9). The study focused on whether the presence of gallstones in patients with biopsy-proven NAFLD was associated with advanced LF and histological non-alcoholic steatohepatitis (NASH), providing preliminary reference for research on hepatology and cholecystectomy. Unfortunately, the main shortcoming of the study was the small sample size which included 41 cases of cholecystectomy and 387 without. On the contrary, another cross-section study using data of the third US National Health and Nutrition Examination Survey (NHANES III, 1988–1994), showed the positive association between NAFLD and cholecystectomy (10). Moreover, no further study on the association between cholecystectomy and LF or LC has been conducted ever since. Recently, ultrasound transient elastography (TE) was widely used to evaluate liver fibrosis in chronic liver diseases in a non-invasive and reproducible manner (11). Notably, transient elastography was first conducted in the NHANES 2017–2018 cycle, providing opportunity to assess the weak connection between cholecystectomy and LF or LC.

Consequently, the present study sought to examine the association between cholecystectomy and LF or LC using a nationally representative sample of US adults from the NHANES 2017–2018.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline Phosphatase; ALB, albumin; BMI, body mass index; CAP, controlled attenuation parameter; CI, confidence interval; GGT, gamma glutamyl transferase; HBV, hepatitis B virus; HCV, hepatitis C virus; IQR, interquartile range; LF, liver fibrosis; LC, liver cirrhosis; LSM, median liver stiffness; NAFLD, non-alcoholic fatty liver disease; NHANES, National Health and Nutrition Examination Survey; OR, odds ratio; PSM, propensity score matching; TC, total cholesterol; TB, total bilirubin.

MATERIALS AND METHODS

Study Population

This study analyzed data from NHANES 2017–2018, where ultrasound TE of the liver was first conducted. The NHANES was a national, cross-sectional survey that assessed the health and nutritional status of individuals in the United States. A detailed description of NHANES has been published elsewhere (12). During the 2017–2018 cycle of NHANES, 9,254 participants finished the survey. However, the present study excluded individuals who were <20 years old and could not undergo TE ($N = 4{,}744$). Patients with autoimmune hepatitis and those lacking data of cholecystectomy were also excluded from further analysis ($N = 13$). Consequently, 4,497 participants were enrolled for further analysis. Moreover, written informed consent was obtained from all the participants and the survey protocol was approved by the Research Ethics Review Board of the National Center for Health Statistics. Additionally, specific informed consent was not required for this secondary analysis of the publicly available data. This report was also drafted according to the reporting guidelines for cross-sectional studies, stipulated by Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) (13).

Primary Exposure

Patients who had undergone cholecystectomy were identified based on self-reports and this information was acquired from the "medical conditions" section of the questionnaire. In addition, 8,897 participants who were older than 20 years answered the question; "Ever had cholecystectomy?" Notably, 641 (7.2%) individuals answered "Yes" while 4,925 (55.4%) participants answered "No".

Outcomes

During NHANES 2017–2018, TE was first conducted by educated health technicians. Additionally, liver stiffness was measured using the FibroScan® model 502 V2 Touch, which used ultrasound and vibration-controlled TE. Notably, TE is a widely used, noninvasive and reliable method of evaluating LF or LC (14, 15). All participants older than 12 years of age were eligible except for individuals who could not lie on the exam table, had an implanted electronic medical device, were pregnant or had a lesion at the site of examination. In addition, only individuals with complete tests (a fasting time of 3 hours, complete stiffness ≥10 measures and interquartile range of liver stiffness/median stiffness <30%) were enrolled in this study. Moreover, LF was defined as F0-F4, with the cutoff values of median liver stiffness (LSM) being 6.3, 8.3, 10.5 and 12.5 (KPa), respectively (16). Furthermore, Significant LF and LC was defined as LSM ≥ 6.3 KPa (fibrosis grade ≥ F1) and LSM ≥ 12.5 KPa (fibrosis grade ≥ F4), respectively (16, 17).

Covariates

Covariates were selected based on known confounders from previous literature and clinical practice. Briefly, demographic factors such as age, sex and race/ethnicity were included first. In addition, levels of education, alcohol use, diabetes, HBV infection, HCV infection, physical activity status, serum cotinine

levels, Body Mass Index (BMI), and the poverty income ratio were also evaluated through interviews.

In this study, age was classified into six categories: 20–29, 30–39, 40–49, 50–59, 60–69, and 70–80 years. In NHANES 2017–2018, race/ethnicity was classified as Hispanic (referring to all Hispanics), non-Hispanic White (referring to whites with no Hispanic origin), non-Hispanic Black (meaning blacks with no Hispanic origin), non-Hispanic Asian (meaning Asians with no Hispanic origin) or other races including Alaska Natives or American Indians, Native Hawaiians or other Pacific Islanders and multiracial individuals. In addition, the BMI was categorized into three groups: under/normal weight (<25.0 kg/m^2), overweight (25.0–29.9 kg/m^2 and obesity (≥30.0 kg/m^2). Participants with diabetes were also defined as those with a self-reported history of diagnosis with diabetes or glycohemoglobin ≥ 6.5% (18). Moreover, individuals with HCV or HBV infections were identified based on positive diagnostic tests (19, 20) or self-reported infection.

Furthermore, current alcohol use was categorized as none, moderate (>0 to ≤2 drinks/d for men or >0 to ≤1 drink/d for women), heavy (>2 to <5 drinks/d for men or >1 to <4 drink/d for women) or binge (≥5 drinks/d for men or ≥4 drink/d for women) based on recommendations from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) in the National Institute of Health. On the other hand, smoking was categorized according to the serum cotinine levels into low (<0.015 ng/ml), moderate (0.015–3 ng/ml) and high levels (>3 ng/ml) (21). Moreover, the participants were categorized into three groups: active (≥the recommended level of activity), less active (<the recommended level of activity) and inactive (no activity), based on evidence that more than 75 min of vigorous or 150 min of moderate physical activity per week is recommended for Americans (22). In addition, the level of income was measured using the poverty income ratio (ratio of family income to poverty threshold) and was classified into three categories: <1.3, 1.3–1.8, and >1.8. The level of education (more than high school education, high school education, less than high school education) and laboratory-measured levels of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Albumin (ALB), Alkaline Phosphatase (ALP), γ-glutamyl Transpeptidase (GGT), Total Cholesterol (TC), Total Bilirubin (TB) and platelet were also evaluated through interviews.

Statistical Analysis

Continuous variables were expressed as the weighted mean (standard deviation) and comparisons between two groups were made using the independent samples *T*-test or Mann-Whitney test. In addition, categorical variables were described by weighted percentages (95% confidence interval, 95% CI) and compared using the χ^2 test. Multivariate logistic regression analysis was also performed to evaluate the correlation between LF, LC and cholecystectomy. The final model was adjusted for age, gender, race, level of education, alcohol use, diabetes, HBV infection, HCV infection, physical activity status, serum cotinine levels, BMI, and the poverty income ratio.

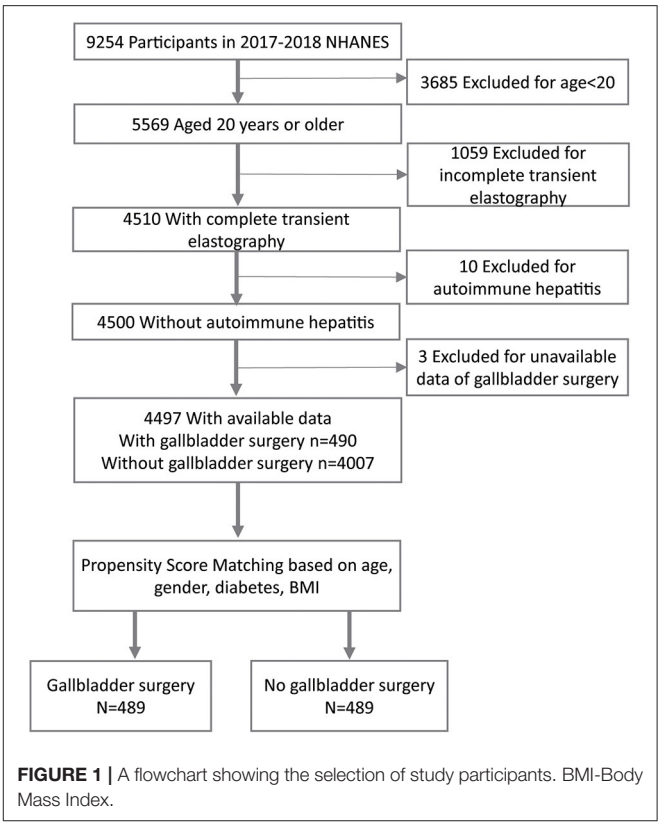

FIGURE 1 | A flowchart showing the selection of study participants. BMI-Body Mass Index.

Additionally, subgroup analyses were conducted by examining age, gender, race/ethnicity. Propensity Score Matching (PSM) was also applied to match two groups, with a ratio of 1:1 and a clipper of 0.00 using SPSS version 25.0 (IBM, New York, USA).

All the statistical analysis were performed using the R software (http://www.R-project.org, The R Foundation) and Empowerstats (http://www.empowerstats.com, X&Y Solutions, Inc), with appropriate interview/examination weights to represent the complex survey design. Moreover, 2-sided tests were used to obtain all the p values and statistical significance was set at $p < 0.05$.

RESULTS

Overall Characteristics of the Participants

A total of 4,497 participants who were older than 20 years in NHANES 2017–2018, were included in this analysis. Herein, 490 individuals had undergone cholecystectomy while 4,007 participants had not (**Figure 1**). The overall characteristics of the included participants are shown in **Table 1**. **Table 1** showed that individuals who had undergone cholecystectomy were mostly older (57.21 ± 14.62 years vs. 46.68 ± 16.95 years, $p < 0.001$), female [77.3% (95% CI, 73.6–81.0%) vs. 47.1% (95% CI, 45.6–48.6%), $p < 0.001$], non-Hispanic Whites [73.2% (95% CI, 69.3–77.1%) vs. 61.1% (95% CI, 59.6–62.6%), $p < 0.001$], obese [57.8% (95% CI, 53.4–62.2%) vs. 38.8% (95% CI, 37.3–40.3%), $p <$

TABLE 1 | General characteristics of included participants ($n = 4497$) by the presence or absence of a history of cholecystectomy in the NHANES 2017–2018.

Characters	Yes ($n = 490$)	No ($n = 4007$)	*p*-Value
Age (years)	57.21 ± 14.62	46.68 ± 16.95	<0.001
20–29	4.3 (2.5–6.1)	20.6 (19.3–21.9)	
30–39	10.6 (7.9–13.3)	19.1 (17.9–20.3)	
40–49	17.0 (13.7–20.3)	15.6 (14.5–16.7)	
50–59	18.9 (15.4–22.4)	19.2 (18.0–20.4)	
60–69	27.5 (23.6–31.5)	14.6 (13.5–15.7)	
70–80	21.8 (18.1–25.5)	10.9 (9.9–11.9)	
Gender			<0.001
Male	22.7 (19.0–26.4)	52.9 (51.4–54.4)	
Female	77.3 (73.6–81.0)	47.1 (45.6–48.6)	
Race/ethnicity			<0.001
Hispanic	11.7 (8.9–14.6)	16.3 (15.2–17.4)	
Non-Hispanic White	73.2 (69.3–77.1)	61.1 (59.6–62.6)	
Non-Hispanic Black	6.5 (4.3–8.7)	11.8 (10.8–12.8)	
Non-Hispanic Asian	2.2 (0.9–3.5)	6.3 (5.5–7.1)	
Other races[a]	6.4 (4.2–8.6)	4.5 (3.9–5.1)	
Education			0.184
More than high school	58.3 (53.9–62.7)	62.6 (61.1–64.1)	
High school or equivalent	30.4 (26.3–34.5)	26.3 (24.9–27.7)	
Less than high school	11.3 (8.5–14.1)	11.0 (10.0–12.0)	
Not recorded	0.0	0.1 (0.0–0.2)	
Poverty-income ratio			0.928
<1.3	17.1 (13.8–20.4)	17.6 (16.4–18.8)	
1.3–1.8	8.4 (5.9–10.9)	8.2 (7.4–9.0)	
>1.8	65.0 (60.8–69.2)	63.9 (62.4–65.4)	
Not recorded	9.5 (6.9–12.1)	10.3 (9.4–11.2)	
BMI group			<0.001
<25	13.8 (10.8–16.9)	28.9 (27.5–30.3)	
25–30	27.9 (23.9–31.9)	31.8 (30.4–33.2)	
≥30	57.8 (53.4–62.2)	38.8 (37.3–40.3)	
Not recorded	0.6 (−0.1, 1.3)	0.6 (0.4–0.8)	
Physical activity level			<0.001
Inactive	51.2 (46.8–55.6)	50.8 (49.3–52.3)	
Less active	12.5 (9.6–15.4)	7.1 (6.3–7.9)	
Active	36.4 (32.1–40.7)	42.1 (40.6–43.6)	
Daily alcohol drinking status			<0.001
Non-drinkers	7.4 (5.1–9.7)	7.1 (6.3–7.9)	
Moderate-drinkers	30.5 (26.4–34.6)	29.6 (28.2–31.0)	
Heavy-drinkers	17.8 (14.4–21.2)	13.6 (12.5–14.7)	
Binge-drinkers	22.0 (18.3–25.7)	34.4 (32.9–35.9)	
Not recorded	22.3 (18.6–26.0)	15.3 (14.2–16.4)	
History of diabetes			<0.001
Yes	25.0 (21.2–28.8)	11.3 (10.3–12.3)	
Having HBV infection			0.009
Yes	1.0 (0.1–1.9)	0.9 (0.6–1.2)	
Having HCV infection			0.006
Yes	2.9 (1.4–4.4)	2.4 (1.9–2.9)	
Laboratory parameters			
Smoking (serum cotinine levels)			<0.001
<0.015	46.5 (42.1–50.9)	35.7 (34.2–37.2)	
0.015–3	29.5 (25.5–33.5)	36.7 (35.2–38.2)	
≥3	22.6 (18.9–26.3)	23.5 (22.2–24.8)	
Not recorded	1.4 (0.4–2.4)	4.1 (3.5–4.7)	
ALT (U/L)	20.98 ± 14.97	23.65 ± 17.13	<0.001
AST (U/L)	20.15 ± 10.31	22.58 ± 13.29	<0.001
ALP (U/L)	82.89 ± 33.52	75.44 ± 22.99	<0.001
ALB (g/L)	39.68 ± 3.23	41.13 ± 3.06	<0.001
GGT (U/L)	30.79 ± 62.64	30.10 ± 35.28	0.706
TC (mmol/L)	4.83 ± 0.94	4.91 ± 1.03	0.098
TB (umol/L)	7.99 ± 4.87	8.09 ± 4.60	0.632
Platelet ($\times 10^9$/L)	255.77 ± 71.75	243.21 ± 58.68	<0.001
Transient Elastography			
Median stiffness (kPa)	6.71 ± 6.45	5.52 ± 4.27	<0.001
Controlled attenuated parameter (dB/m)	279.84 ± 59.85	261.35 ± 62.53	<0.001
Liver fibrosis status			<0.001
Yes	34.7(30.5–38.9)	19.8(18.6–21.0)	
Liver cirrhosis			0.041
Yes	4.5 (2.7–6.3)	2.8 (2.3–3.3)	

Values are weighted mean ± SD or weighted % (95% confidence interval). P values are weighted. [a]Other races include American Indian or Alaska Native, Native Hawaiian or other Pacific Islander, and multiracial persons.

NHANES, National Health and Nutrition Examination Survey; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline Phosphatase; ALB, albumin; GGT, gamma glutamyl transferase; TC, total cholesterol; TB, total bilirubin.

0.001], had less physical activity levels (12.5 vs. 7.1%, $p < 0.001$), had diabetes [25.0% (95% CI, 21.2–28.8%) vs. 11.3% (95% CI, 10.3–12.3%), $p < 0.001$] and had lower serum cotinine levels [less than 0.015 ng/mL, 46.5% (95% CI, 42.1–50.9%) vs. 35.7% (95% CI, 34.2–37.2%), $p < 0.001$], compared to those who has not received the surgery. Moreover, participants who had undergone cholecystectomy had higher levels of ALP (82.89 ± 33.52 U/L vs. 75.44 ± 22.99 U/L, $p < 0.001$), platelets (255.77 ± 71.75 $\times 10^9$/L vs. 243.21 ± 58.68 $\times 10^9$/L, $p < 0.001$), median liver stiffness (6.71 ± 6.45 KPa vs. 5.52 ± 4.27 KPa, $p < 0.001$), controlled attenuated parameter (279.84 ± 59.85 dB/m vs. 261.35 ± 62.53 dB/m, $p < 0.001$), than those who had not received the surgery.

Moreover, the incidence of LF and LC was higher in participants who had received cholecystectomy [34.7% (95% CI, 30.5–38.9%) vs. 19.8% (95% CI, 18.6–21.0%), $p < 0.001$; 4.5% (95% CI, 2.7–6.3%) vs. 2.8% (95% CI, 2.3–3.3%), $p = 0.041$, respectively].

Characteristics of Participants After PSM

Given the significant differences at baseline between included participants who had undergone cholecystectomy and those who had not, PSM was performed on the individuals using such covariates as age, gender, BMI group and history of diabetes, which were previously associated with metabolic disorder and

TABLE 2 | General characteristics of participants ($n = 978$) by the presence or absence of a history of cholecystectomy after propensity score matching in the NHANES 2017–2018.

Characters	Yes ($n = 489$)	No ($n = 489$)	*p*-Value
Age (years)	57.23 ± 14.61	56.76 ± 15.09	0.624
20–29	4.3 (2.5–6.1)	4.5 (2.7–6.3)	
30–39	10.6 (7.9–13.3)	8.9 (6.4–11.4)	
40–49	16.9 (13.6–20.2)	16.2 (12.9–19.5)	
50–59	18.9 (15.4–22.4)	18.6 (15.2–22.0)	
60–69	27.6(23.6–31.6)	21.9 (18.2–25.6)	
70–80	21.9 (18.2–25.6)	29.9 (25.8–34.0)	
Gender			0.343
Male	22.7 (19.0–26.4)	25.3 (21.4–29.2)	
Female	77.3 (73.6–81.0)	74.7 (70.8–78.6)	
Race/ethnicity			<0.001
Hispanic	11.7 (8.9–14.5)	17.0 (13.7–20.3)	
Non-Hispanic White	73.2 (69.3–77.1)	59.9 (55.6–64.2)	
Non-Hispanic Black	6.5 (4.3–8.7)	12.0 (9.1–14.9)	
Non-Hispanic Asian	2.2 (0.9–3.5)	6.4 (4.2–8.6)	
Other races[a]	6.5 (4.3–8.7)	4.8 (2.9–6.7)	
Education			0.086
More than high school	58.2 (53.8–62.6)	60.9 (56.6–65.2)	
High school or equivalent	30.5 (26.4–34.6)	24.1 (20.3–27.9)	
Less than high school	11.3 (8.5–14.1)	14.8 (11.7–17.9)	
Not recorded	0.0	0.1 (−0.2, 0.4)	
Poverty-income ratio			0.988
<1.3	17.2 (13.9–20.5)	17.2 (13.9–20.5)	
1.3–1.8	8.2 (5.8–10.6)	8.3 (5.9–10.7)	
>1.8	65.1 (60.9–69.3)	65.7 (61.5–69.9)	
Not recorded	9.5 (6.9–12.1)	8.8 (6.3–11.3)	
BMI group			0.417
<25	13.8 (10.7–16.9)	13.7 (10.7–16.7)	
25–30	28.5 (24.5–32.5)	24.9 (21.1–28.7)	
≥30	57.7 (53.3–62.1)	61.5 (57.2–65.8)	
Physical activity level			0.009
Inactive	51.3 (46.9–55.7)	59.8 (55.5–64.1)	
Less active	12.5 (9.6–15.4)	7.7 (5.3–10.1)	
Active	36.3 (32.0–40.6)	32.6 (28.4–36.8)	
Daily alcohol drinking status			0.006
Non-drinkers	7.4 (5.1–9.7)	6.4 (4.2–8.6)	
Moderate-drinkers	30.4 (26.3–34.5)	36.0 (31.7–40.3)	
Heavy-drinkers	17.8 (14.4–21.2)	14.3 (11.2–17.4)	
Binge-drinkers	22.1 (18.4–25.8)	28.2 (24.2–32.2)	
Not recorded	22.3 (18.6–26.0)	15.1 (11.9–18.3)	
History of diabetes			0.900
Yes	24.9 (21.1–28.7)	25.1 (21.3–28.9)	
Having HBV infection			0.128
Yes	1.0 (0.1–1.9)	1.4 (0.4–2.4)	
Having HCV infection			0.053
Yes	2.9 (1.4–4.4)	2.1 (0.8–3.4)	
Laboratory parameters			
Smoking (serum cotinine levels)			0.183
<0.015	46.5 (42.1–50.9)	44.4 (40.0–48.8)	
0.015–3	29.6 (25.6–33.6)	33.5 (29.3–37.7)	
≥3	22.5 (18.8–26.2)	19.3 (15.8–22.8)	
Not recorded	1.4 (0.4–2.4)	2.8 (1.3–4.3)	
ALT (U/L)	20.92 ± 15.17	21.92 ± 14.52	0.314
AST (U/L)	20.10 ± 10.45	21.72 ± 12.15	0.030
ALP (U/L)	83.01 ± 33.97	80.50 ± 25.16	0.216
ALB (g/L)	39.66 ± 3.27	40.46 ± 2.94	<0.001
GGT (U/L)	30.67 ± 63.47	31.63 ± 41.87	0.794
TC (mmol/L)	4.83 ± 0.95	5.11 ± 1.22	<0.001
TB (umol/L)	7.99 ± 4.93	7.14 ± 4.45	0.007
Platelet ($\times 10^9$/L)	255.86 ± 72.11	245.84 ± 60.77	0.023
Transient Elastography			
Median stiffness (kPa)	6.71 ± 6.46	5.03 ± 2.41	<0.001
Controlled attenuated parameter (dB/m)	279.75 ± 59.85	266.38 ± 58.55	<0.001
Liver fibrosis			<0.001
Yes	34.6 (30.4–38.8)	16.4 (13.1–19.7)	
Liver cirrhosis			0.0099
Yes	4.5 (2.7–6.3)	1.5 (0.4–2.6)	

Values are weighted mean ± SD or weighted % (95% confidence interval). p values are weighted. [a]Other races include American Indian or Alaska Native, Native Hawaiian or other Pacific Islander, and multiracial persons.

NHANES, National Health and Nutrition Examination Survey; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline Phosphatase; ALB, albumin; GGT, gamma glutamyl transferase; TC, total cholesterol; TB, total bilirubin; NAFLD, non-alcoholic fatty liver disease.

liver disease (23–25). After PSM, 489 pairs of cases were further analyzed (**Table 2**). The findings showed that gender, age, level of education, diabetes, HBV infection, HCV infection, serum cotinine levels, BMI group, and the poverty income ratio, were comparable between the two groups after PSM. Compared to participants who had not received cholecystectomy after PSM, more individuals who had undergone cholecystectomy were Non-Hispanic Whites [73.2% (95% CI, 69.3–77.1%) vs. 59.9% (95% CI, 55.6–64.2%), $p < 0.001$], heavy-drinkers [17.8% (95% CI, 14.4–21.2%) vs. 14.3% (95% CI, 11.2–17.4%), $p = 0.006$], had higher levels of physical activity (less active: 12.5 vs. 7.7%; active: 36.3 vs. 32.6%; $p = 0.009$), TB (7.99 ± 4.93 umol/L vs. 7.14 ± 4.45 umol/L, $p = 0.007$), platelet [$(255.86 \pm 72.11) \times 10^9$/L vs. $(245.84 \pm 60.77) \times 10^9$/L, $p = 0.023$), and higher values of median stiffness (6.71 ± 6.46 KPa vs. 5.03 ± 2.41 KPa, $p < 0.001$) as well as controlled attenuated parameter (279.75 ± 59.85 dB/m vs. 266.38 ± 58.55, $p < 0.001$). However, those who had undergone cholecystectomy had lower levels of ALB (39.66 ± 3.27 g/L vs. 40.46 ± 2.94 g/L, $p < 0.001$), TC (4.83 ± 0.95 mmol/L vs. 5.11 ± 1.22 mmol/L, $p < 0.001$) and AST (20.10 ± 10.45 U/L vs. 21.72 ± 12.15 U/L, $p = 0.030$). Moreover, the incidence of LF (≥F1) was more than two-fold higher in participants who had received cholecystectomy [34.6% (95% CI, 30.4–38.8%) vs. 16.4% (95% CI, 13.1–19.7%), $p < 0.001$), and LC (≥F4) was threefold

TABLE 3 | Associations between cholecystectomy and liver fibrosis after propensity score matching ($n = 978$), NHANES 2017–2018.

	Model 1 OR (95% CI), P	Model 2 OR (95% CI), P	Model 3 OR (95% CI), P
Cholecystectomy			
No	Reference	Reference	Reference
Yes	2.130 (1.598, 2.839) <0.001	2.149 (1.598, 2.891) <0.001	2.393 (1.738, 3.297) <0.001
Stratified by age			
20–29y	Inf. (0.000, Inf)* 0.996	Inf. (0.000, Inf) 0.997	105266.561 (0.000, Inf) 1.000
30–39y	4.154 (1.341, 12.870) 0.014	3.016 (0.869, 10.465) 0.082	1.134 (0.152, 8.466) 0.903
40–49y	2.375 (1.108, 5.092) 0.026	2.563 (1.155, 5.686) 0.021	4.287 (1.407, 13.060) 0.010
50–59y	3.804 (1.910, 7.576) <0.001	4.380 (2.110, 9.090) <0.001	5.488 (2.238, 13.461) <0.001
60–69y	1.384 (0.790, 2.426) 0.256	1.435 (0.807, 2.553) 0.219	1.728 (0.847, 3.523) 0.132
70–80y	1.600 (0.959, 2.669) 0.072	1.590 (0.934, 2.705) 0.087	1.585 (0.866, 2.901) 0.135
Stratified by gender			
Men	1.810 (1.073, 3.054) 0.026	1.972 (1.130, 3.441) 0.017	2.153 (1.113, 4.166) 0.023
Women	2.309 (1.631, 3.268) <0.001	2.247 (1.572, 3.211) <0.001	2.555 (1.727, 3.778) <0.001
Stratified by race			
Hispanic	1.832 (1.042, 3.218) 0.035	1.751 (0.978, 3.135) 0.060	1.496 (0.736, 3.038) 0.265
Non-Hispanic White	3.253 (1.959, 5.404) <0.001	3.199 (1.918, 5.334) <0.001	3.835 (2.186, 6.730) <0.001
Non-Hispanic Black	1.023 (0.529, 1.978) 0.947	1.038 (0.518, 2.079) 0.916	1.276 (0.559, 2.912) 0.562
Non-Hispanic Asian	2.700 (1.004, 7.263) 0.049	2.174 (0.749, 6.310) 0.153	5.924 (0.912, 38.490) 0.062
Other races	2.422 (0.753, 7.786) 0.138	3.671 (0.946, 14.247) 0.060	106.704 (1.434, 7938.920) 0.034

*Model 1: Non-adjusted model; Model 2 adjusted for: gender; age; race; Model 3 adjusted for: gender; age; race; education; alcohol; diabetes; HBV infection; HCV infection; physical activity status; serum cotinine levels; BMI, and poverty income ratio. *'Inf' means that values can't be calculated.*
NHANES, National Health and Nutrition Examination Survey; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus.

higher [4.5% (95% CI, 2.7–6.3%) vs. 1.5% (95% CI, 0.4–2.6%), $p = 0.0099$].

Associations Between Cholecystectomy and LF

After PSM and unadjusted analysis (**Table 3**), the OR value for the presence of LF in participants who had undergone cholecystectomy was 2.130 (95% CI, 1.598–2.839), compared to those who had not received the surgery. This value remained statistically significant after adjusting for gender, age, and race (OR, 2.149 [95% CI, 1.598–2.891]). In addition, there was an increase in the OR value for the association of LF with cholecystectomy, after full adjustment (2.393 [95% CI, 1.738–3.297]).

In addition, subgroup analyses revealed that cholecystectomy patients who are 40–49 years old, 50–59 years old, female, or Non-Hispanic White are at a higher risk of developing LF regardless of whether PSM was performed. After PSM, the OR value for the association of LF with cholecystectomy remained significant in participants who were 40–49 years old (Full adjustment: 4.287 [95% CI, 1.407–13.060]) and 50–59 years of age (Full adjustment: 5.488 [95% CI, 2.238–13.461]). After stratification by gender, the OR value remained significant especially in females (Full adjustment: 2.555 [95% CI, 1.727–3.778]). Additionally, there were significant associations between cholecystectomy and other covariates, including non-Hispanic Whites (Full adjustment: OR, 3.835 [95% CI, 2.186–6.730]).

Associations Between Cholecystectomy and LC

Finally, the study assessed the association between cholecystectomy and LC (**Table 4**) in participants after PSM. After PSM and unadjusted analysis, the OR value for the presence of LC in participants who had undergone cholecystectomy was

TABLE 4 | Associations between cholecystectomy and liver cirrhosis after propensity score matching ($n = 978$), NHANES 2017–2018.

	Model 1 OR (95% CI), P	Model 2 OR (95% CI), P	Model 3 OR (95% CI), P
Cholecystectomy			
No	Reference	Reference	Reference
Yes	3.020 (1.455, 6.267) 0.003	3.030 (1.435, 6.395) 0.004	3.287 (1.496, 7.218) 0.003
Stratified by Age			
20–29y	1.000 (0.000, Inf)* 1.000	1.000 (0.000, Inf) 1.000	1.000 (0.000, Inf) 1.000
30–39y	Inf. (0.000, Inf) 0.995	Inf. (0.000, Inf) 0.998	Inf. (0.000, Inf) 1.000
40–49y	Inf. (0.000, Inf) 0.996	Inf. (0.000, Inf) 0.999	123814.773 (0.000, Inf) 1.000
50–59y	1.537 (0.418, 5.653) 0.517	1.703 (0.435, 6.664) 0.444	1.494 (0.218, 10.233) 0.683
60–69y	2.069 (0.607, 7.058) 0.246	2.284 (0.628, 8.308) 0.210	3.913 (0.697, 21.985) 0.121
70–80y	5.292 (1.139, 24.588) 0.034	4.401 (0.929, 20.847) 0.062	3.738 (0.679, 20.591) 0.130
Stratified by gender			
Men	3.507 (1.111, 11.066) 0.032	3.009 (0.904, 10.016) 0.073	4.557 (0.937, 22.147) 0.060
Women	2.744 (1.061, 7.093) 0.037	2.708 (1.025, 7.150) 0.044	2.673 (0.962, 7.426) 0.059
Stratified by race			
Hispanic	3.421 (0.884, 13.231) 0.075	3.109 (0.765, 12.625) 0.113	2.272 (0.329, 15.680) 0.405
Non-Hispanic White	1.978 (0.626, 6.248) 0.245	1.833 (0.570, 5.894) 0.309	1.688 (0.461, 6.179) 0.429
Non-Hispanic Black	4.560 (0.466, 44.664) 0.192	3.619 (0.360, 36.410) 0.275	0.157 (0.000, Inf) 1.000
Non-Hispanic Asian	6.300 (0.629, 63.127) 0.118	2.780 (0.228, 33.932) 0.423	Inf. (0.000, Inf) 1.000
Other races	2.667 (0.260, 27.382) 0.409	6.139 (0.461, 81.690) 0.169	Inf. (0.000, Inf) 1.000

*Model 1: Non-adjusted model; Model 2 adjusted for: gender; age; race; Model 3 adjusted for: gender; age; race; education; alcohol; diabetes; HBV infection; HCV infection; physical activity status; serum cotinine levels; BMI, and poverty income ratio. *'Inf' means that values can't be calculated.*
NHANES, National Health and Nutrition Examination Survey; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus.

3.020 (95% CI, 1.455–6.267), compared to those who had not received the surgery. This value remained statistically significant after adjusting for gender, age, and race (OR, 3.030 [95% CI, 1.435–6.395]). In addition, there was an increase in the OR value for the association of LC with cholecystectomy, after full adjustment (3.287 [95% CI, 1.496–7.218]).

In addition, subgroup analyses after PSM revealed that there was no statistically difference in demographic data including race, gender, and age.

DISCUSSION

The results showed that there was a positive correlation between cholecystectomy and LF or LC. In addition, the association remained statistically significant even after adjusting for possible confounders. Moreover, the association was still significant after exact PSM by age, gender, BMI, and diabetes.

Prior to this day, little research had been conducted on the correlation between cholecystectomy and LF or LC. Notably, a retrospective, multicenter study in Turkey showed no independent association between the presence of cholecystectomy and advanced LF (9). On the contrary, another cross-section study using data of the third US National Health and Nutrition Examination Survey (NHANES III, 1988–1994), showed the positive association of NAFLD with cholecystectomy (10). Nonetheless, research on the association between cholecystectomy and LF or LC is largely scarce. The results obtained herein were contrary to those reported in Turkey and may be a good update to understand the association of cholecystectomy and LF/LC (9). Cholecystectomy is the mainstream procedure for treating most gallbladder diseases and is associated with such complications as bile duct injury (0.08–0.5%), bile leak (0.42–1.1%), retained common bile duct stones (0.8–5.7%) and biliary strictures (0.4–0.6%) (26–30). These complications can in turn lead to prolonged hospital stays,

increased morbidity, increased claims and more financial burden (30, 31). Moreover, obstruction of the bile duct caused by bile duct injury or biliary stricture may lead to LF, LC, and portal hypertension (32).

The possibility of correlation between cholecystectomy and LF or LC is further supported by the findings from the present study. After cholecystectomy, changes in bile flow and concentration of bile acid in the bile duct (33) might occur, which may cause chronic cholestasis, NAFLD and metabolic syndrome (10, 34–36). Interestingly, the study showed that participants with cholecystectomy for over 14 years had a higher incidence of LF than those <14 years (**Supplementary Table 4**).

Additionally, numerous studies have shown the positive association between metabolic syndrome and LF (25, 37). Moreover, the discovery of a bile acid shunt pathway between the gallbladder and liver, provided new insights on the protective role of the gallbladder (38). Interestingly, endocrine hormones secreted by the gallbladder, such as FGF19, may provide another possible mechanism for the development of metabolic syndrome after cholecystectomy (39–41).

These results highlighting the positive correlation between cholecystectomy and LF or LC in adults have an important implication in public health. Cholecystectomy is among the most common operations performed worldwide, with 750,000–1,000,000 procedures conducted in the United States, annually (42). Considering the early and delayed complications associated with cholecystectomy, it would be important to reassess the function and importance of the gallbladder (34). Strict surgical indications should also be implemented to reduce unnecessary cholecystectomy (43), given that preoperative evaluation of abdominal pain through gastroduodenoscopy was reported to be able to prevent 3.8% of cholecystectomies (44). Additionally, inexperienced surgeons should undergo standardized and strict training according to the operation protocols in order to reduce cholecystectomy-related bile duct injury (42). More importantly, annual monitoring of cholecystectomy patients should be conducted through liver ultrasound TE, especially those with such risk factors as being 40–59 years old, female, or Non-Hispanic White. This might help with the early diagnosis of LF or LC, hence enabling timely intervention (11). Moreover, further research is needed to identify the exact group of cholecystectomy patients who may be at a higher risk of developing LF or LC.

While the present study uncovered some insightful findings, it had a few limitations. First, the research results are not applicable to individuals younger than 20 years, including children and adolescents because of the age limit in cholecystectomy questionnaires used in NHANES 2017–2018. In addition, the study was not able to collect new data because this was a secondary analysis. Therefore, there might be a risk of residual confounding bias from the non-recorded covariates. Specifically, the results were not adjusted for cholecystectomy-related complications, which are potentially important contributors to LF or LC. Moreover, the study was unable to establish causality based on the cross-sectional data. On the other hand, it is ethically impossible to perform a randomized clinical trial on cholecystectomy in humans. Nonetheless, the study had several strengths, including a large sample size, a nationally representative population and use of exact PSM. As outcome variables, LF/LC were also assessed though the widely used TE in a standardized way, including repeated measurements to maintain accuracy.

In conclusion, the present study showed that cholecystectomy is positively associated with LF and LC in US adults, regardless of PSM. The discovery of this risk factors therefore provides new insights on the prevention of LF, LC.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://wwwn.cdc.gov/nchs/nhanes/.

AUTHOR CONTRIBUTIONS

Z-QX and H-XL: contributed to the conception and design, the acquisition, analysis, interpretation of the data, and the drafting of the article or critical revision for important intellectual content. W-LT, LY, X-WM, W-XL, and Q-BW: collected data. C-ZS and Y-JC: contributed to the conception and design and the reviewing of the article or critical revision for important intellectual content. All authors approved the final version, and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.787777/full#supplementary-material

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REVIEW
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Inflammation and Fibrogenesis in MAFLD: Role of the Hepatic Immune System

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Metabolic (dysfunction)-associated fatty liver disease (MAFLD) is the definition recently proposed to better circumscribe the spectrum of conditions long known as non-alcoholic fatty liver disease (NAFLD) that range from simple steatosis without inflammation to more advanced liver diseases. The progression of MAFLD, as well as other chronic liver diseases, toward cirrhosis, is driven by hepatic inflammation and fibrogenesis. The latter, result of a "*chronic wound healing reaction*," is a dynamic process, and the understanding of its underlying pathophysiological events has increased in recent years. Fibrosis progresses in a microenvironment where it takes part an interplay between fibrogenic cells and many other elements, including some cells of the immune system with an underexplored or still unclear role in liver diseases. Some therapeutic approaches, also acting on the immune system, have been probed over time to evaluate their ability to improve inflammation and fibrosis in NAFLD, but to date no drug has been approved to treat this condition. In this review, we will focus on the contribution of the liver immune system in the progression of NAFLD, and on therapies under study that aim to counter the immune substrate of the disease.

Keywords: NAFLD, MAFLD, liver immunology, immunometabolism, liver fibrogenesis, NAFLD therapies

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INTRODUCTION: DEFINITIONS, CHANGE OF TERMINOLOGY, AND EPIDEMIOLOGY

MALFD stands for "*metabolic (dysfunction)-associated fatty liver disease*" and is a recently recommended term by an international panel of experts (1) to replace the long used NAFLD (non-alcoholic fatty liver disease) and NASH (non-alcoholic steatohepatitis). The latter was coined by Ludwig et al., referring to the fatty liver and inflammation observed in biopsy specimens of patients who had other metabolic disorders, such as obesity or related conditions, and were not alcohol abusers (2), while NAFLD appeared for the first time in a paper by Schaffner and Thaler (3). According to EASL and AASLD guidelines, NAFLD indicates an excessive accumulation of liver fat, corresponding to the presence of steatosis in >5% of hepatocytes, documented by liver histology or imaging, in people who don't drink an at-risk amount of alcohol (nor having other causes of steatosis). The latter specification is believed to represent a weak point in this definition, due to the absence of an international consensus in defining threshold levels for at-risk alcohol consumption and the potential shame associated with the term "alcoholic" (1, 4–6). NAFLD term includes a set of pathological conditions ranging from mild alterations (NAFL) to others conferring a worse

prognosis (NASH, which implies hepatocyte injury and liver fibrosis of increasing severity up to NASH-cirrhosis, and hepatocarcinoma). On the other hand, the Asian Pacific Association for the Study of the Liver (APASL) has already presented guidelines on the diagnosis and management of MAFLD (7). The open challenge is to find out the causes why some people with NAFLD progress to advanced liver disease while others do not (8).

The agreement on the term "MAFLD" originated to emphasize the metabolic etiology of this spectrum of conditions, and consequently avoid the use of a "non-definition" (1). Moreover, with this new term the coexistence of other cofactors for the progression of the disease, including alcohol consumption, is allowed (1, 6). Experts proposed that the term MAFLD should include the set of conditions, overcoming the non-NASH/NASH dichotomy and that it should be enriched with data on the severity of the disease (grade of activity and stage of fibrosis) (1). The issue of the terminology of NAFLD and NASH is not new, in fact was already addressed in the past (1, 9). However, skepticism is not absent regarding the recently proposed change, which according to some authors could be precocious and counterproductive (10). Their doubts concern the use of a term ("metabolic") that likewise may lack specificity; because other liver diseases (also responsible for hepatic steatosis, e.g., Wilson disease) have a metabolic etiology; since, although knowledge of pathophysiology and other aspects of NAFLD has increased, great challenges still exist; furthermore, they believe this change could have negative repercussions for socio-sanitary and scientific reasons (10).

NAFLD is estimated to have a global prevalence of around 25% of the general population and is responsible for high morbidity and mortality, having been found that its prevalence has grown in tandem with the global increase of obesity (8, 11–13). It is an increasingly common cause of liver transplantation and hepatocarcinoma, which in NAFLD can arise even in the absence of cirrhosis (8). In addition to liver-related causes of morbidity and mortality, it has a strong link with the various components of the metabolic syndrome (MetS) (8); in fact, NAFLD showed to have a high prevalence in patients with MetS elements (12, 14). It was also observed that, over the years, people with NAFLD have a high probability of developing other metabolic comorbidities, cardiovascular diseases, and non-fatal or fatal events (the latter representing the leading cause of death for these patients), compared to those without NAFLD (8, 13, 15, 16), and that patients with cardiovascular disease (CVD) risk factors have an increased risk of developing NAFLD compared to people without these risk factors, suggesting a bi-directional relationship between NAFLD and CVD risk factors (15). The cardiovascular risk for patients with NAFLD, which is especially observed for those who have NASH, appears to be independent of the various components of the metabolic syndrome, suggesting a direct role of the liver disease (16, 17). Despite its strong negative impact on human health, to date, there are still no approved therapies to reverse this condition. The chronic inflammation which occurs in NASH is a central pathophysiological event and guides the progression of the disease through increasing degrees of fibrosis toward liver cirrhosis.

HOMEOSTASIS OF THE LIVER IMMUNE SYSTEM IN HEALTH STATE

The liver is crucial in the metabolism of carbohydrates, lipids, and proteins, is responsible for bile formation, detoxification and inactivation of substances, and has storage functions, but it is also an important immune organ (18). In the hepatic parenchyma, a rich variety of elements participating in the immune response exists (19), some of which being not strictly immune cells. Among the latter there are hepatocytes, the most abundant cell population of the liver, which, in addition to their "primary" functions, express Pattern Recognition Receptors (PRRs), can produce acute phase proteins, cytokines, chemokines, complement proteins and other opsonins; they produce proteins involved in iron metabolism, such as hepcidin, the availability of this element being able to affects bacterial proliferation; hepatocytes are the main source of LPS-binding protein, soluble CD14, and soluble MD-2, which participate in the formation of TLR4-MD-2-LPS complex, from which, in turn, starts the signaling that leads to NF-kB activation and inflammatory responses; fibrinogen, produced by hepatocytes, participates in the immune response as it mediates the adhesion of leukocytes, can activate the complement system, and because its active fragment fibrin has antibacterial properties; moreover, they express MHC-I and in some conditions also MHC-II, lacking, however, in the expression of costimulatory molecules (18–22), liver sinusoidal endothelial cells (LSECs, which in addition to offering a physical barrier between the lumen of the sinusoid and the space of Disse, participate in the process of leukocytes transmigration, exhibit scavenger activity, are capable of endocytosis, express TLRs and MHC molecules, and are involved in tolerance mechanisms, by direct action on T lymphocytes, e.g., by PDL1 expression, or through the "veto" function, consisting in vetoing the ability of other APCs, like dendritic cells, to activate T lymphocytes, in a mode requiring physical contact but MHC-independent) (19, 23–28), biliary epithelial cells (BECs, antigen presentation, TLRs expression, production of inflammatory mediators in response to insults; these cells were found capable of "endotoxin tolerance," which was demonstrated after observation that human intrahepatic biliary epithelial cell lines pretreated with LPS developed tolerance to further stimulation with such substance; this effect was attributed to the negative regulation of the TLR signaling mediated by interleukin-1 receptor-associated kinase M, IRAK-M) (19, 29), and hepatic stellate cells (HSCs, the main actors in the fibrogenesis process, also express TLRs, MHC-I, MHC-II, and CD1 molecules, and, as observed for the LSECs, are involved in the induction of T-cell tolerance also through a veto function) (19, 22, 30). These cells are part of innate immunity, but also interact with elements of adaptive responses. Among the innate immune cells housed in liver sinusoids there are myeloid- (Kupffer cells, KC, dendritic cells, DCs, myeloid-derived suppressor cells, MDSC) or lymphoid-derived cells (such as natural killers, NK, and innate lymphoid cells, ILCs). Other abundant elements do not reflect either the innate or adaptive system criteria and were therefore defined as "innate-like," or "unconventional" lymphocytes. These include

mucosal-associated invariant T (MAIT) cells, which are today deemed to be a leading share of hepatic T lymphocytes in the healthy liver (31), natural killer T (NKT) cells, and γδ-T cells. Furthermore, the healthy liver also hosts conventional T and B lymphocytes (adaptive immunity) (19). Compared to other lymphoid organs, such as the lymph nodes and the spleen, the liver greatly differs in terms of composition of its resident cells (32).

A complex relationship between the large number of antigens to which cells of healthy liver are continuously exposed and the maintenance of an immune homeostasis exists: the liver occupies a first-line position, filtering more than 2,000 liters of blood per day coming from the portal vein, which in turns carries a large amount of gut-derived food antigens and bacterial products (e.g., LPS), and from the hepatic artery which transports oxygen-rich blood (22). Furthermore, in the liver it occurs the formation of neo-antigens due to the intrahepatic transformation of many compounds (32). Under stationary conditions, the hepatic immune cells maintain tolerance to non-harmful substances (e.g., food-derived antigens), but they must also be able to mount an adequate response against the pathogenetic ones (22, 32). The tolerance state originates in a tolerogenic microenvironment, due to the complex interplay that takes place between different cells. In fact, liver resident cells block adaptive immune responses by inducing states of energy, exhaustion, deviation, or by leading immune cells to apoptosis (22, 33). The concept of hepatic tolerance was initially hypothesized in the 60's by observing long-term survivals of allogeneic pig liver transplants without using immunosuppression (34, 35), a phenomenon subsequently confirmed in other animal models (36). Furthermore, the finding that liver transplanted animals receiving non-hepatic allografts from the same liver donor showed acceptance of such grafts, suggested that the liver can induce systemic T-cell tolerance (36).

Among the mechanisms responsible for liver immune tolerance, there is the expression by liver cells of MHC complexes in the absence of costimulatory molecules (e.g., CD80/CD86); lack of MHC-II expression; release of cytokines with suppressor activity, such as IL 10 or TGF-β; exposure of immune cells to programmed cell death ligand-1 (PD-L1), or Fas-L; phagocytosis by Kupffer cells; inhibition of professional APC activating function (19, 30, 32, 37, 38). In the context of liver transplants specific mechanisms inducing tolerance take part (36). Since tolerance has been observed to be a marked phenomenon in the liver, hypotheses have been formulated to explain this occurrence (33). In the "*graveyard hypothesis*" the liver was conceived as a site where T lymphocytes that are already directed toward apoptosis ("moribund" lymphocytes) are sequestered, whereas the "*killing field hypothesis*" suggests that this organ may be a site in which activated T lymphocytes accumulate, and where tolerance mechanisms lead them to apoptosis (39). The "*school*" model was another suggested theory, and postulates that lymphocytes migrating through the liver are educated (like "students") to have regulatory functions rather than participate in immunosurveillance; in this model, the hepatic antigen presenting cells (hepatocytes, LSECs, KCs, DCs, HSCs) represent the "teachers" who induce such lymphocytes to a regulatory state, this action being favored by the anatomy of the hepatic sinusoids (40). Another modality of hepatic immune homeostasis maintenance was observed to depend on liver draining lymph nodes (LNs), differently depending on which one is considered, having been found that portal LN is a site of regulatory T cells induction, whereas the celiac LN is involved in T cell responses (33, 41).

Bile acids and the extracellular matrix (as described below) can also modulate the immune response in the liver (19, 42, 43). Moreover, cellular metabolism is closely linked to immune properties. In fact, different metabolic patterns have been found associated with different immune cell functions. A predominantly glycolytic metabolism was observed in different types of effector T lymphocytes and other activated immune cells with effector function participating in inflammatory processes, while fatty acid oxidation was observed to be preferred by non-inflammatory immune cells (e.g., regulatory T cells, T_{reg}) (44, 45). Moreover, it was observed that glycolysis induced by HIF-1α on the one hand, and oxidative metabolism induced by IL-4 / STAT6 / PGC-1β on the other hand, drove different types of macrophage phenotypes, proinflammatory (46) vs. alternative (anti-inflammatory) (47), respectively (48).

In contrast to the immune homeostasis of the healthy liver, which nevertheless is capable of effective local or systemic inflammatory responses, in NAFLD, cells with immune functions become key players in the disease progression.

NAFLD IS A MULTIFACTORIAL, SYSTEMIC DISEASE CAUSED BY A SET OF SIMULTANEOUS AND SYNERGISTIC EVENTS

The "two-hit" model for NAFLD progression was proposed in 1998 by C. P. Day and O. F. James. In this theory, the first hit is the excess in the accumulation of lipids within hepatocyte (steatosis), and the second one corresponds to other factors responsible for steatohepatitis (49). The currently accepted theory, "multiple-hit hypothesis," proposed by Tilg and Moschen (50), replaced the two-hit model and indicates that there are multiple synergistic events leading to liver inflammation, proceeding in parallel. In this theory, inflammation not necessarily follows the fat accumulation, being the opposite also plausible: inflammation caused by different insults could exist before steatosis in NASH, and may contribute to its progression (50). Several factors contribute to this pathological condition, including insulin resistance, which is a central event in the NAFLD pathophysiology, excess flow of fatty acids to the liver, lipotoxicity, mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress (50, 51). Altered liver-adipose tissue cross talk (because of the effect on the liver of the imbalance of adipokine production by a dysfunctional adipose tissue) (50, 51) and gut-liver axis, are important dysfunctions occurring in NAFLD and implicated in its pathogenesis (50). Patients with NAFLD showed to have changes in gut microbiota, a high prevalence of intestinal bacterial overgrowth, and increased gut permeability (52–54). The increased liver exposure to bacterial derived products

(e.g., endotoxemia), proved to cause liver fat accumulation and inflammation mediated by immune system cells (e.g., Kupffer cells *via* TLR-4) (51, 55). Among the genetic factors conferring susceptibility to NAFLD there are polymorphisms in *patatin-like phospholipase domain containing-3* (*PNPLA3*) gene, which is the most studied in NAFLD, *transmembrane 6 superfamily, member 2* (*TM6SF2*) gene, *membrane bound O-acyltransferase domain containing 7-transmembrane channel-like 4* (*MBOAT7*) gene, *glucokinase regulator (GCKR)* gene, and *17-beta hydroxysteroid dehydrogenase-13 (HSD17B13)* gene (8, 56); moreover, variants of genes regulating the mitochondrial activity, insulin signaling, and immune response have also been shown to be involved in such disease (57). Epigenetic changes, such as altered DNA methylation and miRNA expression, have recently been investigated in NAFLD and linked to disease progression (58–60). Environmental risk factors affect the onset and progression of fatty liver and include dietary styles like Western diet (high in saturated fats), high consumption of fructose (e.g., that contained in some sweetened beverages or high fructose corn syrup) and refined carbohydrates, and sedentary lifestyle. The prevalence of NAFLD also varies in relation to age, sex, and ethnicity (4, 8, 51, 61). It should be noted, however, that not a single risk factor but the interplay of many elements causes NAFLD progression; in fact, not all obese or people with risk factors for NAFLD are affected by this condition, and NAFLD can develop in non-obese, non-diabetic people (8).

The concept of metabolic flexibility (opposed to metabolic inflexibility) indicates the ability to adjust the utilization of substrates depending on different conditions (e.g., changes in their availability) (8, 62). The typical alterations observed in NAFLD patients (high triglycerides, FFAs, and insulin) led to the hypothesis that it could be a condition characterized by metabolic inflexibility (8). A key element for the pathogenesis of NAFLD is the excess of fat and lipotoxicity (51, 63, 64). The latter, rather than the excess of fat alone, is associated with disease progression (65). The excess of circulating FFAs and the consequent abnormal liver uptake and fat accumulation typical of NAFLD, derives from abnormal lipolysis (hydrolysis of triglyceride) in the adipose tissue, mediated by insulin resistance, which is the event responsible for the largest share of hepatic fat accumulation, increased *de novo* lipogenesis (starting from glucose or fructose), and excess in dietary fat intake (64, 66, 67). Fatty acids in the liver are addressed to oxidation (mitochondrial β-oxidation, or oxidation in peroxisomes, or microsomes) or are esterified to triglycerides (TGs), to form very low-density lipoprotein (VLDL) particles, which will be secreted, or lipid droplets, which will be stored in the hepatocytes (63, 64, 67). Triglycerides formation, although associated with steatosis, is thought to be a protective response to an excess of fats, as it will be stored in an inert, non-toxic form (50, 63, 67, 68). Saturation of the processes responsible FFAs handling, due to the large amount that reaches the hepatic parenchyma, leads to alterations in mitochondrial function and an increase in the production of reactive oxygen species (ROS) (69). This ROS increase is not effectively counteracted and in NAFLD it was found an inefficiency of the ROS detoxification systems (70). The resulting oxidative stress also causes lipid damage by lipid peroxidation, which results in the formation of compounds (e.g., 4-hydroxy-2-nonenal, 4-HNE, and malondialdehyde, MDA) that contribute to the disease progression (69, 71, 72). However, it is still unclear whether mitochondrial dysfunction is a consequence of NAFLD-associated alterations or an upstream condition that predisposes to NASH (66, 73). The oxidative stress that occurs in NAFLD is in close association with activation of the immune system, e.g., ROS are a stimulus for the activation of Kupffer cells (KCs), which in turn will become ROS producers (74, 75). Lipotoxicity refers to cell dysfunctions and injury caused by lipids; saturated fatty acids such as palmitic acid and stearic acid, lysophosphatidylcholine, free cholesterol, and ceramides are considered lipotoxic species (65, 76, 77). Lipotoxicity leads to endoplasmic reticulum stress, altered autophagy, release of extracellular vehicles (EVs), and, ultimately, to activation of cell death pathway (64, 67, 78, 79). EVs, which are distinguished by size in exosomes (up to 100 nm in diameter) and microparticles (from 100 to 1,000 nm), are involved in cell-cell communication (80), and during lipotoxicity-induced hepatocytes injury they would contribute to the liver damage by eliciting pro-inflammatory responses [e.g., by inducing the release of inflammatory cytokines in macrophages (65, 81–83); moreover, they were found to be internalized by HSCs and cause their activation (81)]. Given their role in NAFLD, EVs were proposed as a marker of diseases progression (65).

Figure 1 illustrates the risk factors for NAFLD, the molecular events underlying its progression, and the histological features found in the distinct entities of its spectrum.

THE FIBROGENESIS PROCESS IN NAFLD

The hepatocytes injury and death, caused in NAFLD by metabolic dysfunctions, lead to the release of warning signals which are responsible for recruitment and activation of immune and fibrogenic cells. These cells amplify the pathological process by releasing pro-inflammatory and pro-fibrogenic factors, thus creating a vicious circle (69, 84–88).

Fibrogenesis has the physiological role of repairing a damaged tissue, so acting as a wound healing response. However, regardless of etiology, chronic liver injury and inflammation and the consequent fibrogenesis, over the years, can lead to progressive fibrosis, which in turn can evolve to liver cirrhosis, a silent condition until its complications appear, which is associated with high morbidity and mortality (8, 65). Abnormal hepatic fibrogenesis is a dynamic process in which an excess of production and a progressive accumulation over time of extracellular matrix (ECM) components takes part. In fact, in pathological conditions, the regulation of the amount of matrix, as a result of deposition and reabsorption processes, is not guaranteed (65).

Normal ECM is composed of different classes of components, including several types of fibrillary and non-fibrillar collagens, non-collagenous proteins (such as fibronectin, laminin, and elastin), and proteoglycans (89). In a proteomics study of healthy liver tissue samples, it was observed that the ECM is made up of more than 100 distinct ECM proteins (90). In physiological conditions, ECM is directly produced by many cell types

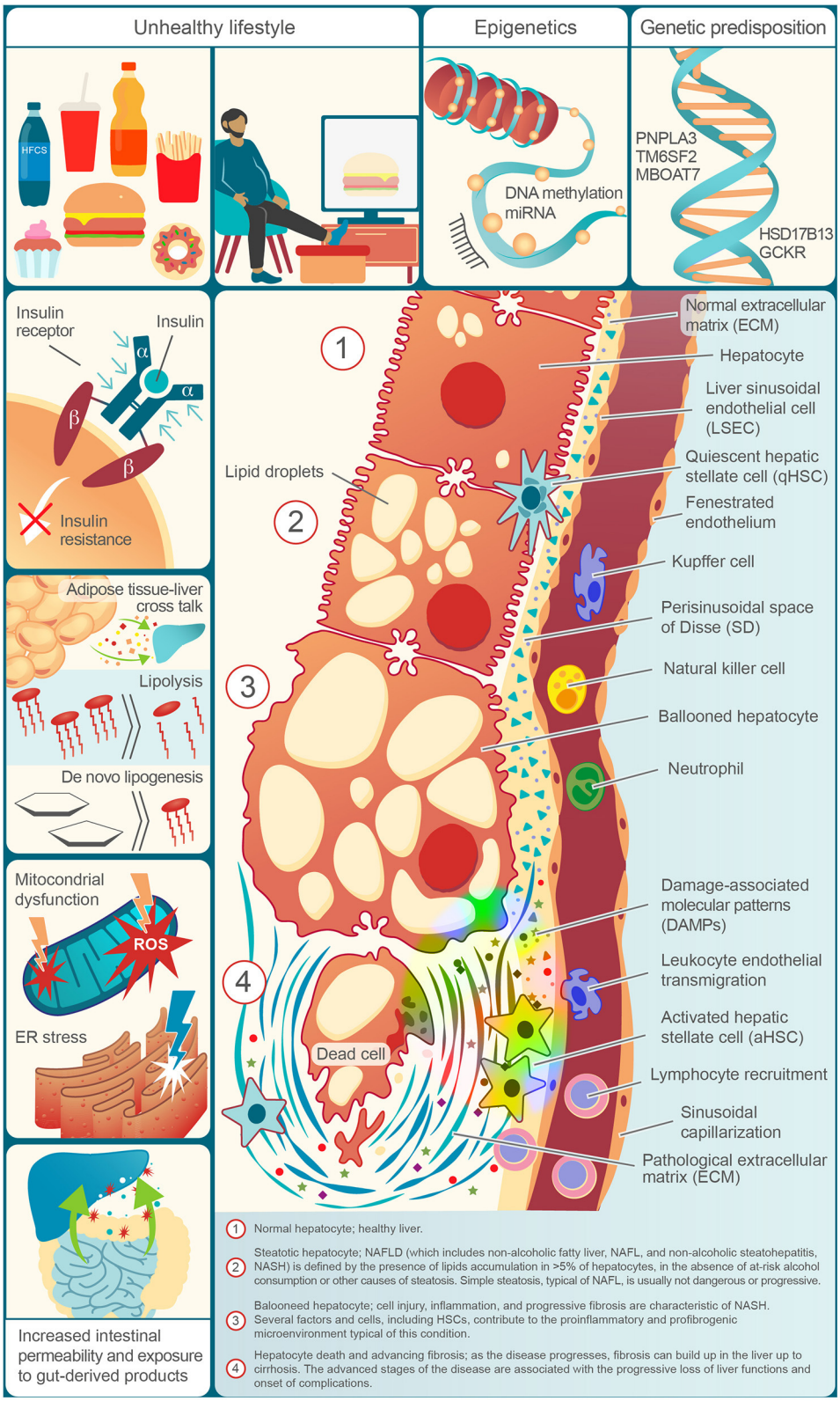

FIGURE 1 | Risk factors, physiopathological molecular events, and typical elements of the NAFLD spectrum in a decorative, cell by cell, succession.

(HSCs, hepatocytes, LSECs, cholangiocytes) (91). Furthermore, these cells release matrix metalloproteinase (MMPs), the major class of enzymes responsible for ECM degradation, and tissue inhibitors of metalloproteinases (TIMPs). For the maintenance of homeostasis, there is a fine balance between the activity of MMPs and that of TIMPs (92). In an experimental model of liver fibrosis, increased activity of TIMP-1 was found to be associated with a decreased spontaneous hepatic fibrosis resolution (93). In the healthy liver, anyway, the ECM occupies only a small part of the entire parenchyma; in the space of Disse, it forms a thin and discontinuous layer (94).

HSCs are the main source of ECM-producing fibroblasts (65, 89). In normal liver, these cells are localized in the space of Disse, and by their dendritic processes, they interact with hepatocytes and other adjacent elements of the liver parenchyma (65). Here they are involved in ECM homeostasis, work as a deposit of vitamin A (of which they are the main repository), and have immune functions (65, 95). After activation and trans-differentiation, they transform into myofibroblast-like cells (HSC/MFs) which abundantly proliferate and produce EC matrix, migrate in response to chemoattractants, produce proinflammatory mediators, thus directly contributing to the "profibrogenic environment," and have more marked contractile properties (65). Their contraction can also influence the portal pressure (96). In addition to HSCs, a smaller proportion of fibrogenic cells derives from portal fibroblasts, but other cells of origin have also been described, such as bone marrow-derived precursor, hepatocytes and cholangiocytes (reflecting a process of "epithelial-to-mesenchymal transition," EMT) (97), or mesothelial cells (MCs), although the contribution of these cells to liver MFs is believed to be minor or questionable (65). It has been hypothesized that ECM and the activity of the HSCs during NASH could have a beneficial role in the early stages of disease being, on the other hand, detrimental in later stages (89).

Activation of HSCs includes initiation and progression phases, occurs in an inflammatory context, and depends on the interaction with many elements, including immune system cells which promote and sustain the fibrogenesis process by producing several mediators (65, 91). Among these, a crucial role is played by profibrogenic cytokines. Transforming growth factor-β (TGFβ) is released by different cell types and is considered the most potent fibrogenic cytokine and activator of HSCs, leading to the production of type I collagen through a signaling pathway that involves Smad proteins (65, 89, 98–100). Phagocytosis of apoptotic cells by macrophages was found *in vitro* to increase the release of TGFβ (101), and also the HSCs were found capable of phagocytizing apoptotic bodies, this event having been found to be causative of profibrogenic responses (102); these results defined a link between hepatocytes death and fibrogenesis (102). Platelet-derived growth factor (PDGF) is another pro-fibrogenic cytokine, which leads to the proliferation and migration of HSCs (100, 103). Other cytokines involved in HSCs activation or proliferation include VEGF, CTGF, and IL-17 (65, 89, 100, 104). Furthermore, leptin showed to exert profibrotic effects, while adiponectin exhibited antifibrogenic properties (105).

Damaged or dead hepatocytes during NAFLD release damage-associated molecular patterns (DAMPs), which can activate the HSCs by toll-like receptors (TLRs) (89, 106), and TLRs on HSCs also perceive microbial products (such as LPS), which increase due to the altered intestinal permeability associated with NAFLD, resulting in activation of these cells (100, 107, 108). Hepatocyte derived hedgehog (Hh) ligands and osteopontin (OPN) were found capable of activating HSCs in NAFLD (109, 110). Another emerging signaling pathway for HSCs activation is the Hippo pathway, which involves the Yes-associated protein (YAP) (89, 111). Among the different stimuli that have been found to activate the HSCs (65, 112), there is the accumulation in these cells of free cholesterol (FC), which was found to lead to an increase of TLR4 expression and to sensitize HSCs to the action of TGFβ (113).

ECM is considered an active biological system, with immunomodulating properties. In fact, it can directly influence the activity of cells participating in the progression of NAFLD. Some components of the ECM include domains that can interact with immune system receptors, having anti- or pro-inflammatory effects. For example, collagen is recognized by the leukocyte associated immune receptor (LAIR)−1, which is expressed by most immune cells and induce a state of immunosuppression, but depending on its expression level and interaction with other molecules (e.g., soluble LAIR-2), it may also lead to pathological states (91, 114). ECM components were also found to directly influence the activity of HSCs through integrins and discoidin domain-containing receptors (DDRs) (100, 115). Moreover, ECM fragments produced during tissue damage, or components actively secreted, can act as DAMPs being recognized by immune system cells through PRRs (91, 116); DAMP-ECM derived responses were found to be mediated primarily by TLR2 and TLR4 (116). ECM components that have been associated with pathological responses include versican (whose mRNA was found to be upregulated in rats with NAFLD, and in biopsies of patients with advanced fibrosis; circulating versican levels were found increased in serum of patients with advanced fibrosis) (117, 118), thrombospondin-1 (TSP-1; in an *in vitro* NAFLD model intracellular lipid accumulation was found associated to TSP mRNA upregulation) (119), cysteine-rich protein 61 (CCN1, which induced hepatic inflammation and injury in a mouse model of NAFLD) (120), lumican (whose hepatic expression was found to be high in patients with progressive NAFLD) (121), and periostin (whose circulating and tissue levels were found to be higher in NAFLD patients than controls) (122, 123). These ECM components can induce the release of pro-inflammatory cytokines, recruitment and activation of immune cells (91). Other components, on the other hand, have shown anti-inflammatory and anti-fibrotic properties (e.g., Extracellular Matrix Protein-1, ECM1, and High Molecular Weight-Hyaluronic Acid, HMW-HA) (91). Studies have shown a link between their genetic depletion and liver fibrosis progression or their immunosuppressive properties, e.g., through the support of the function of regulatory T lymphocytes (91, 124, 125). Moreover, other ECM constituents may have both a pro- or an anti-inflammatory role based on temporal (i.e., stage of the disease) and spatial factors, and depending on the type of receptor or cell from which they are recognized (91). In addition to these effects, ECM is a storage site for cytokines and growth factors (126).

In case of fibrosis and cirrhosis beyond the quantity, also the composition of the ECM is altered (65). In fact, in a healthy liver, the ECM that surrounds the hepatocytes was found to be formed mainly by type IV collagen, laminins, and proteoglycans, while in a liver with fibrosis fibrillar collagen types I and III become prevalent (65, 127). Although in the fibrogenesis process several factors are etiology-independent (127), the progression of fibrosis in chronic liver disease proceeds differently based on the cause. NAFLD, as well as alcoholic steatosis progressing to steatohepatitis, typically has a perisinusoidal (matrix deposition around the sinusoid) and pericellular (around groups of hepatocytes) pattern of fibrogenesis (65, 127).

THE IMMUNE SYSTEM IN THE PROGRESSION OF NAFLD

The immune system plays a key role in hepatic fibrogenesis, as it supports the inflammation that precedes and accompanies the fibrogenic process (128). Recent works have highlighted the link between metabolic dysregulation and activation of the immune system (129–131). As mentioned above, different functions of the immune system are associated with different cellular metabolic activities (e.g., glycolytic vs. oxidative). As hypothesized in a recent review by Cai et al., the altered systemic metabolism that is found in metabolic diseases, characterized by changes in the availability of substrates or presence of specific compounds, could affect the activity of the immune cells by changes in cellular metabolism (132). The recently described *trained immunity* (TI) or *innate immune memory* (long lasting, although less than the adaptive immune system memory, increased responsiveness of cells of the innate system, e.g., monocytes, following secondary stimulations with an exogenous or endogenous insult, due to epigenetic changes and not to permanent genetic rearrangements) (133–135), which challenges the historical assumption that the innate system is devoid of memory, was found to be closely interconnected with cellular metabolism (132). Given the important role of the innate system in the pathogenesis of NAFLD, as already proposed (75), TI could be an interesting subject of study in such a disease.

DAMPs and pathogen-associated molecular patterns (PAMPs) are released following NAFLD-associated damage and dysfunctions, they act as a signal of danger and can start an inflammatory process (136). PAMPs are exogenous danger signals made up of various microbially derived molecules, e.g., lipopolysaccharide (LPS), peptidoglycans, bacterial genetic material, etc., which can reach the liver due to the altered intestinal permeability associated with NAFLD (137). DAMPs correspond to endogenous molecules released by damaged cells, which can act as warning signals. DAMPs is a functional definition, and various molecules, with great diversity, are part of this family (83, 136), including HSPs, sp100 protein, HMGB1, DNA, RNA, etc. DAMPs and PAMPs are recognized by PRRs, which include TLRs, NOD-like receptors (NLRs), retinoic acid inducible gene I (RIG-I) -like receptors (RLRs), and others (132, 136). In this way, they activate cells of the innate immune cells, not purely immune cells, and DAMPs can also regulate adaptive immunity (138, 139). TLR4 (receptor for LPS) (55) and TLR9 (DNA) (140) were found implicated in NAFLD progression; TLR5 (flagellin) was hypothesized to have a protective role in liver disease induced by diet (141); TLR2 (cellular components of Gram-positive bacteria) has instead shown contradictory roles (142).

Some lipid species have also been shown that they can directly activate immune cells: saturated fatty acids (SFAs) were observed to induce COX-2 via TLR4 and NFκB in a macrophage-like cell line (143), as well as the activation of inflammatory responses mediated by macrophages and involving the liver was observed following exposure to peroxidized fatty acids (144) and free cholesterol (130, 145). As already mentioned, adipose tissue dysfunction in NAFLD was proposed as another factor inducing hepatic immune system activation, due to the imbalance in cytokine production (51, 137, 146).

In addition to the innate immunity, which was believed to play a prevalent role, adaptive cells are also greatly involved in NAFLD (147).

As DAMPs can initiate an inflammatory process without the participation of infective agents, they are actors of a sterile inflammation. More precisely, the inflammatory response which occurs in NAFLD is due to metabolic alterations, such as insulin resistance, excess of fat, and lipotoxicity, therefore it can be called "metabolic inflammation." This process is characterized by a chronic low-grade immune activation, which does not resolve (148). This contrasts with an acute insult like microbial infection, in which the immune response is strong, limited in time, and has the purpose of eliminating the pathogen and making the person survive. Prolonged, unresolved, and low-grade inflammation gets no advantage to the host (149), and in NAFLD it causes the onset of scars responsible for liver cirrhosis. Differences in frequency and phenotype of several immune cells were described in NAFLD compared to healthy liver (150). Although the specific role of some of these in NAFLD is far from clear, it is likely that in addition to contributing to inflammation and disease progression, some elements play a protective role, e.g., NK cells through inhibitory cytokines and induction of apoptosis.

Innate Immune System

The innate immunity is capable of very rapid, although not specific, responses and their subset are important players in the pathogenesis of NASH. As mentioned, non-strictly immune cells, such as hepatocytes, also are included in this field. Innate and innate-like cells predominate in the liver and constitute the first line of defense against danger signals.

Macrophages and Monocytes

The liver comprises the largest proportion (80–90%) of resident macrophages in the human body (151). The hepatic macrophages consist of different cell populations including the resident macrophages named Kupffer cells (KC) after their discoverer by Karl Wilhelm von Kupffer (152) and the infiltrating bone marrow derived monocytes (130, 153).

The KCs originate from the yolk sac and act as the dominant liver phagocyte. They localize inside the sinusoids directly in contact with blood circulation (154) and can migrate

through the tissue along sinusoidal walls independently, and in different directions from those of neighboring Kupffer cells (155). The diverse origins of the macrophages reflect the high levels of phenotypical heterogeneity of this cell population (153, 156, 157). Recent studies, using single-cell RNA sequencing, revealed distinct hepatic macrophages with inflammatory and tolerogenic/non-inflammatory phenotypes (158, 159). The different macrophage populations are involved in both hepatic homeostasis and inflammation. KCs promote immune tolerance (160) and play a role in the early response to injury and infection (161), while the infiltrating macrophages are responsible for inflammation and fibrosis progression (153, 162).

Through the polarization process, the macrophages differentiate into subpopulations with specific biological functions. Simplifying, they can be divided into M1 macrophages with pro-inflammatory and antimicrobial activity and M2 with anti-inflammatory and reparative functions (153).

Both KCs and infiltrating monocytes play an essential role in various liver diseases. Several reviews have described their role in liver diseases, such as acute liver failure (163), liver fibrosis (164, 165), non-alcoholic fatty liver disease (130, 157, 159), viral hepatitis (166), and hepatocellular carcinoma (167, 168). Macrophages have been demonstrated to be implicated also in NAFLD development and severity (130, 161). In NAFLD subjects the infiltration of portal macrophages is observed at an early stage before the evidence of inflammation and their activation contributes to disease initiation and progression (169). Another study revealed an increase of activated KCs within the hepatic sinusoids in children with NASH (170). In addition, activated KCs modulate the severity of inflammation in NASH (171).

Alternatively, it was described also an anti-inflammatory role for hepatic macrophages; in fact, activated M2 macrophages can favor liver remodeling and tissue repair in NAFLD and initiate the apoptosis of inflammatory KCs (161). Moreover, NAFLD can increase the risk of development of HCC and tumor associated macrophages secrete inflammatory cytokines and growth factors involved in tumor development and progression. Toll-like receptor (TLR) 4 on macrophages has been shown to contribute to HCC proliferation (167, 172).

As macrophages play a central role in NAFLD, they might be a suitable target for therapies and a biomarker of diseases severity. In the liver, KCs produce the cytokine TNF-α in response to infections; elevated levels of TNF-α in patients without evidence of NAFLD have been demonstrated to be associated with a high risk of fatty liver development (173). Macrophages produce also other proinflammatory cytokines such as IL1 and IL18. Different studies have proven that IL-1α and IL-1β have a significant role in the progression of NAFLD (174, 175). Another cytokine potentially applicable in the diagnosis of NAFLD is IL-18, which is produced by macrophages and KCs. Circulating IL-18 levels correlate with metabolic syndrome (176), but, on the other hand, it has been also demonstrated that IL-18 production negatively regulates NASH progression *via* modulation of the gut microbiota (177).

Another cytokine secreted by KCs is TGF-β; the patients with elevated levels of isoform TGF-β3 show a higher risk of NAFLD development (178). Interestingly the soluble macrophage activation marker CD163 has been reported to correlate with liver injury and demonstrated good predictive ability for advanced fibrosis, which was further increased in combination with the NAFLD fibrosis score (179). However, this marker showed poor associations with liver histology in pediatric NAFLD subjects suggesting a possible different role for macrophages in the pathogenesis of adult and pediatric NAFLD (180). Another study demonstrated that the serum macrophage-derived deaminase ADA2 activity can predict NAFLD and liver fibrosis (181).

Dendritic Cells

Dendritic cells (DCs) have been described as interstitial and non-phagocytic cells. They localize periportally, around central veins and in the liver capsule (157). DCs function as antigen-presenting cells (APCs) recruiting other phagocytic cells to the injury site. DCs play an important role to initiate the immune response by capturing, processing, and presenting the antigens to T cells (182). During homeostasis, DCs display a predominant tolerogenic and immature phenotype. While, in the context of inflammatory state, they maturate and enhance the production of proinflammatory cytokines. Mature DCs activate natural killer T cells and promote T-cell proliferation (183). In NASH mice models hepatic DCs exhibit increment of the production of pro-inflammatory cytokines and chemokines (171). Liver DCs are also implicated in adipogenesis, lipid metabolism and synthesis, and hepatic accumulation (184). Human hepatic DCs are composed of two distinct populations that contain different concentrations of lipid, which regulates immunogenic vs. tolerogenic responses. The increased concentration of toxic lipid plays an important role in the pathogenesis of acute and chronic liver diseases (160, 185).

Neutrophils

Neutrophils are the most abundant group of white blood cells circulating in healthy adults and a key component of the innate response. These cells, which have a limited life span (1–2 days), act by phagocytosis, the release of substances (defensins) contained in their granules including neutrophil elastase (NE), myeloperoxidase (MPO), and lysozyme, the production of reactive oxygen species (ROS), and through the NETs (neutrophil extracellular traps) (186). In addition to microbial invasion, metabolic insults can also induce the recruitment and activation of neutrophils (187). In fact, they are part of the inflammatory infiltrate which characterizes the histology of NAFLD (169), and the extent of the infiltration was found to correlate with the severity of the disease (187). They migrate from the blood circulation to the focus of the inflammation, driven by chemokines and chemotactic agents, which are released creating a gradient within the hepatic compartment (188). Neutrophils are among the first cells to invade the liver in NAFLD, and in this site can attract other immune cells (187, 189). The invasion begins soon after damage, following the release of DAMPs by the damaged hepatocytes (190); furthermore, danger signals derived from the gut also contribute to the recruitment and activation of neutrophils in NAFLD (191). In NASH it was documented a hepatocyte upregulation of the main chemokines that attract

neutrophils (186). Neutrophil-to-lymphocyte ratio (NLR) has been observed to correlate with advanced inflammation and fibrosis in NAFLD patients (192). Moreover, NAFLD patients showed an increase in MPO (193), NETs (194), NE, and PR3 (195) circulating levels. The hepatic concentrations of the latter were associated with advanced stages of the disease (195). MPO showed that it could activate HSCs promoting liver fibrogenesis; its pro-fibrogenic role was also linked to the induction of polarization to M2-macrophages (186, 191). Furthermore, NE showed to be a regulator of insulin signaling, and its deletion improved insulin sensitivity in a mouse model of obesity (196).

Natural Killer Cells

Natural killer cells (NK cells) belong to the innate immune system and act through the production of granules containing perforin and granzymes (197), but they can also play an important role in shaping the adaptive immune response (198). In the liver, they are found within the hepatic sinusoids. These cells can be distinguished into $CD56^{dim}$ NK cells (which represent the most abundant group in peripheral blood) and $CD56^{bright}$ (197). NKG2D is an activating receptor expressed by NK cells, but also by others of the immune system such as T lymphocytes, and is involved in the identification and elimination of damaged cells (199), so acting as a receptor for danger signals. *In vitro* and *in vivo* models showed that NK cells can kill human and mouse HSCs by mechanisms dependent on RAE1, NKG2D, TRAIL, NKp46/NCR1, and p38/PI3K (200–202). In a study of NAFLD patients, NASH ones were found to have higher hepatic levels of NK cells and NKG2D mRNA (203). Furthermore, the NK cells showed different levels of activation based on the levels of fibrosis. $CD56^{dim}$ NK cells circulating levels were found to be high in advanced fibrosis (F3/F4) than in healthy controls, differently from patients with early stages of the disease; moreover, they were found in an inactive state in patients with NAFLD and advanced fibrosis (204). The increased number observed in advanced disease was hypothesized to be a compensatory event to NK cells impairment. For these reasons, NK cells have been linked to a protective role in liver fibrosis.

Innate-Like, "Unconventional," T Lymphocytes

Mucosal-Associated Invariant T (MAIT) cells are currently defined as MR1-Ag restricted cells which have a TCR including Vα7.2 segment paired with Jα33, Jα12, or Jα20; these α-chains associate with a limited repertoire of β-chains. The most studied antigen which MAIT cells recognize by their TCR is a metabolite of riboflavin biosynthesis (205). In healthy people, circulating MAIT cells are 1–10% of total T cells, whereas in the liver they reach up to 45% of intrahepatic T lymphocytes. They are generally CD3+, DN or CD8+, Vα7.2+, CD161+, IL-18Rα+, CD26+, PLZF+ (205). It was observed that in patients with NAFLD related cirrhosis circulating levels of MAIT cells were reduced; in the same study MAIT cells were found to cause proliferation of human hepatic myofibroblasts (HMFs) and release of proinflammatory cytokines by HMFs and macrophages; moreover, CCl4-exposed MAIT cell-deficient mice resulted protected from fibrosis whereas CCl4-exposed MAIT cell-enriched mice showed an increase in fibrosis (compared with WT ones) (206). Another study showed that circulating MAIT cells were reduced and functionally impaired (decreased production of IFN-γ and TNF-α), in NAFLD patients; MAIT cells were increased in the liver of NAFLD patients, and their number was found to positively correlate with the NAS values (NAFLD activity score); *in vitro*, activated MAIT cells induced macrophages differentiation toward M2 phenotype, and MAIT cells-deficient MCD-fed mice showed enhanced liver steatosis and inflammation than WT mice, thus suggesting a protective role for these cells in disease progression (207). Given the conflicting results and the limited availability of studies, the role of MAIT cells in NAFLD appears still unclear.

Natural killer T (NKT) cells are CD1d restricted lymphocytes, which recognize lipid antigens. This definition is due to their expression of both the classic T lymphocyte (CD3) and natural killer cell markers (e.g., CD56) (148). These cells can be divided into two subtypes: invariant NKT (iNKT), or NKT type 1, which possess a semi-invariant TCR-α chain (which in humans includes the Vα24/Jα18 region), and type 2, non-invariant NKT (type 2), with a more variable TCR. They produce cytokines associating with T helper 1 and T helper 2 cells, and also utilize Fas and TNF-a to induce apoptosis, guiding the immune system into tolerance or inflammation (208). Regarding the role of NKTs in NAFLD, contradictory data emerged on their effects on hepatic steatosis, inflammation, and fibrosis. In fact, it was observed that in wild-type mice fed with MCD diets, NKT cells had a profibrogenic role by production of osteopontin (OPN) and hedgehog (Hh) ligands, and by activation of HSCs (209). In another study, reduced steatosis, fibrosis, HSCs activation, and hepatic infiltration of inflammatory cells were observed in iNKT cell–deficient mice on CDAA diet (210). However, improvement in NASH associated with an increase in the intrahepatic population of NKT in leptin-deficient ob/ob mice model (211), increase in liver fat in CD1d −/− (lacking NKT cells) mice following HFD (212), and of liver inflammation and fibrosis in iNKT-lacking, HFD-fed mice (193), were also observed.

γδ-T cells express a TCR formed by γ and δ chains (instead of α and β) and are another T cell population, which can be found in the liver. This group represents 15–25% of all intrahepatic T cells (213) and was predominantly found in portal infiltrates and areas of bile duct proliferation or fibrogenesis (214). These cells recognize non-peptide bacterial antigens, and other ligands, and are IL17A producers (215). γδ-T cells were observed to be increased in the liver of HFD-induced obesity and NAFLD mice; reduced liver damage and steatohepatitis were observed in γδ T cell-deficient mice. Moreover, the gut microbiota showed to support disease progression by γδ-T IL17+ cells (216). In another study on MCD-fed mice, it was observed that γδ-T depletion protects against steatohepatitis, thus demonstrating their pathogenetic role in NAFLD; in this work, however, the progression of the disease appeared IL-17 independent (217). Further studies are needed to clarify the effect of these cells in the progression of NAFLD.

Adaptive Immune System

Adaptive immune cells are recruited by events initiated by innate immunity, but they trigger a more effective, specific response. Although innate immunity has been considered a key player in NAFLD, recent evidence also sheds light on the adaptive system in this condition. After all, NASH is characterized by an intense lymphocytic infiltrate (148), and aggregates of both T and B lymphocytes can be found in NAFLD (218, 219).

CD4+ Helper T Lymphocytes

CD4+ T lymphocytes are further divided into subpopulations based on their functions and cytokines production (220). Among these, there are Th1 cells (proinflammatory cells with a critical role in defense against intracellular pathogens, producing IFN-γ, IL-2, TNFα), Th2 (involved in allergic diseases and response against parasites, producing IL-4, IL-5, IL-13, IL10), Th17 (proinflammatory cells with a defensive role against extracellular bacteria, but also fungi, producing IL17A, IL17F, IL21, IL22, IL23), Th22 (antibacterial functions, producing IL-22), Treg (key elements in the maintenance of self-tolerance, suppressing T-cell activation and releasing IL-10, TGF-β, IL-4) (220). Liver recruitment of CD4+ T lymphocytes was observed in patients and mice models of NASH (221, 222). It was observed that methionine and choline-deficient high-fat (MCDHF) fed, IFN-γ-deficient mice showed less steatosis, inflammation, and fibrosis than WT counterparts. In the same study, it was also observed, *in vitro*, that IFN-γ induced TNF-α production by macrophages in a dose-dependent manner (223). Other studies also suggested a role of Th1 in NAFLD, showing an increase of these cells, Th1 proinflammatory cytokines, or genes toward a Th1 phenotype polarization in patients with NASH (218, 224, 225). In a study of 112 patients with NAFLD (of whom 51 had biopsy-proven NAFL and 30 biopsy-proven NASH) a higher frequency of IFN-γ+ and/or IL-4+ cells was observed in peripheral blood of patients with NAFL and NASH than healthy controls, and a marked increase in intrahepatic IL-17, IL-4, and IFN-γ-producing T cells in NAFLD patients, compared to peripheral blood. In addition, an increase in activation of CD4+ T lymphocytes was documented both in peripheral blood and liver (based on the expression of HLA-DR) (226); Th17 was found to be more abundant in the liver of patients with NASH than in those with NAFL and in circulating blood of NASH patients Th17/Treg ratio was found to be higher than that of NAFL ones. These difference, as well as the histology improved, was found attenuated 1 year after bariatric surgery. Therefore, the authors hypothesized that the balance between Th17 and Treg plays a key role in the pathogenesis of NASH (226). Temporal changes in the frequency of T CD4 lymphocyte populations during NAFLD progression have also been observed: in a study on MCD-fed mice, it was observed an increase in Th17 cells in the first phases of the disease, and in the NASH-fibrosis transition, while Th22 increased between the two Th17 expansions. In the same study, an *in vitro* model of hepatocyte lipotoxicity documented that IL-17 exacerbated, while IL-22 prevented hepatocyte lipotoxicity (221). The pathogenetic role of IL-17 in progression from NAFL to NASH has also been documented in other studies (227), while the role of IL-22 in chronic liver disease is not so clear (228). IL-17 has been shown to be able to stimulate Kupffer cells to produce inflammatory and fibrogenic cytokines (including TGF-β) and to directly stimulate HSCs by promoting their activation and the production of type 1 collagen by STAT3 (229). For these reasons, as stated in a recent review, Th1 and Th17 lymphocytes are generally attributed a pathogenetic role in the progression of NAFLD (218).

CD8+ Cytotoxic T Lymphocytes

These effector cells act by releasing cytokines, cytolytic substances such as perforin and granzymes, and cell-cell contact. Cytotoxic T lymphocytes increase in the liver of people with NAFLD, where they are more activated. Their depletion was observed to be associated with a reduction in steatosis, inflammation, fibrosis, and insulin resistance (148, 220, 230). Furthermore, CD8 + T lymphocytes (as well as NKTs) were found to promote the transition from NASH to HCC (231). Their role in the progression of NAFLD, however, needs to be better investigated with further studies.

B Lymphocytes

B lymphocytes are responsible for various immunological functions, including production of antibodies, antigen presentation, cytokines secretion, and regulation of immune responses. However, their biological function in the liver is still not fully elucidated. Only a small number of B cells are residing in the healthy liver and, maybe since hepatic B cells comprise only ∼5% of intrahepatic lymphocytes, there are experimental difficulties in isolating and analyzing specifically these cells (232). This lymphocytes population has been shown to infiltrate the liver parenchyma of NASH patients. These cells may contribute to the progression of the disease through the production of inflammatory mediators and antigen presentation (218); they showed to exert a profibrogenic role through the release of inflammatory cytokines stimulating HSCs (233). In mouse models of NAFLD, it was observed that B lymphocytes were activated early in the course of the disease and resulted important for recruitment and activation of T lymphocytes (219). Circulating levels of the cytokine BAFF were found to be higher in patients with NASH than in those with simple steatosis, and the higher levels of this cytokine correlated with hepatocyte ballooning and advanced fibrosis (234). In a study in which biopsy-proven NAFL and NASH patients had serum immunoglobulin measurements, it was also observed that IgA levels were elevated more frequently in NASH patients compared to those with simple steatosis (235).

THERAPEUTIC APPROACHES ACTING ON THE IMMUNE SYSTEM TO COUNTER THE PROGRESSION OF NAFLD

Several drugs have been studied to reduce liver inflammation and fibrogenesis in NAFLD, resolution of steatohepatitis and improvement in liver fibrosis representing two key endpoints of current trials (236). Moreover, it is also being studied the effect of the combination of molecules acting on different targets. However, despite the advances in knowledge of the fibrogenic

process leading to cirrhosis, to date there are no approved and specific pharmacotherapy to resolve NASH, and targeting the predisposing factors (by lifestyle modifications and weight loss) is considered the best therapeutic option (237). The regression of fibrosis is already obtainable in some conditions, such as in chronic viral hepatitis after antiviral therapy, or for obese NAFLD patients, following bariatric surgery (65, 238). Given the key role of the immune system in the progression of NAFLD, therapeutic approaches aimed at counteracting its harmful role in pathogenesis have also been tested (239). Although many extensively examined or new molecules under study for NAFLD not acting directly on the immune system cells, for example having a primary antioxidant effect (e.g., vitamin E) (240), acting on bile acid metabolism [e.g., OCA, which is the most advanced molecule in the race for drug approval to treat NASH (236)] or on glucose or lipid metabolism (e.g., Elafibranor), or having other primary targets, spill over their action to the immune system (86), below they will be summarized only approaches directly engaging the immune substrate of NAFLD (a list is provided in **Table 1**).

Cenicriviroc (CVC) is a C-C chemokine receptor type 2 and 5 (CCR2 and CCR5) antagonist, expressed mainly on monocytes the former, and on various immune system cells (including lymphocytes) and HSCs the latter. Following the recognition of their ligands, these receptors participate in the recruitment and activation of various immune cells, which were linked to amplification and perpetuation of the inflammatory response in NAFLD (241). Therefore, the rationale for the use of CVC was a reduced migration and hepatic infiltration of monocytes/macrophages (due to the blockade of CCR2), and a reduced migration and activation of HSCs (due to the parallel inhibition of CCR5). Preclinical studies have shown its effectiveness in reducing liver fibrosis (241). In a study (CENTAUR trial) involving 289 subjects with NASH and hepatic fibrosis in which 145 received CVC and 144 placebo, it was observed that it was safe and well-tolerated, but the primary outcome of improvement in NAS by $\geq$2 points without worsening of fibrosis after 1 year, was not met. However, this drug improved liver fibrosis in a significantly higher percentage of cases than placebo (20 vs. 10%) (242). After 2 years of treatment, most people who achieved improvement in fibrosis maintained this result (243). It was being tested in a randomized, double-blind, placebo-controlled phase 3 trial (AURORA) to evaluate its efficacy in the treatment of liver fibrosis in adults with NASH (244), but this study was stopped early due to lack of efficacy (245).

Galectin inhibitors are a class of compounds that interfere with galectins. The latter are carbohydrate-binding proteins that are located inside the cells, in the cytoplasm, in states of quiescence, but can be externalized. In fact, in case of tissue damage, the cytosolic galectins are actively secreted by the cells, and act as DAMP. The main galectin produced during damage is Galectin-3 (Gal-3), which is primarily produced by macrophages (246). It is involved in several inflammatory processes, including the adhesion of neutrophils, opsonization, and macrophages chemoattraction (247, 248). Moreover, Gal-3 was found to lead to myofibroblast activation (249), and was linked to the fibrogenesis process in different liver diseases (246). Galectin-3 inhibitors resulted effective in preclinical studies of NASH and liver fibrosis (250). Among the galectin inhibitors, there is belapectin (GR-MD-02), a natural plant derived molecule that binds to Gal-3 (but also to galectin-1). In a phase 1 study, GR-MD-02 was shown to be safe and well-tolerated in patients with NASH or advanced fibrosis proven by biopsy (251). Therefore, its efficacy was studied in a randomized placebo-controlled trial in patients with liver cirrhosis and portal hypertension; 162 participants were randomized to receive belapectin, 2 or 8 mg/kg, or placebo, but neither dose was found to reach the primary endpoint (HVPG reduction), nor improve liver fibrosis, or reduce the incidence of complications of cirrhosis. However, this drug showed to be associated with an improvement in hepatocyte ballooning. It was also observed that belapectin could have a favorable effect on HVPG and the development of varices in a specific group of patients (NASH-cirrhosis without varices at baseline) (246). A study to evaluate the safety and efficacy of belapectin vs. placebo for the prevention of esophageal varices in patients affected by NASH cirrhosis with signs of portal hypertension but without esophageal varices (NAVIGATE) is currently ongoing (252).

Hepatic macrophages are an interesting target for novel therapeutic approaches for liver diseases. However, there are some important challenges to be faced, like the quite opposing functions of macrophage subsets depending on the experimental condition observed in the animal models, the not complete comparability between animal and human diseases, and the complex human macrophages heterogeneity. However, the increasing understanding about macrophages allowed the identification of several pathways that regulate their recruitment, differentiation/polarization and activation, offering promising starting points for novel therapeutic intervention. Different approaches include inhibition of KCs activation, dampening of monocyte recruitment into the liver, and modulation of macrophage polarization/differentiation. KCs activation can be influenced by several approaches. Using antibiotics, it is possible to reduce the bacterial infection and the consequent TLR4-dependent macrophage activation, ameliorating steatohepatitis, fibrosis, and hepatocarcinogenesis in mice models (253, 254). Antibiotics act influencing the gut barrier and microbiota. Also the probiotics could potentially alleviate pathogenic Kupffer cell activation in the liver (255). Probiotics have several beneficial properties, including interaction with the enterohepatic axis. It has been shown that the use of preparations containing different strains of bacteria and a probiotic in patients with NAFLD is associated with a significant reduction of hs-CRP, TNF-α, and TNFκ-B p65 (256). Beneficial effects of other multiprobiotic compounds have been observed in patients with NAFLD (257). Inflammatory monocytes recruitment to the liver is driven by chemokines. Therefore, different pharmacological strategies have been generated to interfere with chemokine signaling, including monoclonal antibodies, receptor antagonists, inhibition of chemokines (258); an example of this type of pharmacological approach is the aforementioned cenicriviroc. KCs have a high scavenging capacity, which can be used for drug delivery. In fact, dexamethasone has

TABLE 1 | Summary of the drugs recently studied for NAFLD therapy which have a mechanism of action that involves immune system modulation.

Drug name (study reference)	Drug type	Mechanism of action	Expected effect	Administration route	Experimental stage reached	Efficacy	Future perspectives
Cenicriviroc (245)	C-C chemokine receptor type 2 and 5 antagonist	Reduction of migration of monocytes/macrophages, reduction of HSCs activation	Antinflammatory, antifibrotic	Daily oral route	Phase-3 double blind RCT	Stopped for lack of efficacy	Not approved in monotherapy, association with Tropifexor ongoing
Belapectin (GR-MD-02) (252)	Galectin inhibitor	Reduction of galectin secretion with reduction of neutrophils adhesion, opsonization, macrophage chemoattraction, myofibroblast activation	Antinflammatory, antifibrotic, portal hypertension reduction	Intravenously	Phase-2b double blind RCT	Only efficacious in reducing HVPG in pts without esophageal varices at baseline	Phase 2b/3 trial on the efficacy on preventing varices in NASH cirrhosis pts without varices ongoing
Protexin capsules (256)	Synbiotic supplement (prebiotic and probiotic)	Attenuation of inflammatory responses	Antinflammatory, antifibrotic	Daily oral route	Double blind RCT	Improved liver biochemistry, reduced transient elastography score	Available for clinical use, effects of longer treatment durations remain to be determined
Symbiter (257)	Multi-probiotic	Reduction of the inflammatory response and hepatic triglycerides content	Antisteatosic, antinflammatory, antifibrotic	Daily oral route	Double blind RCT	Reduced liver fat, AST, GGT, TNF-α, and IL-6 in NAFLD patients	Available for clinical use, long-term studies required
JKB-121 (264)	TLR-4 antagonist	Reduction of TLR-4 mediated liver inflammation and fibrosis	Antinflammatory, antifibrotic	Twice daily	Phase 2b RCT	JKB-121 did not perform better than placebo in improving liver fat content and/or serum ALT in NASH patients	Further studies on the inhibition of TLR-4 are needed
GPR84 Antagonist (266)	GPR84 antagonist	Inhibition of inflammatory responses GPR84 mediated	Antinflammatory, antifibrotic	Orally administered	Preclinical (mouse) NAFLD model	Reduced macrophages and neutrophil infiltration, ameliorated steatohepatitis	Further studies needed
BI 1467335 (271)	VAP-1 inhibitor	Reduction of hepatic accumulation of inflammatory cells	Antinflammatory, antifibrotic	Oral tablets	Phase 2 RCT	Improved NASH biomarkers	Development discontinued (risk of drug interactions)
Sandy-2 (219)	B-cell Activating Factor (BAFF) -neutralizing monoclonal antibody	Prevention of B cells maturation	Antinflammatory, antifibrotic	I.p. injection	Preclinical (mouse) NASH model	Prevented hepatic B cell maturation, reduced Th-1 lymphocytes activation, ameliorated steatohepatitis	Further studies needed
OKT3 Mab (274)	Anti-CD3 monoclonal antibody	Immunomodulatory effect, induction of regulatory T cells (Tregs)	Antinflammatory, antifibrotic	Oral once daily	Phase 2a RCT	Improved liver, metabolic, and immunologic parameters	Further trials are needed

been demonstrated to reduce fibrosis in mice models through a macrophage-targeted delivery (259, 260). A fascinating alternative to treat liver disease in a murine model is the infusion of KCs expanded *in vitro* to ameliorate liver fibrosis (261). Moreover, macrophages can be isolated from apheresis derived CD14 monocytes of cirrhotic patients and differentiated into macrophages with a pro-resolution phenotype (262, 263).

JKB-121 is an antagonist of TLR-4, which was linked to liver inflammation and fibrosis. Encouraging results derived from preclinical studies on the antagonism of TLR4, but, in a trial on patients with biopsy-proven NASH, grade 1-3 fibrosis, and hypertransaminasemia JKB-121 did not reach the endpoint of reducing the liver fat content by MRI-PDFF and/or serum ALT after 24 weeks (264).

GRI0621, a natural killer T (NKT) cells antagonist, has been investigated in a study on patients with chronic liver disease including NASH to test its effects, but the study was discontinued for administrative decision (265).

G protein-coupled receptor 84 (GPR84) is a surface receptor for medium-chain fatty acids (MCFA). This receptor is expressed by several cells of the innate immune system and showed proinflammatory functions (266). In GPR84-deficient mice, LCFA diet did not cause an increase in liver mass as was observed in WT counterparts (267). In a recent study, it was observed that GPR84 expression was increased in the liver of mice and humans with NAFLD and was associated with inflammation and fibrosis; GPR84 antagonists were found to reduce chemotaxis of monocytes and neutrophils. Moreover, these molecules showed to reduce macrophages accumulation and to improve inflammation and fibrosis in mouse models of NASH. The therapeutic effects in ameliorating steatohepatitis and fibrosis of GPR84 antagonists were linked to the inhibition of the migration of myeloid cells, and not to effects on HSCs, which were not found to express GPR84 (266). Further studies are needed to validate the effectiveness of targeting this system.

Vascular adhesion protein-1 (VAP-1) is a glycoprotein, which has amine oxidase activity and is involved in endothelial adhesion and transmigration processes of leukocytes (268). There is also a soluble form of VAP-1 (sVAP-1), whose levels were found to be elevated in patients with cardiovascular, metabolic (e.g., diabetes and obesity) and hepatic diseases (269). In the liver, it was found involved in the adhesion and transendothelial migration (through the sinusoids) of lymphocytes. It has been observed that sVAP-1 is increased in NAFLD patients and that VAP-1 hepatic expression is increased in patients affected by steatohepatitis compared to those with simple steatosis (269). Moreover, in mouse models of liver damage, inhibition of VAP-1 (by VAP-1–deficient mice or VAP-1 neutralizing antibodies) reduced hepatic migration of inflammatory cells (T cells, NKT cells, and myeloid cells) and attenuated fibrosis (269). Results of another study suggested that VAP-1 may contribute to the progression of NAFLD (270). Hence it has been proposed as a target to limit the progression of NAFLD. A phase II trial was started to document the effects of inhibiting VAP-1 (BI 1467335) in patients with NASH (271); however, the research company announced that it has stopped developing this molecule in NASH due to the risk of drug interactions[1].

The role of B lymphocytes in the progression of NAFLD has been documented (218). Furthermore, the cytokine B-cell Activating Factor (BAFF), necessary for survival and maturation of B lymphocytes, has also been studied in patients with NAFLD. Circulating BAFF levels were found to be higher in patients with steatohepatitis than in those with NAFL (234). BAFF neutralization through BAFF-neutralizing monoclonal antibody Sandy-2 was shown to improve steatosis, inflammation, and fibrosis in transgenic (NASH model) mice overexpressing a soluble form of a BAFF/APRIL receptor (TACI-Ig) (219).

CD3 molecule is associated with the TCR receptor, and this complex is found on the surface of T lymphocytes. Unlike TCR, CD3 is not variable. Muromonab (OKT3) has been the first approved monoclonal antibody and was used to treat organ transplant rejection, but its application is limited by high toxicity. Hence, humanized anti-CD3 antibodies were developed to improve tolerability (272). In a preclinical study on ob/ob mice, anti-CD3 mAb showed to reduce liver fat, adipose tissue inflammation, and blood glucose (273). OKT3 was tested at different dosages (0.2, 1.0, 5.0 mg/day) in a phase II trial in patients with biopsy-proven NASH to determine its effects. This drug, administered for 30 days, was well-tolerated and led to the induction of regulatory T lymphocytes (274).

CONCLUSIONS

Inflammation and fibrogenesis in NAFLD are multifactorial processes involving a multitude of interrelated mechanisms, and in which the immune system plays a key role. Although challenges about its pathogenesis still exist, the knowledge on NAFLD is increasing, leading, also for this reason, to the recent proposal of rename. Therapies aimed at directly fighting the immune substrate of NAFLD are already being studied. In any case, the precise characterization of some elements of the immune system has only occurred in recent years, and the specific role of many subsets in the pathogenesis of NAFLD, as well as that of many other human diseases, is still far from clear. Furthermore, the relationship between cellular metabolism and immune cell functions, termed "immunometabolism," is a candidate for future studies in the field of NAFLD. This knowledge could allow scientists to further elucidate the pathophysiology of this complex disease and to hypothesize new therapeutic approaches.

AUTHOR CONTRIBUTIONS

PT and BMM wrote the paper. All authors participated in revising the manuscript.

[1]Available online at: https://www.boehringer-ingelheim.us/press-release/boehringer-ingelheim-discontinues-development-bi-1467335-nash.

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Hepatic Unsaturated Fatty Acids Are Linked to Lower Degree of Fibrosis in Non-alcoholic Fatty Liver Disease

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Background: The hepatic lipidome of patients with early stages of non-alcoholic fatty liver disease (NAFLD) has been fairly well-explored. However, studies on more progressive forms of NAFLD, i.e., liver fibrosis, are limited.

Materials and methods: Liver fatty acids were determined in cholesteryl esters (CE), phospholipids (PL), and triacylglycerols (TAG) by gas chromatography. Cross-sectional associations between fatty acids and biopsy-proven NAFLD fibrosis ($n = 60$) were assessed using multivariable logistic regression models. Stages of fibrosis were dichotomized into none-mild (F0–1) or significant fibrosis (F2–4). Models were adjusted for body-mass index (BMI), age and patatin-like phospholipase domain-containing protein 3 (PNPLA3 rs738409) (I148M) genotype. A secondary analysis examined whether associations from the primary analysis could be confirmed in the corresponding plasma lipid fractions.

Results: PL behenic acid (22:0) was directly associated [OR (95% CI): 1.86 (1.00, 3.45)] whereas PL docosahexaenoic acid (22:6n-3) [OR (95% CI): 0.45 (0.23, 0.89)], TAG oleic acid (18:1n-9) [OR (95% CI): 0.52 (0.28, 0.95)] and 18:1n-9 and vaccenic acid (18:1n-7) (18:1) [OR (95% CI): 0.52 (0.28, 0.96)] were inversely associated with liver fibrosis. In plasma, TAG 18:1n-9 [OR (95% CI): 0.55 (0.31, 0.99)], TAG 18:1 [OR (95% CI): 0.54 (0.30, 0.97)] and PL 22:0 [OR (95% CI): 0.46 (0.25, 0.86)] were inversely associated with liver fibrosis.

Conclusion: Higher TAG 18:1n-9 levels were linked to lower fibrosis in both liver and plasma, possibly reflecting an altered fatty acid metabolism. Whether PL 22:6n-3 has a protective role, together with a potentially adverse effect of hepatic 22:0, on liver fibrosis warrants large-scale studies.

Keywords: lipids, biomarkers, fatty acids, fibrosis, NAFLD

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects more than 25% of the world's population (1). Its wide clinical spectrum encompasses simple isolated steatosis, non-alcoholic steatohepatitis (NASH) with varying degree of fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). NAFLD is closely associated with the incidence of type-2 diabetes mellitus, cardiovascular disease, liver-related morbidity and all-cause mortality and is currently the second leading cause of end-stage liver disease (2). Large observational studies have indicated that a higher stage of fibrosis, ranging from significant fibrosis (stage 2) to cirrhosis (stage 4), is the strongest histological predictor of liver-related and all-cause mortality in NAFLD (3, 4). It is thus important to enhance our understanding of the underlying pathophysiology of fibrotic scarring in patients with NAFLD.

Fatty acids and other lipid species have been shown to be implicated in human NAFLD development, and more specifically in the early stages of liver fat accumulation (5–10). Rosqvist et al. showed that a hypercaloric diet rich in saturated fatty acids (SFA) resulted in a marked increase in intrahepatic fat in comparison to a diet rich in polyunsaturated fatty acids (PUFA) (7, 8). The SFA-rich diet also increased circulating ceramides, lipid molecules that may trigger fibrogenesis by activating lipogenic and pro-inflammatory pathways and hepatic stellate cells (HSC) (5). However, the role for dietary fatty acids and/or endogenously synthesized fatty acids in NAFLD fibrogenesis remains unclear. Although several cross-sectional studies have investigated associations between plasma and hepatic fatty acids and the prevalence of NASH (6, 11–16), studies on liver-derived fatty acids and biopsy-proven fibrosis in patients with NAFLD are limited. Furthermore, as liver biopsies are difficult and time-consuming to obtain, prone to sampling variability and may put patients at risk of complications, it is important to find reliable and reproducible non-invasive biomarkers (e.g., circulating fatty acids) of liver fibrosis. Such biomarkers, either alone or as complements to existing scores, may add important value in diagnosing NAFLD fibrosis and for monitoring disease progression and treatment response.

The primary aim of this study was therefore to investigate associations between liver fatty acids measured in three different lipid fractions and biopsy-proven liver fibrosis in patients with NAFLD. A secondary aim was to examine whether associations between liver fatty acids and fibrosis could be confirmed in plasma-derived fatty acids.

MATERIALS AND METHODS

The current analysis is part of the AM-02 NASH study, a cross-sectional study with the main objective to evaluate the ability of non-invasive imaging biomarkers to discriminate between NASH and NAFL for the purpose to use in future clinical trials of NASH therapeutics. Subjects were recruited from the Departments of Gastroenterology and Hepatology and from the Swedish CArdioPulmonary BioImage Study "SCAPIS" (17). A total of $n = 134$ individuals were screened for eligibility. Inclusion criteria included a signed informed consent, an age between 18 and 70, clinically suspected NAFLD and at least one of the following: imaging indicative of NAFLD, alanine aminotransferase (ALT) more than 1.5 × upper limit of normal, caspase-cleaved fragment of cytokeratin 18 (CK18 M30) concentration >180 U/L and/or a biopsy showing NAFLD within 3 months prior to screening visit. Exclusion criteria included clinical or histological evidence of alcoholic liver disease, regular and excessive use of alcohol (>30 grams/day for men and >20 grams/day for women), drug addiction, history of any other liver disease, treatment with corticosteroids or immunosuppressive therapy within 10 weeks before screening visit, human immunodeficiency virus (HIV) infection, standard exclusion criteria for magnetic resonance imaging (MRI) (BMI > 40, claustrophobia, metal in body, renal insufficiency) and liver biopsy (any bleeding disorder or medication with anticoagulants) and pregnant and/or breastfeeding women. A total of $n = 68$ subjects ($n = 15$ with NAFL and $n = 53$ with NASH) were eligible for continuation in the study. From these, a sample size of $n = 60$ from those subjects who provided liver tissue for fatty acid composition analysis was included in the current analysis of the AM-02 NASH study. A complete flow-chart of the study is depicted in **Supplementary Figure 1**. The study was approved by the Swedish Ethical Review Authority.

Liver and Plasma Fatty Acids (Exposure)

Hepatic and plasma fatty acids were analyzed using gas chromatography (Agilent Technologies system, 7890B), as described previously (18). A total of 14 fatty acids in both cholesteryl esters (CE) and triacylglycerols (TAG) and 19 fatty acids in phospholipids (PL) were analyzed. For each lipid fraction, five fatty acid ratios reflecting the activity of desaturase and elongase enzymes were analyzed: stearoyl-coA desaturase 1 index (SCD-1) [palmitoleic acid (16:1n-7)/palmitic acid (16:0)], Δ5 desaturase (D5D) [arachidonic acid (AA) (20:4n-6)/dihomo-γ-linolenic acid (20:3n-6)], Δ6 desaturase (D6D) [γ-linolenic acid (18:3n-6)/linoleic acid (LA) (18:2n-6)], stearic acid (18:0)/palmitic acid (16:0) and AA/LA (20:4n-6/18:2n-6). In addition, for each lipid fraction individual fatty acids were pooled into five separate fatty acid classes: total SFA, total monounsaturated fatty acids (MUFA), total PUFA, total n-3 PUFA, and total n-6 PUFA.

Liver Fibrosis (Outcome)

The main outcome measure of this analysis was liver fibrosis. After a fast of at least 6 h, the study subjects underwent an ultrasound-guided liver biopsy. Liver biopsies were assessed by two experienced liver pathologists individually blinded to clinical, radiology and biomarker data using the steatosis, activity and fibrosis (SAF) histological scoring system (19). If the score differed between the two pathologists, the sample was re-evaluated in consensus. The stage of fibrosis was assessed as F0 (none), F1 (1a or 1b perisinusoidal zone 3 or 1c portal fibrosis), F2 (perisinusoidal and periportal fibrosis without bridging), F3 (bridging fibrosis) and F4 (cirrhosis). For the purpose of this study, stages F0 and F1 [none to mild fibrosis (F0–1)] as well as F2, F3, and F4 [significant fibrosis (F2–4)] were combined into two separate groups.

BMI, Age, and PNPLA3 (I148M) (Covariates)

Potential confounders were chosen based on the background literature and a directed acyclic graph (DAG). The DAG was constructed in Dagitty and can be found in **Supplementary Figure 2** (20). Sex, age, body mass index (BMI), patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 (I148M, C/G) genotype, transmembrane 6 superfamily member 2 (TM6SF2) rs58542926 (E167K, C/T) genotype and diet were identified as potential confounders. However, due to the small sample size of $n = 60$ and the risk of overfitting the logistic regression model, only the most relevant confounders were included in the final model. Included covariates were: BMI (continuous), age (continuous) and PNPLA3 rs738409 (I148M, C/G) genotype (categorical). BMI was calculated as the weight (kg) divided by the height (m) squared. Age was captured at the first screening visit, together with other demographics using a self-administered questionnaire. PNPLA3 rs738409 (I148M) was genotyped after DNA extraction from blood samples collected at visit 3 using the TaqMan® PCR method, according to manufacturer's instructions. Genotypes were subsequently dichotomized into CC or CG/GG groups. Sex was excluded based on conflicting findings regarding its association with liver fibrosis (20). TM6SF2 rs58542926 (E167K, C/T) was excluded based on the lack of studies examining the association of this single nucleotide polymorphism (SNP) on hepatic fatty acid composition. Lastly, diet was excluded due to missing information on this variable.

Liver Fat, NASH and Clinical Laboratory Measures

Liver fat was assessed by MRI (Achieva, Philips Healthcare, Best, Netherlands) after 6 h of fasting, as described previously (21). Histological grading of steatosis, lobular inflammation and hepatocyte ballooning was assessed based on the SAF histological scoring system (19). Stages of steatosis were classified as 0 (<5% of hepatocytes with large or medium-sized lipid droplets), 1 (5–33% of hepatocytes with large or medium-sized lipid droplets), 2 (34–66% of hepatocytes with large or medium-sized lipid droplets) and 3 (>67% of hepatocytes with large or medium-sized lipid droplets). Ballooning was categorized as 0 (normal hepatocytes with cuboidal shape and pink eosinophilic cytoplasm), 1 (presence of clusters of hepatocytes with a rounded shape and pale cytoplasm usually reticulated) and 2 (same as 1 with some enlarged hepatocytes, at least 2-fold that of normal cells). Lobular inflammation was defined as a focus of two or more inflammatory cells within the lobule, where foci were counted at 20x magnification (0: none; 1: ≤2 foci per 20x; 2: >2 foci per 20x). NASH was defined when at least one point was given to both lobular inflammation and ballooning in the presence of steatosis (at least one point). Clinical variables from fasting blood samples collected at the same clinic visit as the liver biopsy (**Supplementary Figure 1**), were assessed by standard laboratory techniques at Uppsala University Hospital.

Statistical Analysis

The primary analysis of this study was to examine associations between liver-derived fatty acids and NAFLD fibrosis. A secondary analysis was to examine whether potential associations between liver-derived fatty acids and fibrosis could be confirmed in plasma fatty acids. Both of these analyses were decided upon *a priori*. Further *post-hoc* analyses included (1) pooling individual liver and plasma fatty acids into their respective fatty acid classes and examine their associations with liver fibrosis (2) performing principal component analyses (PCA) for both liver and plasma fatty acids and model the first principal component (PC1) for each lipid fraction with liver fibrosis in multivariable logistic regression analyses. Normal distribution was assessed using the Shapiro-Wilk W test and when appropriate, skewed distributed continuous variables ($W < 0.95$) were logarithmically transformed before analysis or analyzed non-parametrically. Two-sample *t*-tests or non-parametric Mann-Whitney U tests were performed for between-group comparisons (F0–1 vs. F2–4) in population characteristics and liver fatty acid composition. Homogeneity of variance was assessed through visual inspection of the standard deviations or by box-plots. Spearman rank correlations were performed between liver- and plasma-derived fatty acids.

Multivariable logistic regression analyses, adjusted for age, BMI and PNPLA3 (I148M) genotype, were performed for each liver-derived fatty acid, fatty acid ratio and pooled fatty acids in the three lipid fractions (CE, PL, TAG) and the prevalence of significant fibrosis (F2–4) (dichotomized outcome). BMI, age and all fatty acids were treated as continuous variables whereas the PNPLA3 (I148M) genotype was treated as a categorical nominal variable (CC vs. CG/GG). Odds ratios with 95% confidence intervals were calculated from the multivariable logistic regression models for each standard deviation change in fatty acid proportion. Further *post-hoc* sensitivity analyses including both sex (categorical) and TM6SF2 (E167K) (dichotomized into CC or CT/TT) in addition to the *a priori* determined confounders; BMI, age and PNPLA3 (I148M) were performed for those hepatic fatty acids that were statistically significantly associated with liver fibrosis in the primary analysis and for all plasma fatty acids in the secondary analysis. Multicollinearity was inspected using correlation matrices between covariates in the logistic regression model and a correlation coefficient of 0.8 or more was predetermined to be indicative of multicollinearity. None of the covariates in the model had any missing data, hence imputation was not needed.

No correction for multiple hypothesis testing was applied due to the exploratory nature of this study. All statistical analyses were performed using JMP software version 15.1.0 (SAS Institute, Inc) and a $P < 0.05$ was set as the significance level.

RESULTS

Population characteristics are shown in **Table 1**. The mean ages of F0–1 ($n = 36$) and F2–4 ($n = 24$) were 58.5 and 57.0 years, respectively. The proportions of males and females were

TABLE 1 | Population characteristics[a].

	F0–1 ($n = 36$)	F2–4 ($n = 24$)	*P*-value
Age (years)	58.5 (53.5–63.0)	57.0 (42.8–66.5)	0.95*
Sex [*n* (%) male/female]	21 (58)/15 (42)	15 (63)/9 (38)	0.75
BMI (kg/m^2)	30.3 ± 3.8	30.8 ± 3.0	0.57
Type-2 diabetes [*n* (%)]**	13 (43)	10 (45)	0.88
Hypertension [*n* (%)]**	17 (57)	13 (59)	0.86
Hyperlipidaemia [*n* (%)]**	8 (27)	4 (18)	0.47
Platelets (10^9/L)	232.6 ± 53.5	235.0 ± 61.6	0.87
Albumin (g/L)	39.1 ± 2.9	40.3 ± 2.9	0.12
Liver fat (%)	14.7 (8.8–24.1)	17.9 (11.2–20.4)	0.32
SAF steatosis [*n* (%) 1/2/3]	14 (39)/11 (31)/11 (31)	6 (25)/11 (46)/7 (29)	0.41
PNPLA3 (I148M) [*n* (%) CC/(CG/GG)]	16 (44)/20 (56)	11 (46)/13 (54)	0.92
NASH [*n* (%)]	25 (69)	21 (88)	0.11
SAF ballooning [*n* (%) 0/1/2]	3 (8)/30 (83)/3 (8)	0/20 (83)/4 (17)	0.32
SAF lobular inflammation [*n* (%) 0/1/2]	10 (28)/26 (72)/0	3 (13)/20 (83)/1 (4)	0.16
Fibrosis stage [*n* (%) 0/1/2/3/4]	4 (11)/32 (89)/0/0/0	0/0/19 (79)/3 (13)/2 (8)	

[a]*Data are presented as mean ± SD, % or as median (IQR) for skewed distributed variables. BMI, Body mass index; F0–1, Fibrosis stages 0–1; F2–4, Fibrosis stages 2–4; NASH, Non-alcoholic steatohepatitis; PNPLA3, Patatin-like phospholipase domain-containing protein 3; SAF, steatosis, activity and fibrosis.*

SAF ballooning categories 0–1 and SAF lobular inflammation categories 1–2 were combined into one category to satisfy the assumptions of the Chi-2 test.

**Analyzed using a non-parametric Mann-Whitney U test. **n = 52.*

similar between the groups (58% males in F0–1 and 63% males in F2–4). There were no statistically significant differences in any of the clinical (i.e., platelets and albumin) or histological variables (i.e., liver fat, NASH prevalence, SAF ballooning and lobular inflammation) related to NAFLD severity between F0–1 subjects and F2–4 subjects, except for the degree of liver fibrosis. Mean BMI was 30.3 kg/m^2 and 30.8 kg/m^2 for F0–1 and F2–4, respectively. Prevalence of type-2 diabetes, hypertension and hyperlipidaemia was 43, 57, and 27% for F0–1 and 45, 59, and 18% for F2–4. The distribution of the PNPLA3 genotype [CC/(CC/CG)] was 44/56% for F0–1 and 46/54% for F2–4.

Liver Fatty Acid Proportions Between F0–1 and F2–4

Liver fatty acids and fatty acid ratios were analyzed in three lipid fractions (CE, PL, TAG) and were subsequently compared between subjects with F0–1 and F2–4. There were no statistically significant differences in proportions of liver CE fatty acids between F0–1 and F2–4. However, proportional differences were observed for PL docosahexaenoic acid (22:6n-3) (F0–1: 7.92 (SEM 0.25), F2–4: 6.97 (SEM 0.37), $P = 0.04$) and TAG oleic acid (18:1n-9) [F0–1: 46.13 (SEM 0.43), F2–4: 44.61 (SEM 0.61), $P = 0.047$] (**Figure 1**).

Associations Between Liver Fatty Acids and Liver Fibrosis

In multivariable logistic regression analyses adjusted for BMI, age and PNPLA3 (I148M) genotype, PL behenic acid (22:0) (OR: 1.86, 95% CI: 1.0, 3.45, $P < 0.05$) was directly associated with liver fibrosis whereas PL 22:6n-3 (OR: 0.45, 95% CI: 0.23, 0.89, $P = 0.02$), TAG 18:1n-9 (OR: 0.52, 95% CI: 0.28, 0.95, $P = 0.03$) and TAG 18:1n-9 combined with vaccenic acid (18:1n-7) (18:1) were inversely associated with liver fibrosis. Pooling individual fatty acids into their respective fatty acid classes demonstrated a positive association between total PL SFA and liver fibrosis (OR: 2.13, 95% CI: 1.10, 4.12, $P = 0.03$) and inverse associations between total PL PUFA (OR: 0.39, 95% CI: 0.20, 0.76, $P = 0.006$), total TAG MUFA (OR: 0.52, 95% CI: 0.28, 0.96, $P = 0.04$) and liver fibrosis (**Figure 2**).

Associations Between Plasma Fatty Acids and Liver Fibrosis

The corresponding plasma fatty acids to those liver-derived fatty acids that were associated with liver fibrosis in **Figure 2**, were further included in multivariable logistic regression models, adjusted for BMI, age and PNPLA3 (I148M) genotype. Both TAG 18:1n-9 (OR: 0.55, 95% CI: 0.31, 0.99, $P = 0.048$) and TAG 18:1 (OR: 0.54, 95% CI: 0.30, 0.97, $P = 0.04$) demonstrated similar associations with liver fibrosis in plasma as for in the liver. Plasma TAG 18:1n-9 correlated strongly with liver TAG 18:1n-9 (Spearman rho = 0.73, $P < 0.0001$). Interestingly, PL 22:0 showed the opposite relationship with liver fibrosis in plasma as for in the liver (OR: 0.46, 95% CI: 0.25, 0.86, $P = 0.02$). Plasma PL 22:0 was not correlated with liver PL 22:0 (Spearman rho = 0.07, $P = 0.58$). No association was observed for PL 22:6n-3. Total plasma TAG MUFA was inversely associated with liver fibrosis in *post-hoc* analyses (OR: 0.50, 95% CI: 0.27, 0.93, $P = 0.03$). No associations were observed for plasma PL PUFA or plasma PL SFA and liver fibrosis (**Figure 3**).

Principal Components of Fatty Acids in Plasma and Liver

Although a trend was observed between PC1 in plasma PL and liver fibrosis in multivariable logistic regression analyses (adjusted for BMI, age and PNPLA3 (I148M) genotype) (OR: 1.18, 95% CI: 0.95, 1.45, $P = 0.13$), no further associations were observed (data not shown). PC1 was characterized by lower proportions of pentadecanoic acid (15:0), heptadecanoic acid (17:0) and very long-chain SFA and higher proportions of 16:0, 16:1n-7, 18:1n-9, 18:1, 18:3n-6, D5D and SCD-1.

Post-hoc Sensitivity Analyses

None of the associations between liver or plasma fatty acids and liver fibrosis were markedly attenuated when including sex and TM6SF2 (E167K) as additional confounders in the logistic regression models (**Supplementary Table 1**).

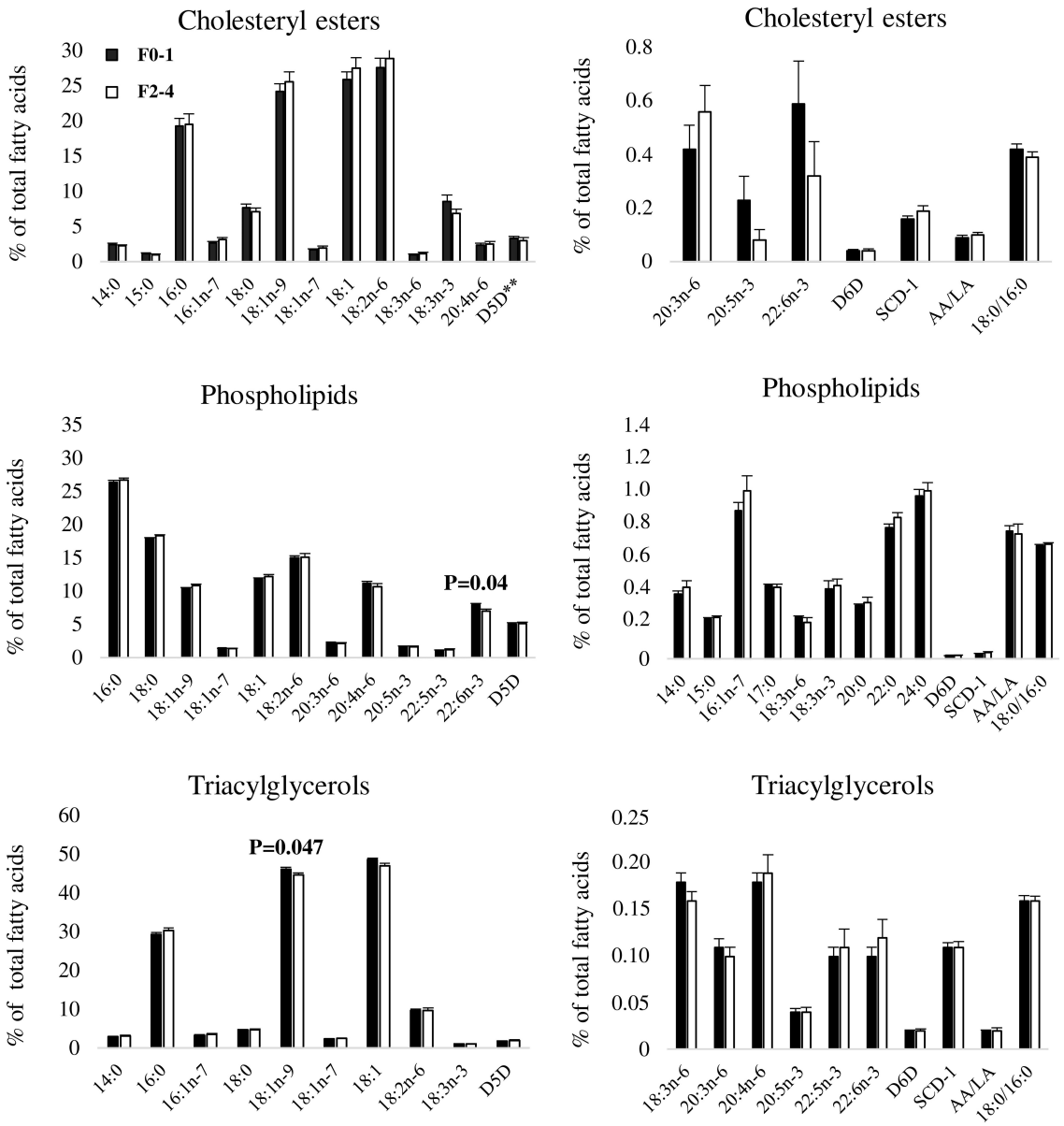

FIGURE 1 | Proportions of liver fatty acids between F0–1 and F2–4. Data are expressed as means ± SEM. Black bars represent fibrosis stages 0–1 (F0–1) and white bars represent fibrosis stages (F2–4), as indicated by the legend in the top left corner of the first bar graph. Due to large differences in proportions of fatty acids, each lipid fraction is divided into two separate graphs to enhance visibility. 14:0, myristic acid; 15:0, pentadecaenoic acid; 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 17:0, heptadecaenoic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:1n-7, vaccenic acid; 18:1, oleic acid combined with vaccenic acid; 18:2n-6, linoleic acid; 18:3n-6, γ-linolenic acid; 18:3n-3, α-linolenic acid; 20:0, arachidic acid; 20:3n-6, dihomo-γ-linolenic acid; 20:4n-6, arachidonic acid; 20:5n-3, eicosapentaenoic acid; 22:0, behenic acid; 22:5n-3, docosapentaenoic acid; 22:6n-3, docosahexaenoic acid; 24:0, lignoceric acid; SCD-1, stearoyl-coA desaturase; D5D, delta 5 desaturase; D6D, delta 6 desaturase; AA/LA, arachidonic acid/linoleic acid. SCD-1, D5D and D6D are estimated using fatty acid product-to-precursor ratios: 16:1n-7/16:0 (SCD-1), 20:4n-6/20:3n-6 (D5D), 18:3n-6/18:2n-6 (D6D). **n(F0–1) = 19, n(F2–4) = 16 due to 25 zero-values of 20:3n-6.

DISCUSSION

In this cross-sectional study of subjects with biopsy-proven NAFLD, we observed several links between the composition of liver fatty acids and fibrosis. A positive association between liver PL 22:0 and inverse associations between liver PL 22:6n-3, TAG 18:1n-9 and TAG 18:1 and liver fibrosis were observed. These associations were confirmed in plasma TAG 18:1n-9 and 18:1, however an inverse association was observed for plasma PL 22:0. Furthermore, *post-hoc* analyses demonstrated a positive association between liver PL SFA and inverse associations between liver PL PUFA and liver TAG MUFA and liver fibrosis. The latter findings were confirmed in plasma TAG MUFA but not in plasma PL PUFA or PL SFA.

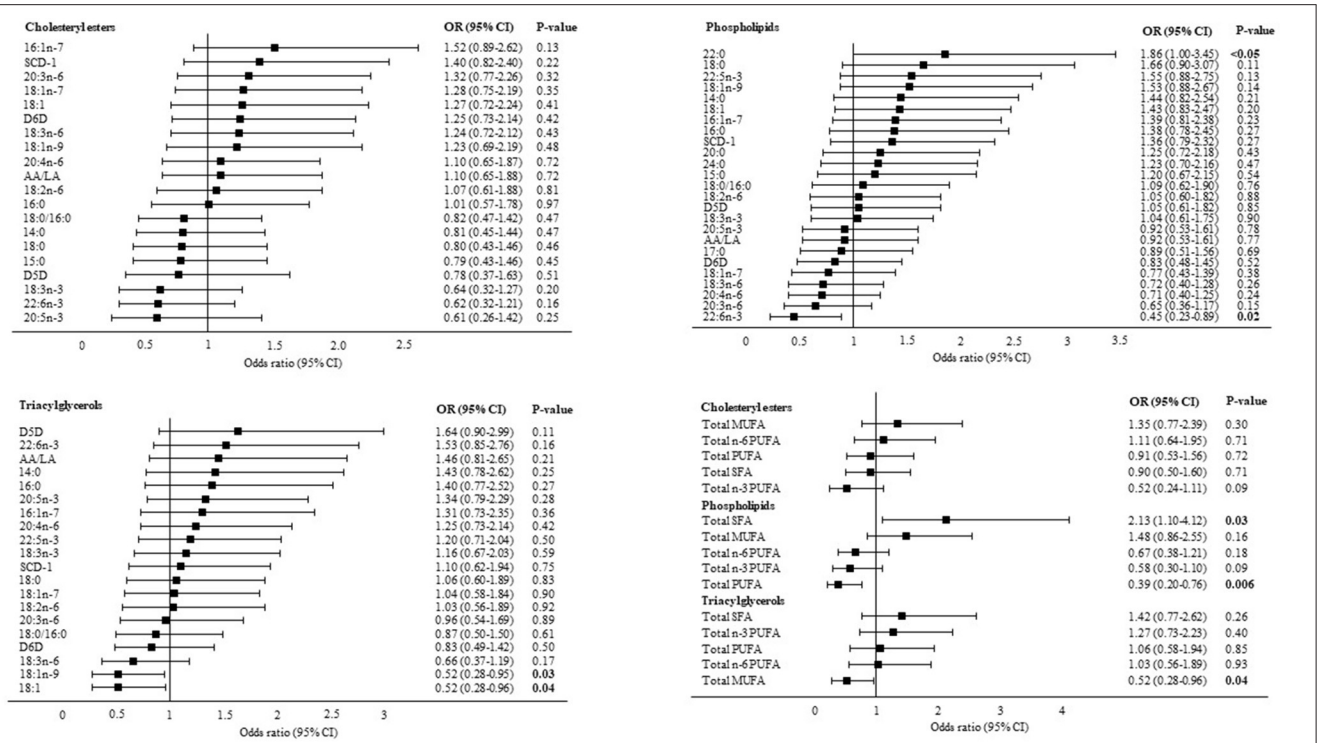

FIGURE 2 | Associations between liver fatty acids and significant liver fibrosis. Data are presented as adjusted odds ratios **(OR)** with 95% confidence intervals **(CI)** and *P*-values for each standard deviation change in liver fatty acid proportions. OR are adjusted for BMI, age and PNPLA3 (I148M) genotype. 14:0, myristic acid; 15:0, pentadecaenoic acid; 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 17:0, heptadecaenoic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:1n-7, vaccenic acid; 18:1, oleic acid combined with vaccenic acid; 18:2n-6, linoleic acid; 18:3n-6, γ-linolenic acid; 18:3n-3, α-linolenic acid; 20:0, arachidic acid; 20:3n-6, dihomo-γ-linolenic acid; 20:4n-6, arachidonic acid; 20:5n-3, eicosapentaenoic acid; 22:0, behenic acid; 22:5n-3, docosapentaenoic acid; 22:6n-3, docosahexaenoic acid; 24:0, lignoceric acid; SCD-1, stearoyl-coA desaturase; D5D, delta 5 desaturase; D6D, delta 6 desaturase; AA/LA, arachidonic acid/linoleic acid. SCD-1, D5D and D6D are estimated using fatty acid product-to-precursor ratios: 16:1n-7/16:0 (SCD-1), 20:4n-6/20:3n-6 (D5D), 18:3n-6/18:2n-6 (D6D). D5D in cholesteryl esters: n(F0–1) = 19, n(F2–4) = 16 due to 25 zero-values of 20:3n-6.

Our finding of a PUFA/MUFA-depleted, SFA-enriched liver in subjects with NAFLD fibrosis, characterized by lower proportions of 22:6n-3 and 18:1n-9 and higher proportions of 22:0 is to some extent in accordance with previous cross-sectional studies on hepatic lipid profiles in NAFLD. A relatively consistent pattern from these studies is that subjects with NAFL/NASH are characterized by PUFA-depleted livers, with lower proportions of 20:4n-6, eicosapentaenoic acid (20:5n-3) and 22:6n-3 (13, 15, 16). Notably, Kotronen et al. demonstrated an inverse correlation between hepatic PUFA (both 22:6n-3 and 18:2n-6) and free fatty acid 16:0 in severely obese patients, suggesting that PUFA might be implicated in the pathogenesis of steatosis (22). However, when comparing liver fatty acid profiles of subjects with NASH vs. NAFL, these differences are largely diminished, possibly indicating a greater role for fatty acids in the early stages of NAFLD (6, 12, 15, 16).

The relationship between fatty acids and NAFLD severity is complex. In the present study, we identified liver-derived fatty acids that were independently associated with liver fibrosis. Among these were 22:6n-3, an omega-3 PUFA that has been reported to be depleted in livers of patients with alcoholic cirrhosis (23), and which potentially could inhibit fibrogenesis through multiple mechanisms. These include inhibition of lipogenic pathways and the production of pro-inflammatory eicosanoids as well as suppression of HSC activation (24, 25). Due to the limited conversion rate from α-linolenic acid (18:3n-3) and 20:5n-3, plasma and tissue levels of 22:6n-3 mainly reflect dietary intake of marine sources, such as fatty fish and fish-oil supplements (26). On the contrary however, human supplementation trials of omega-3 fatty acids in NAFLD fibrosis have been few and findings have been mixed, with durations ranging from 6 to 12 months and with combined doses of both 22:6n-3 and 20:5n-3 ranging from 600 to 3,600 mg/day (27, 28). This apparent heterogeneity makes it difficult to draw any firm conclusions regarding the role of 22:6n-3 supplementation in NAFLD fibrosis. Longer-term follow-up studies with higher doses of isolated 22:6n-3 are warranted (29). Furthermore, as 22:6n-3 is an established biomarker of fatty fish intake, attention should be directed to investigate the potential role for diet in the treatment and prevention of NAFLD fibrosis. Lastly, altered desaturation and elongation of 22:6n-3 in liver fibrosis cannot be ruled out.

Interestingly, we also found opposite directions of associations between liver PL and plasma PL 22:0 and liver fibrosis. 22:0 is

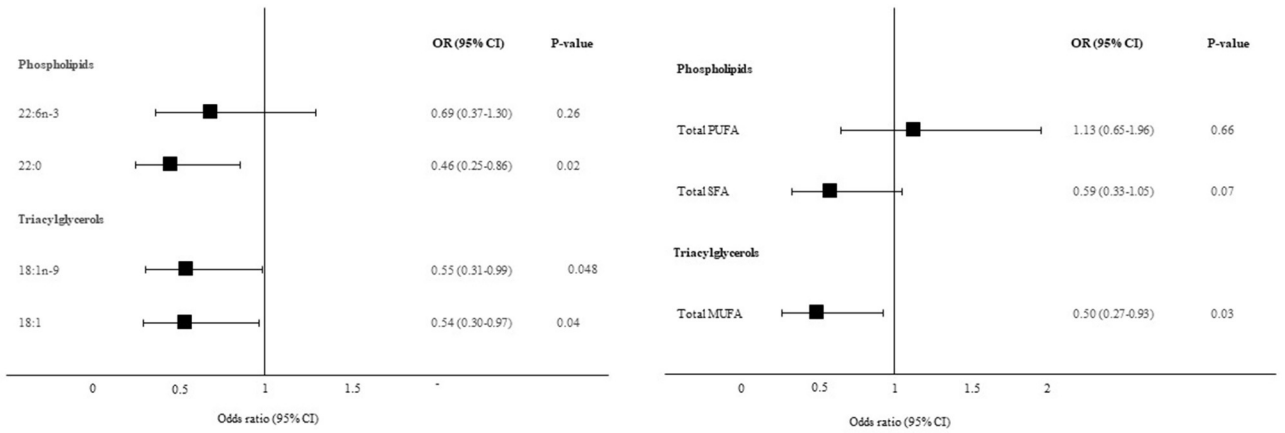

FIGURE 3 | Association between plasma fatty acids and significant liver fibrosis. Data are presented as adjusted odds ratios **(OR)** with 95% confidence intervals **(CI)** and *P*-values for each standard deviation change in plasma fatty acid proportions. OR are adjusted for BMI, age and PNPLA3 (I148M) genotype. 18:1n-9, oleic acid; 18:1, oleic acid combined with vaccenic acid; 22:0, behenic acid; 22:6n-3, docosahexaenoic acid.

a very long-chain SFA that has been inversely associated with the incidence of type-2 diabetes in studies using circulating fatty acids (30), supporting our findings of an inverse association between plasma 22:0 and liver fibrosis. However, no studies have yet assessed these very long-chain SFA in human liver tissue in relation to cardiometabolic diseases. Jang et al. recently showed, using an arteriovenous technique combined with a metabolomics approach in pigs, that 22:0 constituted a significant part of metabolites produced from lung tissue, indicating that circulating very long chain SFA in humans may partially reflect extrahepatic tissue metabolism (31). This finding is further supported by the lack of correlation between liver and plasma PL 22:0 in our study (Spearman rho = 0.07, $P = 0.58$). The relation between very long chain SFA and NAFLD warrants further attention in future studies.

Notably, 18:1n-9 and the combined 18 carbon MUFA 18:1 in both liver and plasma TAG were inversely associated with liver fibrosis. Although circulating 18:1n-9 might partially reflect dietary intake of MUFA-rich sources (e.g., olive oil or rapeseed oil) (32, 33), 18:1n-9 in plasma is primarily endogenously synthesized from SFA by SCD-1 and has been associated with the incidence of type-2 diabetes in a pooled sample of 17 prospective studies and with elevated liver enzymes in one cross-sectional study (34, 35). However, the vast majority of these studies have assessed 18:1n-9 in either PL or CE and not in TAG. In the Finnish METSIM cohort however, 18:1n-9 was measured in circulating TAG, demonstrating a non-significant inverse association with the incidence of type-2 diabetes (37). These findings are indirectly supported by the non-significant positive associations between 18:1n-9 and liver fibrosis in CE and PL in our study. This highlights the importance of cautious interpretation when extrapolating fatty acids from one lipid fraction to another. As further support, Araya et al. observed an increase of 18:1n-9 in total lipids, but not in TAG, in patients with NAFL and NASH vs. controls (15). Importantly, TAG 18:1n-9 in the liver correlated strongly with TAG 18:1n-9 in plasma in our study (Spearman rho = 0.73, $P < 0.0001$). The inverse association between TAG 18:1n-9 and liver fibrosis could be considered contradictory, however, it might reflect an enhanced desaturation and elimination of lipotoxic 16:0 through enhanced SCD-1 activity (36). Taken together, our findings suggest that plasma fatty acids could potentially be used as biomarkers for discriminating patients with NAFLD fibrosis and encourage further large-scale studies in the area.

There are several limitations worthy of consideration. First, the cross-sectional design makes it impossible to infer causality from our findings. Secondly, since the AM-02 NASH study was not designed to primarily address research questions posed in this study, lack of associations between fatty acids and liver fibrosis might be explained by small sample sizes and hence lower statistical power, indicated by the wide confidence intervals of the estimates. Lastly, missing information on diet might have contributed to residual confounding and thereby distorted the associations between fatty acids and liver fibrosis. At the same time, there are several strengths worth highlighting. Firstly, liver fibrosis was diagnosed from liver biopsies by two independent liver pathologists, and although biopsies are prone to sampling errors, histological grading remains the gold standard in assessing liver fibrosis in NAFLD. Secondly, fatty acids were measured in three different lipid fractions in both liver tissue and plasma, thereby allowing us to examine associations over multiple fatty acid compartments frequently used in epidemiological studies. Lastly, the homogenous fibrosis groups, as indicated in **Table 1** (clinical characteristics, biochemistry and histological scores except for fibrosis) might have contributed to reducing the risk of residual confounding. Importantly, due to the small sample size and multiple hypotheses tests, our findings should be interpreted as exploratory and hypothesis generating.

In conclusion, TAG 18:1n-9 and the combined TAG MUFA 18:1 demonstrated inverse associations with significant liver fibrosis in both liver and plasma, whereas PL 22:0 showed the opposite relationships in these compartments. Large-scale

studies are warranted to further investigate the role for these fatty acids as potential diagnostic biomarkers of NAFLD fibrosis. In addition, PL 22:6n-3, a biomarker of fatty fish intake, was inversely associated with fibrosis in the liver. Whether dietary modifications using marine sources of 22:6n-3 may have therapeutic implications in NAFLD fibrosis prevention needs to be investigated in longitudinal studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://osf.io/89b3n/, https://osf.io/89b3n/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Swedish Ethical Review Authority. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF, UR, FRos, FRor, and JV designed the research. FRor, JV, HA, HN, CS, PH, JH, AG, AW, and MF conducted the research. FRor, JV, HA, HN, CS, PH, and JH provided databases. MF analyzed data. MF, FRos, UR, FRor, and JV wrote the manuscript. UR had primary responsibility for final content. All authors reviewed, revised, and approved the final manuscript.

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REVIEW
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An Update in Epigenetics in Metabolic-Associated Fatty Liver Disease

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Metabolic-associated fatty liver disease (MAFLD) is characterized by hepatic steatosis accompanied by one of three features: overweight or obesity, T2DM, or lean or normal weight with evidence of metabolic dysregulation. It is distinguished by excessive fat accumulation in hepatocytes, and a decrease in the liver's ability to oxidize fats, the accumulation of ectopic fat, and the activation of proinflammatory pathways. Chronic damage will keep this pathophysiologic cycle active causing progression from hepatic steatosis to cirrhosis and eventually, hepatocarcinoma. Epigenetics affecting gene expression without altering DNA sequence allows us to study MAFLD pathophysiology from a different perspective, in which DNA methylation processes, histone modifications, and miRNAs expression have been closely associated with MAFLD progression. However, these considerations also faced us with the circumstance that modifying those epigenetics patterns might lead to MAFLD regression. Currently, epigenetics is an area of great interest because it could provide new insights in therapeutic targets and non-invasive biomarkers. This review comprises an update on the role of epigenetic patterns, as well as innovative therapeutic targets and biomarkers in MAFLD.

Keywords: MAFLD, NASH, DNA methylation, histone modification, miRNAs

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INTRODUCTION

Metabolic-associated fatty liver disease (MAFLD) is characterized by hepatic steatosis accompanied by one of three features: overweight or obesity, T2DM, or lean or normal weight with evidence of metabolic dysregulation (1).

MAFLD, as with the previous term NAFLD, represents the hepatic manifestation of a multisystem disorder, whose incidence is 20–30% in the western countries (2). Currently, there is no FDA-approved therapeutic agent for MALFD, and changes in diet and increase in physical activity are the first-in-line treatment of hepatic steatosis (3).

Gene expression is ultimately influenced by diverse epigenetic processes, including DNA methylation, histone modification, and expression of non-coding RNA molecules, like miRNAS. Epigenetic changes are reversible, and lifestyle and environmental exposure can define epigenetic patterns throughout life (4).

Epigenetic variations differ in the same individual among cell types and are associated with disease susceptibility by producing long-term changes in gene transcription (5). Alterations in

hepatic epigenetics significantly contribute to MAFLD development by altering transcriptional networks implicated in redox homeostasis, peroxisome and mitochondria function, inflammation, insulin sensibility, and fat homeostasis. Most important epigenetic mechanisms implicated in the development of metabolic associated fatty liver disease are described in the next sections.

DNA METHYLATION

DNA methylation is the covalent addition of a methyl group to the C5 position of cytosine generating a 5-methylcytosine (5mC), usually in cytosine–guanine dinucleotides-rich regions known as CpG islands. In general, hypermethylation of CpG islands is associated with gene repression, since the methyl group may physically block binding of transcription factors to the DNA, or it can act as a binding site for transcriptional repressors such as histone deacetylases; whereas hypomethylation is permissive to transcription (6). DNA methylation is catalyzed by a family of enzymes called DNA methyltransferases (*DNMTs*) that transfer the methyl group from an S-adenyl methionine (SAM) to DNA (7). *DNMT1* accounts for the recognition of the hemimethylated strand after a cell cycle. *DNMT3a* and *DNMT3b* are responsible for *de novo* methylation (8). The ten–eleven translocation (Tet) enzymes remove the methyl group in DNA (9).

DNA methylation is the most studied epigenetic mechanism in MAFLD. Detection of aberrant DNA methylation patterns could provide therapeutic targets and molecular tools for diagnosis and prediction of MAFLD (10). Several studies have analyzed genome-wide methylation changes associated with MAFLD, showing alterations in the methylation signature of many genes including regulatory loci for key metabolic and inflammatory pathways. For example, a study in humans using liver biopsies from obese patients with MAFLD showed methylation and expression differences in nine key enzymes implicated in intermediate metabolism and insulin signaling: pyruvate carboxylase (*PC*), ATP citratelyase (*ACLY*), phospholipase C-gamma-1 (*PLCG1*), insulin-like growth factor 1 (*IGF1*), insulin-like growth factor binding protein 2 (*IGFBP2*), and protein kinase C epsilon (*PRKCE*), putative polypeptide *N*-acetylgalactosaminyl-transferase-like protein 4 (*GALNTL4*), glutamate receptor delta-1 (*GRID1*), and inositol hexaphosphate kinase 3 (*IP6K3*) (11). A similar study founded that 41 genes responsible for lipid homeostasis were significantly and differentially methylated, including members of the APO family (lipid transport), genes involved in cholesterol transport like intracellular cholesterol transporter 1 (*NPC1L1*), acyl-CoA, sterol regulatory element binding transcription factor 1 (*SREBF1*), StAR-related lipid transfer domain containing 5 (*STARD5*), and solute carrier family 2 member 4 (*SLC2A4*) (12). Insulin resistance (IR) is part of the pathophysiology of MAFLD and its progression to NASH (13). An increased hepatic methylation of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (*PPARGC1A*) has been correlated with high plasma fasting insulin levels ($r = 0.51$, $p < 0.01$) and HOMA-IR ($r = 0.58$, $p < 0.003$) in patients with MAFLD (14, 15).

DIET AND DNA METHYLATION

"Western diet" is characterized by excessive fat and sugar consumption and seems to contribute to MAFLD pathogenesis (16). Preclinical studies demonstrated that the consumption of high-fat diet alters DNA methylation of gene clusters (17) and induces hypermethylation in promoter regions of peroxisome proliferator-activated receptor alpha (*PPARA*) (18), whereas, high fructose induces hypermethylation of carnitine palmitoyltransferase 1A (*CTP1A*) and *PPARA* genes (19) and global hypomethylation of mitochondrial DNA (20). *PPARA* is a transcriptional regulator of genes involved in mitochondrial beta-oxidation, fatty acid transport, and hepatic production of glucose, and *PPARA* hypermethylation decreased its gene expression and induced fatty accumulation in the liver. On the other hand, peroxisome proliferator-activated receptor gamma (*PPARG*) is upregulated in diabetes, obesity, and MAFLD. Mice fed a high-fat diet (HFD) showed a reduction of the level of cytosine methylation *Pparg* promoter, DNMT activity, and induction of hepatic *Pparg* expression (21).

Furthermore, Wang et al. proposed a regulatory pathway for sugar leading to induction of lipid accumulation; Huh-7 cells administered with high-glucose showed a close relationship between an increase in nuclear 25-hydroxycholesterol and activation of *DNMT1*, which methylates cytosine of CpG in promoter regions, suppressing expression of genes involving in MAFLD diseases (22).

It is challenging to confirm these studies in humans; however, a human study examined the effect of lifestyle interventions on DNA-methylation. The participants received a regimen of either low-fat or Mediterranean-low carbohydrates for 18 months. At baseline, intrahepatic fat was inversely correlated with DNA-methylation in calcium release activated channel regulator 2A (*CRACR2A*), alpha-2-macroglobulin pseudogene 1 (*A2MP1*), and ARH/RhoGEF and pleckstrin domain protein 1 (*FARP1*) genes. In conclusion, patterns in DNA-methylation changed in *A2MP1* gene after lifestyle interventions (23).

DNA methylation patterns can be modified also by bioactive food components. For example, methyl-group donors (B9, B12, methionine, betaine, and choline) are required for SAM synthesis in one-carbon metabolism. One-carbon metabolism comprises a series of interlinking metabolic pathways that include the methionine and folate cycles that are central to cellular function, providing methyl groups for the synthesis of DNA, polyamines, amino acids, creatine, and phospholipids (24). Several studies have demonstrated that CH3 deficiency in one-carbon metabolism is strongly associated with MAFLD (25). In animal models, a deficient methyl-donor diet is associated with reduced hepatic global DNA methylation and altered DNA-methylation patterns of lipid genes associated with fatty-liver-like phenotype such as ATP binding cassette subfamily A member 1 (*Abca1*), acetyl-CoA acetyltransferase 1 (*Acat1*), 1-acylglycerol-3-phosphate O acyltransferase 3 (*Agpat3*), and angiotensin II receptor type 1 (*AGTR1*) (26, 27). In contrast, dietary methyl donor-supplementation prevents liver fat accumulation by modifying the methylation of specific gene promoters like *Srebf2*, *Agpat3,* and estrogen receptor 1 (*Esr1*) (28). Recently,

these results were corroborated in humans; hepatic global DNA methylation levels were significantly lower in patients with MAFLD than in the control group, and also among participants who were overweight. These data correlate negatively with histological disease severity. In addition, MAFLD group had a significant higher serum homocysteine concentration (an indicator of methyl donor–deficient diet). This suggests that global DNA methylation and serum one-carbon metabolites may be markers of MAFLD status or severity (29). In patients with type 2 diabetes, a correlation between a high number of hypomethylated CpG sites and reduced levels of folate in the circulation was found (30). Another study was conducted in obese patients, associated low folate intakes with lower calcium/calmodulin-dependent protein kinase 2 (*CAMKK2*) gene methylation and IR (31).

DNA METHYLATION AS PREDICTIVE BIOMARKERS OF DISEASE

DNA methylation in peripheral cells or ccf-DNA is a potential biomarker to diagnose MAFLD. Hypomethylation in promoters of protein kinase C epsilon (*PRKCE*) and *SEC14* like lipid binding 3 (*SEC14L3*) is associated with MAFLD by genome-wide DNA methylation profiling in peripheral blood leukocytes (32). Ma et al. reported differential methylation in 22 CpG in genes like *SLC7A11*, *CPT1A*, *SREBF1*, zinc finger RNA binding protein 2 (*ZFR2*), and *SLC9A3R1* associated with increase hepatic fat in European Ancestry participants (33). Similarly, in patients with histologically confirmed MAFLD, six differentially methylated CpG sites were identified in the Acyl-CoA synthetase long-chain family member 4 (*ACSL4*), cardiolipin synthase 1 (*CRLS1*), carnitine palmitoyltransferase 1A *(CTP1A)*, single Ig and TIR domain containing (*SIGIRR*), single-stranded DNA binding protein 1 (*SSBP1*), and zinc finger protein 622 (*ZNF622*) genes compared with healthy controls (34). Nano et al. reported an association between DNA methylation in *SLC7A11*, *SLC1A5*, *SLC43A1*, phosphoglycerate dehydrogenase (*PHGDH*), psoriasis susceptibility 1 candidate 1 (*PSORS1C1*), *SREBF1,* and ankyrin repeat and sterile alpha motif domain containing 3 (*ANKS3*) with gamma-glutamyl transferase (GGT) levels; while DNA methylation in *SLC7A11* was associated with alanine aminotransferase (ALT) (35). MAFLD may progress to advanced liver disease with the presence of fibrosis, a key histological determinant of long-term prognosis. An observational study compared liver biopsies from patients with mild vs. advanced fibrosis, identifying significant more methylation in gene regulatory regions of transforming growth factor beta 1 (*TGFB1*) and platelet-derived growth factor subunit A (*PDGFA*) in patients with mild fibrosis, whereas *PPARA* and *PPARD* showed considerably less methylation (36).

A previous study has demonstrated that *PPARG* promoter hypermethylation correlated with severe fibrosis in liver biopsies (37), and more recently Hardy et al. found a similar degree of hypermethylation in the *PPARG* promoter in plasma ccf-DNA and hepatocyte-rich tissue captured by laser capture microdissection, suggesting that plasma DNA methylation of *PPARG* could potentially be used as a noninvasive method to determinate liver fibrosis severity in patients with MAFLD (38). Also, hypomethylation in a branched chain amino acid transaminase 1 (*BCAT1*) has been reported inversely associated with fibrosis degree (39). Hypomethylation of fibroblast growth factor receptor 2 (*FGFR2*), caspase 1 (*CASP1*), and hypermethylation of methionine adenosyltransferase 1A (*MAT1A*) were associated with advanced MAFLD in a study of Murphy et al. (40). Parvin beta variant 1 (*PARVB*) (hypomethylated in CpG26) and patatin like phospholipase domain containing (*PNPLA3*) (hypermethylated in CpG99) have also been associated with MAFLD (41). **Figure 1** describes differential DNA methylation patterns associated with MAFLD, some of them proposed as biomarkers.

HISTONE MODIFICATIONS IN MAFLD

Histones are a family of basic proteins whose positive charges allow them to associate with DNA in the nucleus and help them condense it into a chromatin. The basic structural unit of chromatin, the nucleosome (42), is formed by a pair of each H2a, H2b, H3, and H4 histones, an octamer (43). These histones are small globular proteins containing an N-terminal tail that can undergo acetylation, methylation, phosphorylation, SUMOylation, ubiquitination, or ADP-ribosylation. Multiple histones modifying enzymes can carry out more than 60 chemical histone-modifications that affect specific DNA binding sites, causing transcription activation or silencing of specific genes (44).

Lysine acetylation or methylation in the N-terminal tail stands out as the histone modifications with greatest repercussion in gene expression (45). Acetylation is mediated by histone acetyltransferases (HAT) and is usually associated with active gene transcription due to its ability to decompress chromatin. For this reaction, acetyl CoA acts as a cofactor, and subsequently HAT catalyzes the transfer of an acetyl group to the epsilon-amino group of lysine (46), neutralizing the positive charge of lysine and weakening histone and DNA interactions (47). In the opposite way, histone deacetylases (HDAC) remove acetyl groups from lysine and thus restores the compacted form of chromatin (48).

On the other hand, histone methylation in residues in the N-terminal tail of histones causes silencing of chromatin and the inactivation of transcription. However, in particular cases, methylation of histone activates gene transcription and is associated with the initiation of chromatin remodeling (49). The precise effect of methylation is linked to the specific residue where the reaction takes place. The methylation process is carried out by histone methyltransferases (HTM), which have the ability to add one, two, or three methyl groups to lysine or arginine residues of histones. Histone demethylases (HDM) have the ability to remove methyl groups from histone, thus beginning the remodeling of chromatin toward a decompressed or active state. HDMs have been classified into two classes, the FAD-dependent amino oxidases (LSD) and the jumonji C demethylase (JMJD) (50). Imbalance in histone modifications causes a disproportion in transcriptional activity associated

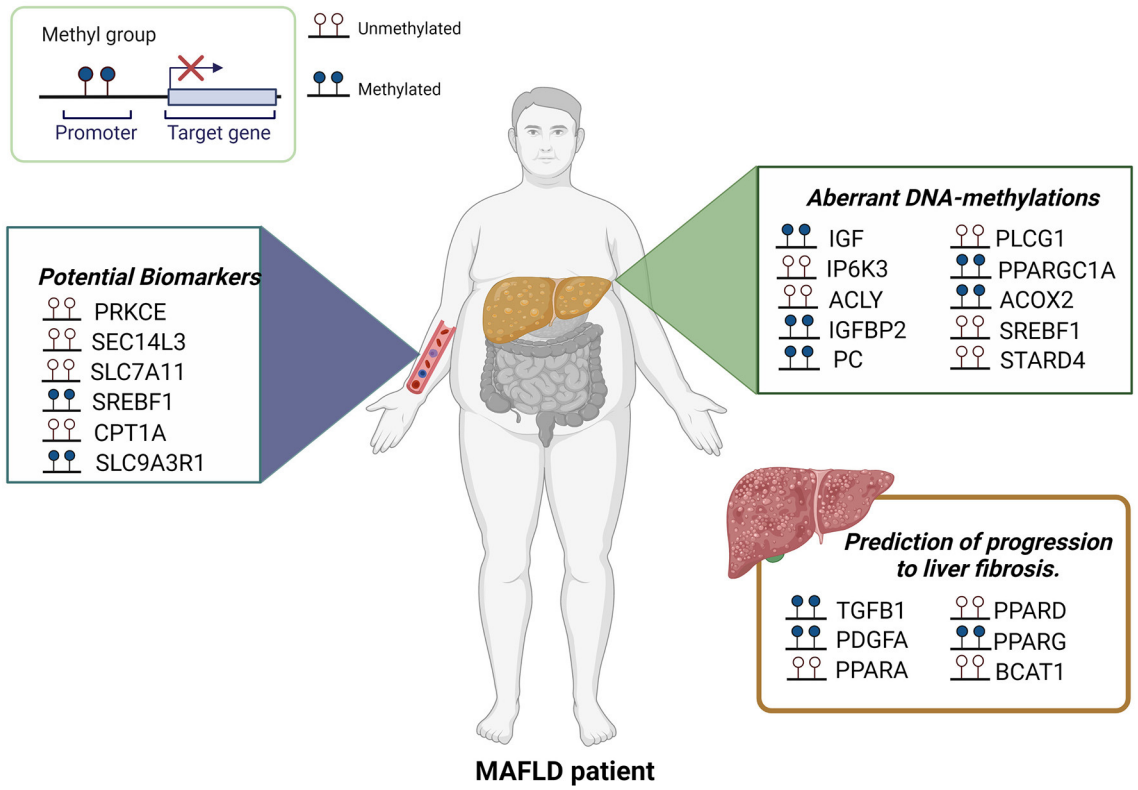

FIGURE 1 | Aberrant DNA-methylations in MAFLD. Several studies using liver biopsies, ccf-DNA or peripheral blood leukocytes have been shown differential DNA methylation patterns associated with MAFLD. Certain CpGs also represent attractive biomarkers for MAFLD and the prediction of progression to fibrosis.

with the development of diseases such as type 2 diabetes mellitus, obesity, and consequently MAFLD (51). Main enzymes involved in histone modifications that are implicated in MAFLD development are enlisted in **Figure 2**.

HISTONE ACETYLATION

During IR or DM2 the risk to develop non-alcoholic fatty liver increases due to inflammatory factors; where nuclear factor enhancing kappa of activated B cells (*NFkB*) or elements of carbohydrate metabolism that affect lipogenesis like element binding protein carbohydrate response (*ChREBP*) stand out. These factors are upregulated by some HAT (52). P300, a member of the HAT family, is a transcriptional regulator that plays a very important role modifying *NFkB* pathway. It has been shown that inhibition of p300 improves MAFLD in mice and restores biochemical parameters, decreases activity of genes involved in lipogenesis, and therefore, the aberrant activity of p300 favors MAFLD development (53). One of the main factors that is altered by p300 is *ChREBP*, a protein essential for the accumulation of fat in the liver. Bricambert et al. corroborated the interaction of these two molecules, activating or inhibiting kinase inducible by serine/threonine kinase 2 (*SIK2*), an element that regulates the activity of p300. In HepG2 cells and mice, SIK2 inhibited p300 activity by direct phosphorylation, and therefore also decreased the lipogenesis mediated by *ChREBP*. *SIK2* depletion caused an overexpression of p300 increasing lipogenesis and causing insulin resistance, hepatic steatosis and inflammation (54). HDACs have 4 families (class I, IIa, IIb, and IV) that differ in structure, enzymatic function, and location. HDACs play an important role in the development of MAFLD, some with more evidence than others. For example, *HDAC1*, a member of the class I family-depleted HepG2 cells decreased sterol regulatory element binding protein (*SREBP1c*) as well as, liver tissue of P50 *NFkB*-subunit KO mice (55). In addition to *HDAC1*, the activity of *HDAC3* has also been evaluated in MAFLD and in obesity and insulin resistance. *HDAC3* regulates hepatic lipid metabolism in the opposite way to *HDAC1*. *HDAC3* is an important lipid homeostatic regulator in the liver, and its loss leads to severe hepatic steatosis in mice (56). It is key to highlight that *HDAC3* also has direct interaction with molecules that participate in the development of hepatic steatosis, such as *SREBP1*, key molecule in the lipogenic process (57). In addition, *HDAC3* has a specific role in the circadian pattern of hepatic lipogenesis, a dysregulation in this cycle mediated by *SREBP1* increases the lipogenic process (58, 59).

A well-known group of deacetylases are silent information regulatory proteins (SIRTs), also known as Sirtuins. SIRTs are members of the class III HDAC family and use NAD^+ as a

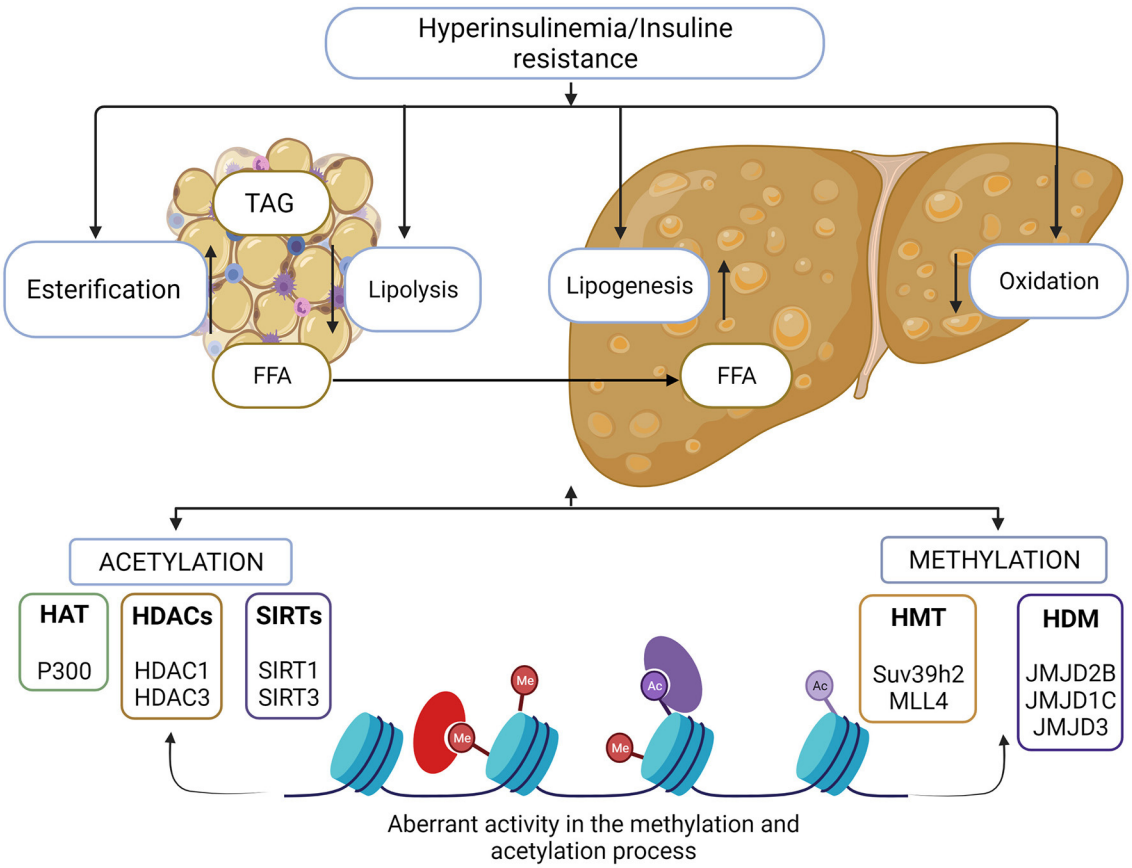

FIGURE 2 | Histone modifications in MAFLD. Metabolic imbalance in lipids and carbohydrates are involved in the development of MAFLD. Adipose tissue storage and insulin resistance triggers the accumulation of free fatty acids in the liver and liver homeostasis is lost. There is a direct association between aberrant chromatin modifications and this metabolic imbalance. Histone methylation and acetylation process, allows the activation of genes associated with the lipogenic and inflammatory process, as well as the reduction in expression of the genes involved in the oxidation of fatty acids, enzymes responsible for these events are possible therapeutic target for MAFLD control.

cofactor. They can interact with histones as well as non-histone proteins and have gained interest in metabolic diseases since they are involved in lipid homeostasis, oxidative stress, and insulin resistance, all events implicated in MAFLD development. Sirtuin family has 7 members characterized by their structure, enzymatic function, and localization. *SIRT 1, 2, 3, 6*, and *7* are mainly found in the nucleus, *SIRT1* and *SIRT2* are also in the cytoplasm, and *SIRT4* and *SIRT5* in mitochondria (60).

The sirtuins with greatest association to nonalcoholic fatty liver disease development are *SIRT1* and *SIRT3*. Recent evidence showed that SIRT1 is an important piece in lipid homeostasis in the liver, and it is an agonist ligand of peroxisome proliferator-activated receptor alpha (*PPAR*), promoting oxidative activity in lipids. Sandoval-Rodriguez and Monroy-Ramirez et al. used synthetic inhibitors and activators of *SIRT1* and *PPARA* in cultured HepG2 cells, demonstrating positive feedback between both proteins, which leads to the fact that a decrease in *SIRT1* favors the development of MAFLD in part due to decrease in *PPARA* activity (61). The effect of *SIRT1* on lipid metabolism has been an important part of the discussion of whether it could function as a therapeutic target for MAFLD. The activation of *SIRT1* during MALFD decreases lipids and TGs accumulation in the liver, decreasing inflammation and lipogenic process (62). For its part, *SIRT3* is also important in MAFLD. Mice deficient in *SIRT3* and fed with HFD increased lipid levels in the liver, promoting development of MAFLD. *SIRT3* deficiency leads to less DNA binding activity in *PPARA*, thereby decreasing the production of molecules activated by *PPARA*; promoting fatty acids oxidative status (63). In addition, regarding oxidative stress and mitochondrial damage, events involved in hepatic steatosis, *SIRT3* deficiency increased oxidative stress and activation of caspase-9 pathway. However, overexpression of *SIRT3* decreases reactive oxygen species and promotes the activation of the *ERK-CREB-Bnip3* pathway improving mitophagy (63).

HISTONE METHYLATION

Transcription silencing is linked with a compacted state of chromatin, generally, associated with methylation of histone

tail. Histone 3 lysine 9 (*H3K9*) has been associated with the development of MAFLD, and the aberrant activity of some methyltransferases have been associated with this process (51).

Histone–lysine *N*-methyltransferase SUV39H2, an enzyme capable of adding mono, di, and trimethylated labels to H3K9, has a fundamental role in the activation of inflammatory pathways. Also, it can reduce the activity of SIRT1 causing NASH progress. SUV39H2 activity was analyzed in KO mice fed a HFD, and they developed hepatic steatosis of less severity compared with the wild type for this enzyme (64).

In addition, there is also a relationship between the development of hepatic steatosis and methylation of histone 3 lysine 4 (*H3K4*) by myeloid/lymphoid or mixed-lineage leukemia 4 (*MLL4*) methyltransferase. It was shown that in overnutrition conditions, *MLL4* provokes *H3K4* methylation facilitating interaction with targets of peroxisome proliferator-activated receptor gamma 2 (*PPARy2*), which promotes lipogenesis (65).

On the other hand, the activity of methylases has also been studied in MAFLD. Clear evidence of the direct effect of *JMJD2B* on histone mark H3K9 has demonstrated the importance of this enzyme in the lipogenic process during MAFLD, with the interaction of *PPARG2* and the ligand-activated liver X receptor alpha (*LXRa*). JMJD2B removes the trimethylated and dimethylated marks, leaving the monomethylated mark of H3K9, causing activation of *PPARG2* and its target genes increasing the hepatic lipogenic process (66). The same situation occurs with Liver X receptor alpha (*LXRA*). It has been shown that the overexpression of JMJD2B increases the activity of this receptor, inducing intracellular accumulation of triglycerides and thus MAFLD development (67).

Another molecule that has a demethylase function and that has been associated with the progression of MAFLD is JMJD1C. In the same way, the interaction of this enzyme with the histone mark H3K9, removing repressive marks, promotes the transcription of genes, inducing lipogenesis and accumulation of hepatic fatty acids. It has been shown that the mammalian Target of Rapamycin (mTOR) complex phosphorylates JMJD1C, allowing interaction with upstream stimulatory factor 1 (USF1), a molecule that activates lipogenic genes and is associated with familial hyperlipidemia (68).

On the contrary, the activity of JMJD3 has been associated with the disease improvement; it removes the repressive mark of histone 3 lysine 27 (H3K27) leaving it in its dimethylated form (H3K27me2), promoting chromatin remodeling, and in turn, working together with *SIRT1*, to promote *PPARA* activation. This evidence was obtained in fasting conditions, and genes involved in gluconeogenesis pathway had no relevant activity, but these facts open up the possibility of a new therapeutic target (69).

microRNAs

microRNAs (miRNAs) are single-stranded non-coding RNAs of 18–25 nucleotides long that can regulate gene expression at posttranscriptional level by inhibiting translation or inducing degradation of target mRNAs through complementary base-pairing (70). miRNAs account for 1–5% of the human genome and regulate at least 50% of protein coding genes in mammals (71). To date, more than 2,800 human miRNAs have been registered in the miRBase 22.1, which are predicted to regulate up to 60% of the human genes. About 50% of miRNAs are transcribed from protein coding genes, mostly intragenic regions particularly introns and few exons. The other half are intergenic, transcribed independently, and regulated by their own promoters. Each miRNA can regulate several target genes, and vice versa, and each target gene can be regulated by various miRNAs, explaining why miRNAs can play crucial functions in essentially all biological processes and in all cell types (72). Evidence have demonstrated that miRNAs are implicated as important mediators in metabolic diseases including obesity, DM2, metabolic syndrome, and metabolic associated fatty liver disease (MAFLD) (73–75). **Figure 3** summarizes upregulated miRNAs involved in pathogenesis and development of MAFLD.

miRNAs IN OBESITY

Several miRNAs including miR-27b, miR-33, miR-34a, miR-122, and miR-223 are important regulators in fatty acid metabolism and cholesterol biosynthesis in the liver (76). Specifically, miR-33 plays a key role in cholesterol homeostasis thought suppression of sterol regulatory element-binding protein 1 (*SREBP1*), high density lipoprotein formation, fatty acid oxidation, and insulin signaling (77).

miR-27b-3p exert regulatory effects in lipid metabolism and is altered in dyslipidemia (78). In high-fat diet model of obesity, miR-27b-3p suppress adipose tissue browning. Due to this key role in promoting body fat accumulation miR-27b-3p should be further explored as a potential target for the treatment of central obesity and linked diseases (79).

miR-122 is the most abundant miRNA in the liver, and has a key role in liver metabolism, cholesterol biosynthesis, fatty acid synthesis, and oxidation (80). It should be noted that miR-122 was the first miRNA to be associated with metabolic regulation (81). Long JK et al. found that miR-122 promoted hepatic lipogenesis inhibiting *LKB1*/*AMPK* pathway by targeting *SIRT1* in HepG2 and Huh-7 cells cultured with free fatty acids (FFA) (82). miR-122 was downregulated in steatotic-FFA-induced hepatocytes, and nonalcoholic steatohepatitis mice model using streptozotocin and HFD (STZ- HFD). Besides, miR-122 showed an important role in hepatic triglyceride accumulation reducing *YY1* mRNA stability causing upregulation in *FXR-SHP* signaling (83).

miR-34a has been reported as a probable tumor suppressor in numerous types of cancers (84). miR-34a is upregulated in MAFLD and is an essential regulator of lipid metabolism (85). In a work by Ding et al., miR-34a levels were increased in L02 cells transfected with miR-34a inhibitor and C57BL/6 mice injected with a miR-34a inhibitor. *Ppara* and *Sirt1,* which are target genes of miR-34a, were downregulated after miR-34a inhibitory treatment, provoking triacylglycerides, liver index, and activated-AMPK pathway decrease (86). In adipose tissue it has been reported that miR-34a expression gradually increases as dietary obesity develops. In miR-34a–KO mice glucose intolerance,

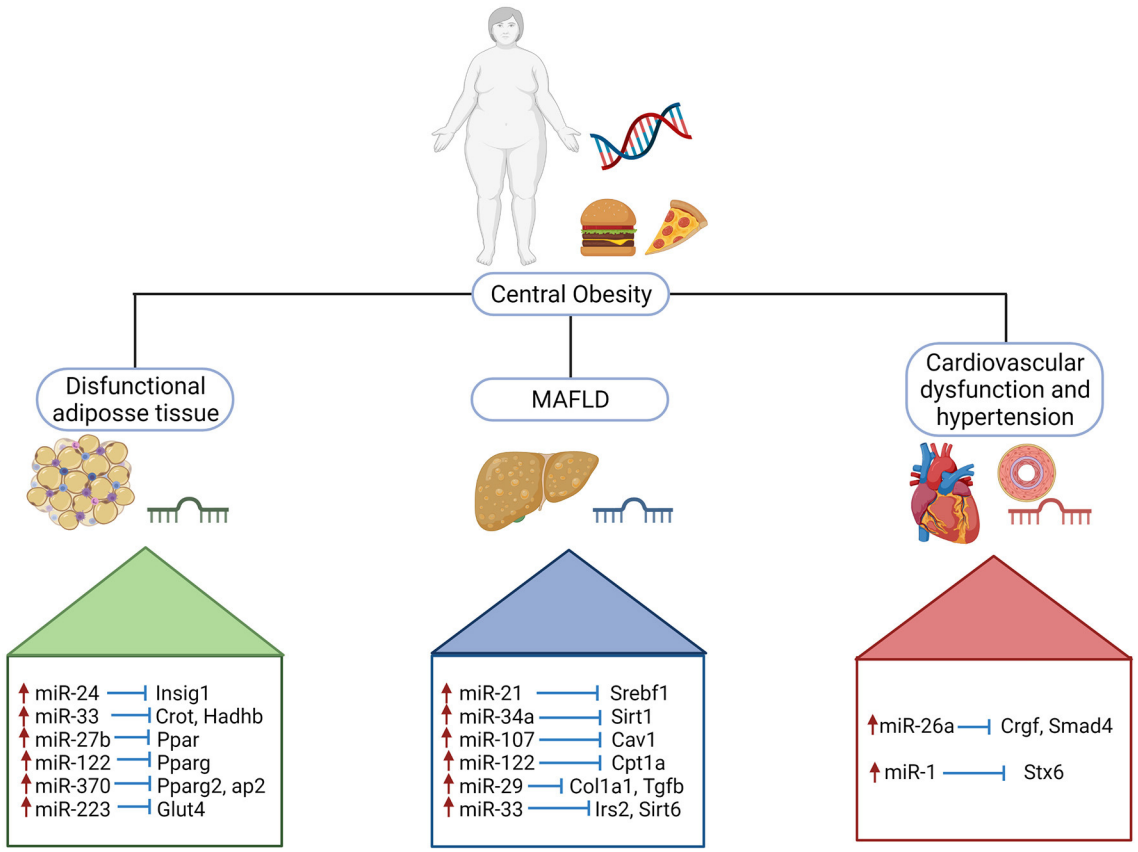

FIGURE 3 | Upregulated miRNAs involved in pathogenesis and development of MAFLD. Schematic illustration of selected miRNAs shows the impact on the stimulatory effect on target genes implicated in obesity, metabolic dysregulation and cardiovascular dysfunction. Insig 1, insulin induced gene 1; Crot, carnitine O-octanoyltransferase; Hadhb, hydroxyacyl-coa dehydrogenase trifunctional multienzyme complex subunit beta; Ppar, peroxisome proliferator activated receptor; Pparg, peroxisome proliferator activated receptor gamma; Pparg2, peroxisome proliferator activated receptor gamma 2; aP2, activating protein 2; Glut4, glucose transporter type 4; Srebf1, sterol regulatory element binding transcription factor; Sirt1; sirtuin 1; Cav1, caveolin 1; Cpt1a, carnitine palmitoyltransferase 1A; Col1a1, collagen type I alpha 1 chain; Tgfb, transforming growth factor beta; Irs2, insulin receptor substrate 2; Sirt6, sirtuin 6; Crgf, teratocarcinoma-derived growth factor 1; Smad4, SMAD Family Member 4; Stx6, syntaxin 6.

insulin resistance, and systemic inflammation were present in epididymal white adipose tissue (epiWAT).

Interestingly, increased miR-34a expression causes adipose inflammation principally by reduced expression of *Klf4*, resulting in suppressive effects on M2 macrophages polarization. Besides, it was found that high expression of miR-34a in visceral fat of overweight/obese patients correlated negatively with diminished *Klf4* (87).

miR-33 is a key regulator of lipid metabolism by targeting genes involved in cholesterol uptake and efflux in the liver, fatty acid metabolism *Cpt1, Crot, Hadhb*, insulin signaling *IRS2* and mitochondrial function *Ampk*, *Pgc1a* (88–90). miR-223 could inhibit cholesterol biosynthesis in mice through negative regulation of the 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*Hmgcs1*) and the sterol-C4-methyloxidase-like protein (*Sc4mol*). Besides, miR-223 decreased high-density lipoprotein-cholesterol (HDL-C) uptake by targeting the scavenger receptor class B member 1 causing *ABCA1* expression increase that rise cholesterol efflux (91). Otherwise, miR-223 targets include inflammatory and oncogenic genes like *CXCL10* and *TAZ*, data obtained in hepatocytes of high fat diet fed mice and in NASH patient livers. Therefore, miR-223 could protect against NASH development 322 (92).

A recent study by Zhang et al. reported that overexpression of miR-802 downregulates insulin transcription and secretion, as well as impairs glucose tolerance, suggesting a role of miR-802 in the development of obesity-associated β cell dysfunction (93). Several studies have reported that miR-221 is upregulated in adipose tissue from obese patients (94, 95). Peng et al. suggested that miR-221 promotes white adipose tissue inflammation and reduces insulin sensitivity in obesity while suppressing *SIRT1* (96).

miRNAs IN METABOLIC ALTERATIONS

Some miRNAs are crucial in MAFLD progression and metabolic alterations including waist circumference, blood pressure, serum triglycerides, and HOMA levels (97).

During adipogenesis, miR-425 expression is controlled by *Pparg* in adipocytes. miR-425 overexpression resulted in a proliferation reduction of 3T3-L1 preadipocytes, but accelerated cellular adipogenic differentiation. miR-425 also influences adipogenesis inhibiting its target gene *Mapk14*,a negative regulator of adipogenesis (98).

miR-107 is known to regulate insulin sensitivity in mouse models mainly by altering liver metabolism. miR-107 has a key role in lipid metabolism, inhibiting *CDK6* expression and its downstream targets, reducing adipogenesis in preadipocytes. Besides, it has been proposed that miR-107 promotes ectopic fatty acid accumulation and reduced glucose tolerance since miR-107 decreased glucose uptake and triglycerides synthesis in mature adipocytes (99). In a work carried out by Okamoto et al., serum miR-379 expression was upregulated in patients with MAFLD compared with healthy individuals. Serum levels of miR-379 showed positive correlations with alkaline phosphatase, total cholesterol, low- density-lipoprotein cholesterol, and non-high-density lipoprotein cholesterol levels in patients with early stage MAFLD (100).

miR-126a has been validated as a biomarker in obesity and related metabolic disease in women by Vonhögen et al. Thus, circulating levels of miR-216a are predictive factors for obesity. Interestingly, they found the obesity predisposition locus, the miR-216a gene that includes CpG islands with differential DNA methylation levels among obese and non-obese children, and is related with differential circulating miR-216a plasma levels in obese and non-obese women (101).

Remarkably, Lin et al. demonstrated that miR-144 targets *Foxo1*, thus reducing its expression and inhibiting its promotional effect on adiponectin, thereby alleviating the inhibitory effect of adiponectin on adipogenesis in an experimental model (102). A study performed by Komaya et al. reported that miR-33b showed high expression in the liver, and its expression was increased in response to cholesterol overload, using genetically modified mice, miR-33 knockout mice, and miR-33b Knock in mice; as a result, miR-33b showed increased atherogenic potential (103).

Basic and clinical evidence has shed light on the association between MAFLD and cardiovascular diseases (CVD) (104); in this context, increased plasma miR-1 was found to be associated with myocardial steatosis and it has been suggested to be a biomarker for diabetic cardiomyopathy (105).

A recent work carried out by Jiang et al. reported that miR-1 expression was increased in liver tissues and primary hepatocytes derived from a diet-induced obese mice, as well as, selective increase of miR-1 expression in EVs derived from steatotic hepatocytes (106). Several studies have shown that miR-26a is highly associated to cardiovascular diseases. Zhang et al. reported that miR-26a prevented blood pressure elevation and inhibited myocardial fibrosis using hypertensive animal models (107). **Figure 3** schematizes crucial miRNAs involved in pathogenesis and development of MAFLD, considering key parameters such as obesity, type 2 diabetes mellitus, and metabolic alterations (hypertension, high level of triglycerides and cholesterol, and HOMA). **Table 1** lists the miRNAs implicated in crucial key process in development of MAFLD and their potential target genes.

TABLE 1 | miRNAs implicated in crucial key process in MAFLD and their potential target genes.

miRNAS	Expression in MAFLD	Potential target genes	References
miR-24	Upregulated	*Insig1, Srb1*	(108, 109)
miR-33a/b	Upregulated	*Crot, Hadhb, Irs2, Sirt6, Dusp1, Tfrc, Abca1, Ski, Hipk2*	(103, 110)
miR-27b	Upregulated	*Ppar, Acot2*	(111)
miR-192	Downregulated	*Scd1*	(82)
miR-122	Upregulated	*Pparg, Agpat1, Dgat1, Cpeb1,Sirt1*	(112, 113)
miR-144	Upregulated	*Abca1*	(114)
miR-148a	Upregulated	*Ldlr, Pgc1a, Insig1*	(115)
miR-223	Upregulated	*Glut4, Nlrp3, Igf1r, Cxcl10*	(116)
miR-145	Downregulated	*Klf4*	(117)
miR-21a	Upregulated	*Srebf1, Smad7, Ppara*	(118, 119)
miR-107	Upregulated	*Cav1, Srebf1, Cpt1a*	(120)
miR-34a	Upregulated	*Sirt1, Hnf4a, Ppara*	(86, 121)
miR-29	Upregulated	*Col1a1, Tgfb, Sirt1*	(122)
miR-26a	Upregulated	*Crgf, Smad4, Eif2a*	(123)
miR-1	Upregulated	*Stx6*	(124)

Insig1, insulin induced gene 1; Srb1, scavenger receptor class B type 1; Crot, carnitine O-octanoyltransferase; Hadhb, hydroxyacyl-coA dehydrogenase trifunctional multienzyme complex subunit beta; Irs2, insulin receptor substrate 2; Sirt6, sirtuin 6; Dusp1, dual specificity phosphatase 1; Tfrc, transferrin receptor; Abca1, ATP binding cassette subfamily A member 1; Ski, SKI Proto-Oncogene; Hipk2, homeodomain interacting protein kinase 2; Ppar, peroxisome proliferator activated receptor; Acot2, acyl-CoA thioesterase 2; Scd1, stearoyl-CoA desaturase 1; Pparg, peroxisome proliferator activated receptor gamma; Agpat1, 1-acylglycerol-3-phosphate O-acyltransferase 1; Dgat1, Diacylglycerol O-Acyltransferase 1; Cpeb1, cytoplasmic polyadenylation element binding protein 1; Sirt1, sirtuin 1; Glut4, glucose transporter type 4; Nlrp3, NLR family pyrin domain containing 3; Igf1r, insulin like growth factor 1 receptor; Cxcl10, C-X-C motif chemokine ligand 10; Smad7, SMAD Family Member 7; Klf4, Kruppel Like Factor 4; Ppara, peroxisome proliferator activated receptor alpha; Cav1, caveolin 1; Srebf1, sterol regulatory element binding transcription factor 1; Cpt1a, carnitine Palmitoyltransferase 1A; Hnf4a, Hepatocyte Nuclear Factor 4 Alpha; Col1a1, collagen Type I Alpha 1 Chain, Tgfb, transforming Growth Factor Beta 1; Crgf: teratocarcinoma-derived growth factor 1; Smad 4: SMAD Family Member 4; Eif2a, eukaryotic Translation Initiation Factor 2A; Stx6: syntaxin 6.

CLINICAL TRIALS INVOLVING miRNAs FOR HEPATIC DISEASES

In the last decade, various miRNA-based therapeutics have been tested in different clinical trials. The first anti-miRNA drug for the treatment of hepatitis C is a locked nucleic acid (LNA) that inhibits miR-122, called Miravisen. Miravirsen inhibits miR-122 biogenesis and repressed HCV infection. miR-122 has a critical role in the life cycle of HCV due to the fact that miR-122 binds to two target sites (S1and S2) at the 5' end of the HCV genome, forming an oligomeric miR-122–HCV complex that protects the HCV genome from nucleolytic degradation or from host innate immune responses. Besides, at least three additional target sites in 3'-untranslated region of HCV genome have not been of

TABLE 2 | Clinical trials using miRNAs for hepatic diseases.

Start year	miRNA source/type	Study type	Characteristics	Status	ClinicalTrials identifier	Authors
2021	Panel of circulating miRNAs (not specific)	Observational cohort prospective	Early detection of hepatocellular carcinoma (HCC): miRNA, microbiome and imaging biomarkers in the evolution of chronic liver disease in a high-risk	Recruiting	NCT04965259	Pierce Chow, et al.
2020	Serum circulating miRNAs	Observational cohort prospective	Hepatic microRNA expression in non alcoholic fatty liver disease	Not yet recruiting	NCT04574557	Nourhan M.Abbas, et al.
2019	miRNA profile (not specific)	Observational case-only prospective	Expression and variance of microRNAs in a cohort of patients with acute decompensation of cirrhosis	Recruiting	NCT03905746	Fanny Lebossé et al.
2017	Serum circulating miRNAs miR-122-5p, miR-126a-3p, miR-193a-5p, miR-222-3p	Interventional clinical trial randomized parallel assignment	Effects of a combination of prebiotic fibers on weight loss during an energy restricted diet in an overweight/obese population	Completed	NCT03135041	Thomas M. Larsen et al.
2016	Plasma circulating miRNA panel	Observational prospective cohort	Comparative study of circulating microRNA changes in patients with liver injury and healthy subjects	Recruiting	NCT03000621	Huang Jian et al.
2016	anti-miR-103/107 (RG-125)	Interventional clinical trial randomized parallel assignment single masking	Study to assess the safety, tolerability, pharmacokinetics and pharmacodynamics of AZD4076 following multiple ascending dose administration to T2DM Subjects with NAFLD	Completed recruiment	NCT02826525	Linda Morrow et al.
2015	Serum miRNAs	Interventional randomized parallel assignment	Impact of IL-28B rs12979860 and rs4803217 gene polymorphisms associated with miRNAs deregulation on HCV-related hepatocellular carcinoma	Not yet recruiting	NCT02507882	Waleed Samir, et al.
2013	Liposomal injection of miR-34a mimic	Interventional clinical trial single group assignment open label	A multicenter phase I study of MRX34, MicroRNA miR-RX34 Liposomal Injection	Completed five immune related serious adverse events	NCT01829971	O'Neill Vincent, et al.
2010	antimiR-122 (Miravirsen)	Interventional clinical trial randomized parallel assignment Double masking	Multiple ascending dose study of miravirsen in treatment-naïve Chronic Hepatitis C subjects	Phase II	NCT01200420	Zeuzem et al.

functional importance (125). Currently, anti-miR-122 safety and effectiveness is being evaluated in a phase II clinical trial (126).

RG-101 is another novel anti-miR 122 for the treatment of hepatitis C virus. It is an N-acetylgalactosamine (GalNAc)-conjugated oligonucleotide. RG-101 repressed replication of HCV genotypes 1a and 1b in replicon systems. However, the precise mechanism of HCV suppression by RG-101 is not yet identified (127). Currently, RG-101 has reached the phase 1B clinical trial (128, 129).

Another miRNA-based therapeutic, a GalNAc conjugated anti-miR 103/107, called RG-125 (AZD4076) is an insulin sensitizer to treat patients with metabolic diseases such as type 2 diabetes and NASH. It has been reported that RG-125 normalized glucose tolerance and improved HOMA-IR in obese-diet induced mice compared with the control group. RG-125 treatment also reversed the extreme hyperglycemia that develops with age in db/db mice (130). **Table 2** lists the clinical trials using miRNAs-based drugs registered in the ClinicalTrials.gov web site (August 2021) for liver diseases.

CONCLUSION AND PERSPECTIVES

Metabolic dysfunction associated fatty liver disease is currently a global health problem, epidemically associated to obesity, metabolic syndrome, and type II diabetes mellitus. MAFLD development and progression involves several genetic and environmental factors including epigenetics. Epigenetics includes an extensive amount of events such as methylation in

CpGs, chemical modification of histones, and posttranscriptional gene regulation by the modification of mRNA stability through short noncoding RNAs such as miRNAs. In latest years, epigenetic modifications in DNA and histone have been studied as essential mechanisms that modify the development of liver diseases including MAFLD. Hence the dysregulation of epigenetic modifications has a critical role in MAFLD progression since it regulates the expression and activity of various genes implicated in lipid metabolism, insulin resistance, DNA repair, and inflammatory process that enhance the pathogenesis of MAFLD (10, 131). Currently, it has been demonstrated that miRNAs involved in lipid synthesis, fatty acid, and glucose catabolism and inflammation are dysregulated in MAFLD being useful as biomarkers (132). Moreover, it has been suggested that precise methylation patterns in DNA may be used as a predictor or diagnostic for MAFLD progression (133). Besides, the crucial paper of numerous micronutrients seems necessary to maintain DNA methylation homeostasis, as they act as cofactors of a variety of enzymes involved in DNA methylation, synthesis, and repair (134). To date, no therapeutic strategy is approved for the treatment of MAFLD, and lifestyle modifications, physical exercise, and weight loss account as the keystone therapeutics for patients with MAFLD. Certainly, a profound understanding of the molecular mechanisms related to gene expression, epigenetic modifications, and environment interactions ought to be a main concern for future studies. Overall, further basic research is necessary to improve mechanistic knowledge of the epigenetic processes and their interactions, their dysregulation in MAFLD, and the molecular and cellular response to epigenetic-based therapies. These studies together with clinical trials will enhance epigenetic-based personalized medicine. In conclusion, research in this area is in constant advance; however, there is still more to study to increase our understanding in MAFLD.

AUTHOR CONTRIBUTIONS

JR-S and RE-G contributed to planning, bibliographic revision, writing of the manuscript, and figures design. RR-C contributed to the writing of the manuscript and literature review. JA-B contributed to figures design and writing and revising of the manuscript. AS-R was responsible for the manuscript planning and revising. All authors have read and agreed to the published version of the manuscript.

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Liver Steatosis: Better Predictor of CKD in MAFLD Than Liver Fibrosis as Determined by Transient Elastography With Controlled Attenuation Parameter

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Background: Changing the term/concept of the non-alcoholic fatty liver disease (NAFLD) to metabolic dysfunction associated fatty liver disease (MAFLD) may broaden the pathological definition that can include chronic renal involvement, and, possibly, changes chronic kidney disease's (CKD's) epidemiological association with liver disease, because CKD is associated with metabolic disorders and almost all patients with CKD present some form of an atherogenic dyslipidemia. Our study explores the relationship between MAFLD and CKD using Transient Elastography (TE) with a Controlled Attenuated Parameter (CAP).

Methods: We evaluated 335 patients with diabetes with MAFLD and with high CKD risk using TE with CAP (FibroScan®). The CKD was defined according to Kidney Disease Improving Global Outcomes (KDIGO) 2012 guidelines. Logistic regression and stepwise multiple logistic regression were used to evaluate the factors associated with CKD. In addition, a receiver operating characteristic curve (ROC) analysis was used to assess the performance of CAP and TE in predicting CKD and its optimal threshold.

Results: The prevalence of CKD in our group was 60.8%. Patients with CKD had higher mean liver stiffness measurements (LSM) and CAP values than those without CKD. We found that hepatic steatosis was a better predictor of CKD than fibrosis. Univariate regression showed that CAP values >353 dB/m were predictive of CKD; while the multivariate regression analysis (after adjustment according to sex, body mass index (BMI), low-density lipoprotein cholesterol (LDLc), and high-density lipoprotein cholesterol (HDLc), and fasting glucose) showed that CAP values >353 dB/m were more strongly

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associated with the presence of CKD compared to the LSM (fibrosis) values.

Conclusion: In patients with MAFLD, CAP-assessed steatosis appears to be a better predictor of CKD compared to LSM-assessed hepatic fibrosis.

Keywords: MAFLD, NAFLD, chronic kidney disease, transient elastography, controlled attenuation parameter

INTRODUCTION

Recent data have proven that fatty liver, associated liver inflammation, and fibrosis [NAFLD and non-alcoholic steatohepatitis (NASH)] increase the risk of chronic kidney disease (CKD) (1). However, the NAFLD diagnosis does not include other liver disfunction/diseases (viral and toxic) associated with fatty liver. Therefore, the term MAFLD (metabolic dysfunction associated fatty liver disease) was recently proposed, which includes in its definition other liver diseases that are also associated with fatty liver. The definition of metabolic dysfunction associated fatty liver disease (MAFLD) is based on the evidence of hepatic steatosis, and the coexistence of overweight/obesity, or type 2 diabetes mellitus or the coexistence of two other risk factors related to metabolic dysregulation (waist circumference ≥102/88 cm in white men/women, low HDL-cholesterol, increase in serum triglyceride levels > 150 mg/dl, blood pressure >130/85, prediabetes, plasma C-reactive protein (CRP) >2.5, homeostatic model assessment (HOMA) score >2.5) (2). MAFLD can be diagnosed regardless of the daily alcohol consumption and other concomitant liver diseases. Furthermore, the relation between NAFLD/NASH and CKD has been explored, and the data have been extensively published (3). However, less is known about the relation between MAFLD and the risk of CKD. Our paper aimed to explore this relation using liver steatosis and liver fibrosis assessments by transient elastography (TE) with controlled attenuation parameter (CAP).

MATERIALS AND METHODS

Patients

Patients who were previously diagnosed with MAFLD, by imaging methods (MRI or/and CAP), were prospectively enrolled in the study conducted in the Departments of Nephrology, Gastroenterology and Hepatology, and Diabetes and Metabolic Diseases in Timisoara Emergency County Hospital for a period of 1 year (January 2018 to December 2018). All patients were Caucasians and underwent transient elastography with controlled attenuation parameters. As inclusion criteria, patients were required to be over 18 years of age and with presence of MAFLD.

Chronic kidney disease (CKD) was defined by an albumin-to-creatinine ratio (A/Cr) >30 mg/g and/or estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2 if persistent for more than 3 months. The eGFR was estimated using the CKD-Epi formula (4).

Exclusion criteria were as follows: pregnancy, ascites, outliers (subjects with inexplicable laboratory data values), decompensated liver disease, cardiac pacemaker, malignancy, end-stage renal disease, heart failure, unreliable or invalid TE and CAP measurements, and elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, which are more than five times the upper limit of normal. Known severe chronic liver disease- patients with liver cirrhosis (hepatitis B virus, hepatitis C virus, autoimmune hepatitis, or alcohol related liver disease) were also excluded from the study due to their previously established diagnostic and well-known etiology to avoid biases. The study protocol was conducted according to Declaration of Helsinki after the approval of "Pius Branzeu" County Emergency Clinical Hospital Ethical Committee (no. 131/25.10.2017). All patients gave their informed consent for the procedures.

Clinical Assessments

Age, body mass index (BMI), waist circumference, and medical history were collected. Laboratory data including serum creatinine, AST, ALT, platelets, glycemia, cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, urine albumin, and urine creatinine were also assessed in the same session with transient elastography, at 1, 2, and 3 months to identify the true CKD.

Transient Elastography With Controlled Attenuation Parameter

Transient elastography was performed with a FibroScan® device (EchoSens, Paris, France). At the time of the procedure, patients were in fasting condition for more than 4 h. Patients were placed in a supine position, with their right arm in a maximum abduction, by intercostal approach, in the right liver lobe. In each patient, we aimed for 10 valid liver stiffness measurements (LSMs). The examination was performed using the M probe (standard probe, transducer frequency 3.5 MHz) or the XL probe (transducer frequency 2.5 MHz). The M and XL probes were used according to the European recommendation on M and XL probe selection (5). A median value of 10 valid LSMs was calculated, and the results were expressed in kilopascals (kPa). A reliable measurement was defined as the median value of 10 valid LSMs, with an interquartile range/median ratio (IQR/M) of < 30% (6).

To discriminate between fibrosis and steatosis stages, we used the TE and CAP cut-off values from a published multicentric trial compared with biopsy (TE: 8.2 kPa for F≥2, 9.7 kPa for F≥3, and 13.6 kPa for F = 4; CAP: 302 dB/m for S≥1, 331 dB/m for S≥2, and 337 dB/m for S3) (7).

Statistical Analysis

MedCalc software (version 19.3.1) and Microsoft Excel 2019 were used for the statistical analysis. The distribution of numerical variables was tested with Kolmogorov–Smirnov test. In addition, we used *t*-test and ANOVA (for normal distributions), and

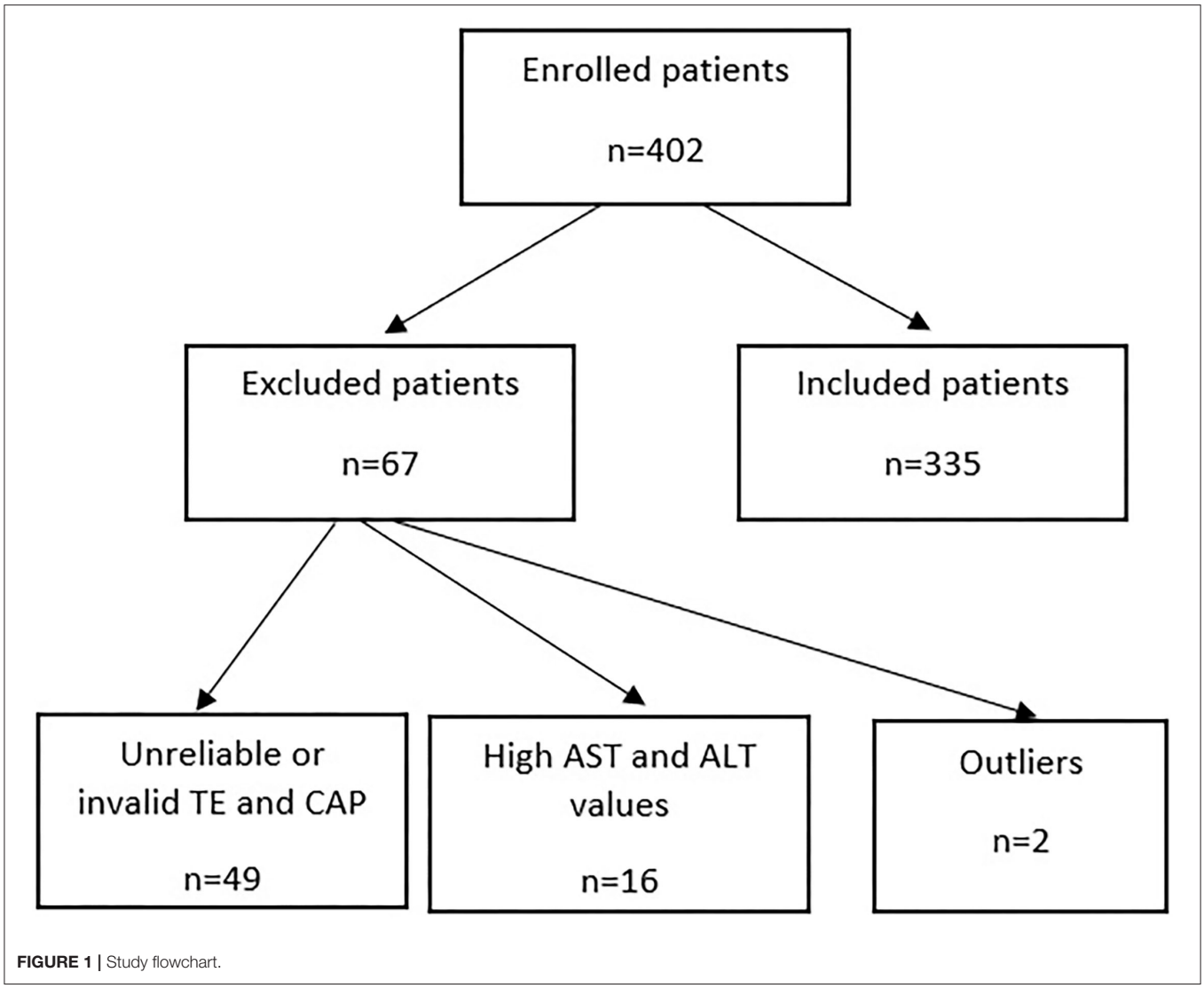

FIGURE 1 | Study flowchart.

Mann–Whitney *U*-test or Kruskal–Wallis tests (for non-normal distributions) to assess the differences between numerical variables. The proportions were analyzed using the chi-square test. Logistic regression and stepwise multiple logistic regression were used for the evaluation of the factors associated with CKD. The ROC analysis was used to assess the CAP and TE performance in predicting CKD and the optimal thresholds.

RESULTS

Baseline Characteristics

From the 402 patients, 355 patients met the inclusion criteria (**Figure 1**); of these, 44.1% were males. The average age was 60.84 ± 9.11 years. Among all patients, 60.8% had chronic kidney disease, and 83.80% had a history of hypertension. The characteristics of the patients are presented in **Table 1**.

When comparing the two subgroups, with CKD and without CKD, subjects with CKD were older (age, $p < 0.0001$); more likely to be men ($p < 0.0001$); and had higher values of triglycerides ($p = 0.04$), fasting glucose, HbA1c ($p = 0.01$, $p = 0.0008$, respectively), mean LSM values ($p = 0.04$), mean at CAP ($p = 0.03$), and similar rates of hypertension, steatosis, and significant fibrosis ($p > 0.05$) (**Table 2**).

No significant differences between the two subgroups were found regarding the waist circumference, BMI, AST, ALT, cholesterol, platelets, LDL, or HDL (**Table 2**).

Mean fibrosis LSM and CAP values were significantly higher in patients with CKD than in those without (8.64 ± 4.30 vs. 8.03 ± 6.57, $p = 0.04$; and 320.09 ± 57.12 vs. 306.29 ± 61.21, $p = 0.04$, respectively).

The ROC curves were used to determine if the transient elastography with controlled attenuation parameter could predict the presence of chronic kidney disease by assessing liver stiffness measurements or liver steatosis. The area under the receiver's operating characteristic curve of CAP was higher (AUC = 0.60, $p = 0.01$) than in TE (AUC = 0.51, $p = 0.98$), $p = 0.001$. The

TABLE 1 | Baseline characteristics of MAFLD patients.

Parameter	*n* = 335
Age (years)	60.84 ± 9.11
Gender (% male)	148 (44.1%)
BMI (kg/m²)	31.44 ± 5.98
Waist circumference (cm)	107.62 ± 14.65
AST (IU/L)	25.00 (9–159)
ALT (IU/L)	36.00 (9–200)
Platelets × 10^3/mm^3	243.52 ± 73.38
Total cholesterol (mg/dL)	189.22 ± 64.76
Triglycerides (mg/dL)	150 (30–420)
LDLc (mg/dL)	109.94 ± 40.09
HDLc (mg/dL)	43.16 ± 15.67
eGFR (mL/min/1.73m^2)	71.19 ± 24.03
eGFR < 60 (mL/min/1.73m^2)	193 (54.3%)
A/Cr > 30	178 (53.4%)
Creatinine (mg/dL)	1.09 ± 0.48
Fasting glucose (mg/dL)	180.38 ± 60.63
HbA1c (%)	8.53 ± 1.80
LSM (kPa)	8.03 ± 6.57
CAP (dB/m)	311.69 ± 59.94
Hypertension	281 (83.8%)
Dyslipidemia	125 (40.2%)
Fibrate treatment	108 (32.2%)
ACE inhibitors	97 (28.9%)
Diabetes duration	10.50 ± 8.51
Steatosis	257 (76.7%)
Severe steatosis	195 (58.2%)
Fibrosis stages	239 (71.4%)
F0–1	60 (17.9%)
F2	8 (2.3%)
F3	28 (8.4%)
F4	
Significant fibrosis (>F2)	96 (28.6%)
Advanced fibrosis (>F3)	36 (10.7%)

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; LSM, liver stiffness measurement; CAP, controlled attenuation parameter; ACE, angiotensin converting enzyme.

TABLE 2 | Comparison between chronic kidney disease and non-chronic kidney disease groups.

Parameter	CKD (*n* = 204)	No CKD (*n* = 131)	*p*-value
Age, years	62.64 ± 8.82	60.03 ± 6.88	0.008
Gender (% male)	91 (61.4%)	57 (38.6%)	0.0001
BMI (kg/m²)	31.35 ± 5.76	31.59 ± 6.32	0.72
Waist circumference (cm)	107.15± 15.49	108.32 ± 13.34	0.47
AST (IU/L)	24.50 (9–159)	23 (10–108)	0.05
ALT (IU/L)	41.12 (9–200)	37.00 (13–189)	0.86
Platelets × 10^3/mm^3	247.32 ± 81.01	237.67 ± 59.39	0.24
Total cholesterol (mg/dL)	191.21 ± 72.65	185.93 ± 49.27	0.46
Triglycerides (mg/dL)	157.5 (30–420)	141 (57–356)	0.04
LDLc (mg/dL)	109.34 ± 41.95	110.85 ± 37.20	0.73
HDLc (mg/dL)	41.00 ± 16.5	42.20 ± 14.19	0.49
eGFR (mL/min)	62.26 ± 24.68	85.21 ± 14.47	<0.0001
Creatinine (mg/dL)	1.23 ± 0.56	0.85 ± 0.16	<0.0001
Fasting glucose (mg/dL)	187.00 ± 62.95	170.14 ± 55.54	0.01
HbA1c (%)	8.79 ± 1.79	8.12 ± 1.74	0.0008
LSM (kPa)	8.64 ± 4.30	7.44 ± 3.15	0.04
CAP (dB/m)	320.09 ± 57.12	306.29 ± 61.21	0.03
Diabetes duration	11.10 ± 8.76	9.59 ± 7.51	0.17
Hypertension	174 (85.2%)	107 (81.6%)	0.38
Steatosis	150 (73.5%)	107 (81.6%)	0.008
Severe steatosis	113 (55.3%)	82 (62.5%)	0.19
Significant fibrosis	59 (28.9%)	37 (28.2%)	0.89
Advanced fibrosis	22 (10.7%)	14 (10.6%)	0.97
Dyslipidemia	69 (33.8%)	56 (42.7%)	0.10
Fibrate treatment	60 (29.4%)	48 (36.6%)	0.16
ACE inhibitors	21 (10.2%)	76 (58.0%)	<0.0001

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; LSM, liver stiffness measurement; CAP, controlled attenuation parameter; ACE, angiotensin converting enzyme.

best CAP cut-off value was 353 dB/m, with a sensitivity of 75% and a specificity of 45.1% (**Figure 2**). A CAP value over 353 dB/m was strongly correlated with the presence of CKD ($r = 0.89$, $p < 0.0001$); The correlation between TE and the presence of CKD was $r = 0.52$, $p = 0.12$.

Univariate and Multivariate Regression Analysis of Factors Involved in CKD Prediction

Six variables showed significant associations with CKD in the univariate analysis, including age ($p < 0.0001$), male gender ($p < 0.0001$), HbA1c ($p = 0.002$), fasting glucose ($p = 0.04$), CAP values ($p = 0.03$), angiotensin converting enzyme (ACE) inhibitors ($p = 0.04$), and creatinine ($p < 0.001$) (**Table 3**).

In multiple logistic regression analysis, all variables showed in **Table 3** were included, and after the adjustment of gender, fasting glucose, BMI, LDLc, and HDLc, CAP value, age, and HbA1c remained associated with the presence of CKD (**Table 3**).

DISCUSSION

For more than 35 years, the term NAFLD has covered conditions associated with the hepatic cell fat accumulation in the absence of evident causes like excess alcohol intake, viral hepatitis, drugs, inherited or acquired metabolic disorders, etc. NAFLD has two subtypes: the less severe non-alcoholic fatty liver (steatosis in more than 5% of hepatocytes without inflammation, necrosis, and fibrosis), and the more severe NASH characterized by steatosis, inflammation (lobular and portal), liver cell injury with the possibility of progression to fibrosis, cirrhosis, and end-stage liver disease. Some consider NAFLD a hepatic manifestation of

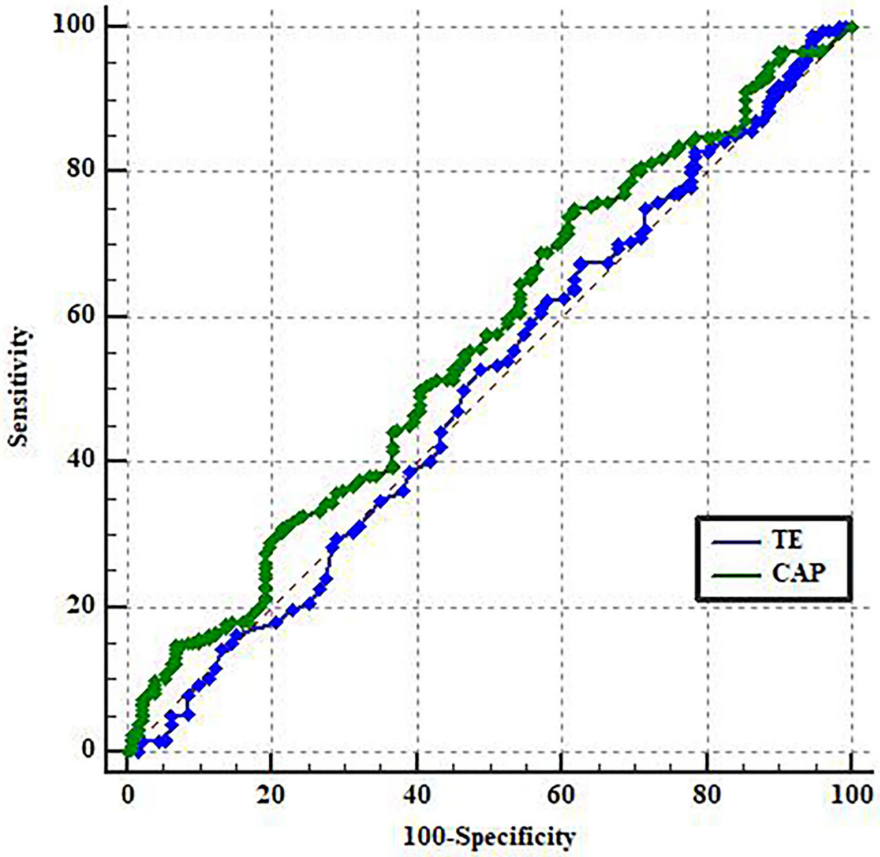

FIGURE 2 | ROC curve for predicting CKD.

metabolic syndrome (8). In the last decade, based mainly on observational studies, NAFLD was associated with an increased risk of cardiovascular disease and cardiovascular mortality. Therefore, it has been proven that the higher the severity of NAFLD, the higher the risk of fatal and non-fatal cardiovascular events (9). Considering the multiple common risk factors in observational studies and in two meta-analyses of those studies, NAFLD was also associated with both incident and prevalent chronic kidney disease. Additionally, it was found that the risk of CKD increased with the severity of the NAFLD (2, 10). The prevalence of CKD in cross-sectional studies varied between 5–47 and 2–29% in the longitudinal studies analyzed by Musso et al., depending on the definition of CKD used by the authors. The prevalence and incidence of CKD were significantly higher in NASH patients (11). A later meta-analysis by Mantovani et al. exploring the risk of incident CKD showed similar results (2). In both studies, both incident and prevalent CKD risk were significantly associated with the severity of liver fibrosis (2). Another studies by Ciardullo et al. (12, 13), Lomonaco et al. (14), and Yeung et al. (15) sustained the same, that in patients with NAFLD, liver fibrosis is associated with CKD and their prevalence of liver steatosis and liver fibrosis is greater in our study, but it may be due to the differences between the study cohorts. Our cohort is based only by participants who are Caucasian with type 2 diabetes, while the studies by Ciardullo et al. (12, 13) and Lomonaco et al. (14) are based on a mixed reced-ethnicity from United States, while the study conducted by Yeung et al. (15) is based entirely on Asian subjects. In recent years, it became evident that the excess accumulation of fat in liver cells may occur in many pathologic events beyond alcohol consumption, hepatitis B and C, autoimmune liver damage, and drugs. Additionally, the proven threshold for liver-safe alcohol consumption is not very clear (11). It has been suggested that even the alcohol-producing gut microbiota (*Klebsiella pneumoniae*) may influence the evolution of fatty liver disease (16). It seems that metabolic disorder is constantly associated with a fatty overload of the liver (17). The increasing number of patients with fatty liver; the possibility of progression to inflammation, fibrosis, cirrhosis, and complications as hepatic carcinoma, and the need for effective etiology-related interventions lead to a need for changing the nomenclature (paradigm?) of this disease. Thus, the term metabolic (disorder) associated fatty liver disease (MAFLD) was proposed (3). The definition of MAFLD requires the presence of hepatic steatosis associated with overweight/obesity (BMI >25 kg/m^2 in white and >23 kg/m^2 in Asian individuals), diabetes, or the presence of

TABLE 3 | Univariate and multivariate logistic regression analysis of factors associated with CKD.

Factor	Univariate analysis			Multivariate analysis		
	OR	95% CI	*p*-value	OR	95% CI	*p*-value
Age	1.08	1.00–1.20	<0.0001	1.04	1.00–1.07	0.04
Male	1.10	0.89–1.15	<0.0001	1.08	0.98–1.09	0.93
BMI	1.02	0.99–1.02	0.72	1.00	0.46–2.50	0.70
Triglycerides	1.00	0.98–1.00	0.44	0.99	0.99–1.00	0.46
LDLc	0.99	0.99–1.00	0.76	0.99	0.96–1.01	0.53
HDLc	0.99	0.99–1.01	0.43	1.01	0.98–1.04	0.33
HbA1c	1.01	1.00–1.10	0.002	1.00	1.00–1.01	0.02
Fasting glucose	1.00	0.99–1.05	0.04	1.00	0.99–1.01	0.11
Cholesterol	1.00	0.98–1.01	0.48	1.00	1.00–1.03	0.45
CAP	1.05	1.00–1.23	0.03	1.07	1.00–1.20	0.01
Creatinine	1.20	1.00–1.35	<0.0001	1.15	0.85–1.56	0.12
ALT	0.99	0.99–1.00	0.05	0.99	0.98–1.00	0.86
AST	0.99	0.97–1.00	0.38	1.00	0.98–1.02	0.67
TE	1.00	0.54–1.35	0.17	1.03	0.40–1.22	0.61
Severe steatosis	1.10	1.00–1.15	0.03	1.29	0.52–1.80	0.65
Advanced fibrosis	1.25	0.78–1.45	0.74	1.32	0.34–1.40	0.61
Hypertension	1.20	0.98–1.25	0.32	1.50	0.52–3.18	0.30
Dyslipidemia	1.00	0.99–1.02	0.59	1.10	0.50–2.39	0.75
Fibrate treatment	1.00	0.99–1.00	0.84	1.04	0.89–1.10	0.79
Duration of diabetes	0.99	0.97–1.00	0.16	0.99	0.95–1.03	0.97
ACE inhibitors	1.00	0.96–1.03	0.04	0.99	0.98–1.00	0.06

BMI, body mass index; HbA1c, hemoglobin A1c; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; CAP, controlled attenuation parameter; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LSM, liver stiffness measurements; OR, odds ratio; CI, confidence interval; ACE, angiotensin converting enzyme.

metabolic dysregulation. To establish metabolic dysfunction, at least two of the following characteristics must be present: waist circumference ≥102/88 cm in white men/women or ≥90/80 cm in Asian men/women, prediabetes, high serum C-reactive protein values, increased BP values or BP under treatment, low HDL-cholesterol, increase in serum triglyceride levels, and a HOMA score of >2.5. Specific individual characteristics such as genetic predisposition, age, sex, ethnicity, diet, metabolic status, and gut microbiota increase the risk of developing MAFLD. More importantly, MAFLD does not exclude other causes of fatty liver (17). The change of name and definition was associated with some changes in epidemiology. Though prevalence did not change significantly, the incidence of MAFLD decreased (by 25%), and it seems that hepatic fatty overload not associated with MAFLD definition criteria is less likely to develop into a significant liver disease (18). Moreover, it seems that the MAFLD criteria identify significant liver fibrosis better when compared to NAFLD (19).

Concerning the risk of CKD in patients with MAFLD, the data are scarce and contradictory. However, in analyzing the NHANES III 1988–94 database, the authors found that MAFLD can identify the patients with CKD risk better and that the risk of CKD and albuminuria is strongly correlated with the severity of liver fibrosis (the prevalence of CKD in MAFLD was 36.2%, and it was 30.2% in NAFLD) (20). However, the cross-sectional NHANES 2017–2018 study did not confirm these results (21).

Our study investigated patients with established MAFLD and its association with CKD (multiple risk factors: DM all cases, poor DM control, HT in 83.8%, average BMI 31.44 ± 5.98 kg/m^2, and hypercholesterolemia). Under these conditions, the prevalence of CKD was high (60.8%). Subjects with CKD, who were older than those without, were more likely to be men, and had higher triglyceride values, poorer glycemic control, and higher rates of hypertension (as expected). Concerning the TE findings with CAP, higher mean LSM values and higher mean CAP were recorded in patients with CKD, and similar rates of steatosis and fibrosis were evidenced in patients with patients (**Table 2**). When ROC curves were used to determine the presence of CKD related to liver fibrosis and steatosis, steatosis showed a higher AUC than fibrosis. Univariate regression analysis showed that the severe steatosis and CAP values (along with age, male gender, HbA1c values, and fasting glucose values), but not severe fibrosis and LSM values, were associated with CKD. The stepwise multiple logistic regression analysis confirmed our hypothesis; that is, after adjustment for gender, fasting glucose, BMI, LDLc, and HDLc, CAP values higher than 353 dB/m were strongly correlated with the presence of CKD (other predictors were age and HbA1C), and not with fibrosis or LSM values.

We explored some regression models for CKD prediction in combination with CAP values to exclude bias factors that may be involved in CKD (i.e., hypertension, dyslipidemia,

fibrate treatment, and duration of diabetes). In all models, the higher CAP values were independently associated with CKD. Although all patients with CKD tend to develop dyslipidemia (more frequently the very atherogenic type) (22), the question of whether CKD may influence steatosis in patients with MAFLD patients remains unanswered. Our study has some limitations: All patients were type two diabetes with steatosis; the lack of a control group to sustain the findings; and the fact that the study was a cross-sectional one.

CONCLUSIONS

In patients with established MAFLD and with multiple metabolic risk factors for CKD, the liver fatty overload evaluated with CAP seems to be a better predictor of CKD than LSM and fibrosis. However, more studies with a higher number of patients are needed to confirm our results. Furthermore, some questions remain to be answered in future research: In the liver–kidney crosstalk, how is CKD influencing the MAFLD outcomes, since CKD is a one-way pathological process? Additionally, does MAFLD influence CKD progression to an end-stage kidney disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by County Emergency Hospital "Pius Branzeu" Timisoara Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM and AS: conceptualization. AM and FB: methodology. RL and BT: software. AS, IS, and RT: validation. IG, FB, and FG: formal analysis. RL, LM, and LC: investigation. AS, RT, and IS: resources. LM, RL, and AM: data curation. LM, RL, and VT: writing—original draft preparation. AS, IG, AM, and FB: writing—review and editing. IG, AM, and NO: visualization. AS: supervision. All authors have read and agreed to the published version of the manuscript.

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MINI REVIEW
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MAFLD/NAFLD Biopsy-Free Scoring Systems for Hepatic Steatosis, NASH, and Fibrosis Diagnosis

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Metabolic dysfunction-associated fatty liver disease (MAFLD), formerly known as nonalcoholic fatty liver disease, is the most prevalent liver disorder worldwide. Historically, its diagnosis required biopsy, even though the procedure has a variable degree of error. Therefore, new non-invasive strategies are needed. Consequently, this article presents a thorough review of biopsy-free scoring systems proposed for the diagnosis of MAFLD. Similarly, it compares the severity of the disease, ranging from hepatic steatosis (HS) and nonalcoholic steatohepatitis (NASH) to fibrosis, by contrasting the corresponding serum markers, clinical associations, and performance metrics of these biopsy-free scoring systems. In this regard, defining MAFLD in conjunction with non-invasive tests can accurately identify patients with fatty liver at risk of fibrosis and its complications. Nonetheless, several biopsy-free scoring systems have been assessed only in certain cohorts; thus, further validation studies in different populations are required, with adjustment for variables, such as body mass index (BMI), clinical settings, concomitant diseases, and ethnic backgrounds. Hence, comprehensive studies on the effects of age, morbid obesity, and prevalence of MAFLD and advanced fibrosis in the target population are required. Nevertheless, the current clinical practice is urged to incorporate biopsy-free scoring systems that demonstrate adequate performance metrics for the accurate detection of patients with MAFLD and underlying conditions or those with contraindications of biopsy.

Keywords: MAFLD, NAFLD (non alcoholic fatty liver disease), scoring-algorithm, biopsy, steatosis, NASH, fibrosis, diagnosis

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INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD), formerly known as nonalcoholic fatty liver disease (NAFLD), is the most prevalent liver disorder worldwide (1, 2). Besides being considered a major public health concern (3, 4), it is expected to become the leading cause of liver failure requiring transplantation by 2030 (5).

Specifically, NAFLD is defined as an increase in hepatic lipid content not associated with chronic hepatitis due to viral infections, autoimmune diseases, or the use of steatogenic medications (6–9). Moreover, NAFLD can progress from steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and eventually, cirrhosis and hepatocellular carcinoma (10). In its early phases, the disease has a silent presentation, thus hindering the diagnosis and placing patients at risk of worse clinical outcomes (11, 12).

Nowadays, NAFLD is considered the hepatic component of metabolic syndrome (metabolic syndrome) (13), a disorder intricately related to type 2 diabetes mellitus (T2DM) (14, 15), insulin resistance, and cardiovascular diseases (16). For this reason, some authors have proposed a new, flexible term, MAFLD (17–19) (**Figure 1**).

Historically, MAFLD/NAFLD diagnosis required liver biopsy (20). Liver biopsy is a painful, invasive procedure that can increase mortality from 0.009 to 0.14%, has a risk of intraperitoneal hemorrhage, and only assesses approximate 1 per 50,000 of the entire liver parenchyma (21). In response, the need for new non-invasive strategies has been evidenced (22–25), especially for patients with underlying conditions (26) or biopsy contraindications (27).

Recently, grouping several non-invasive serological biomarkers has become a trend for the prediction and diagnosis of liver fibrosis (28). Moreover, studies have shown that these systems may avoid up to 38–80% of liver biopsies (29, 30). Currently, no single marker has been used for the precise detection of MAFLD/NAFLD, as isolated biomarkers do not provide sufficiently accurate information for diagnosis (31–33). However, when coupled with clinical features and with each other, accurate diagnosis, staging, and prognosis for this disease become possible (34).

Therefore, this review presents the state-of-the-art biopsy-free scoring systems (BFSS) for the diagnosis of MAFLD/NAFLD. Moreover, it further contrasts, in a stratified arrangement (**Figure 1**) of hepatic steatosis (HS), NASH, and fibrosis, the biomarkers, clinical associations, and discriminating performance metrics (**Table 1**) of such BFSS.

HEPATIC STEATOSIS SCORING SYSTEMS

Defined as a lipid concentration >5% in the hepatic parenchyma (66) without portal or lobular inflammation (67), HS is the mildest form of MAFLD/NAFLD (68). Currently, 4% of patients with HS are expected to develop fibrosis in their lifetimes (69). Thus, the BFFS proposed to aid in the prompt diagnosis are discussed in this section.

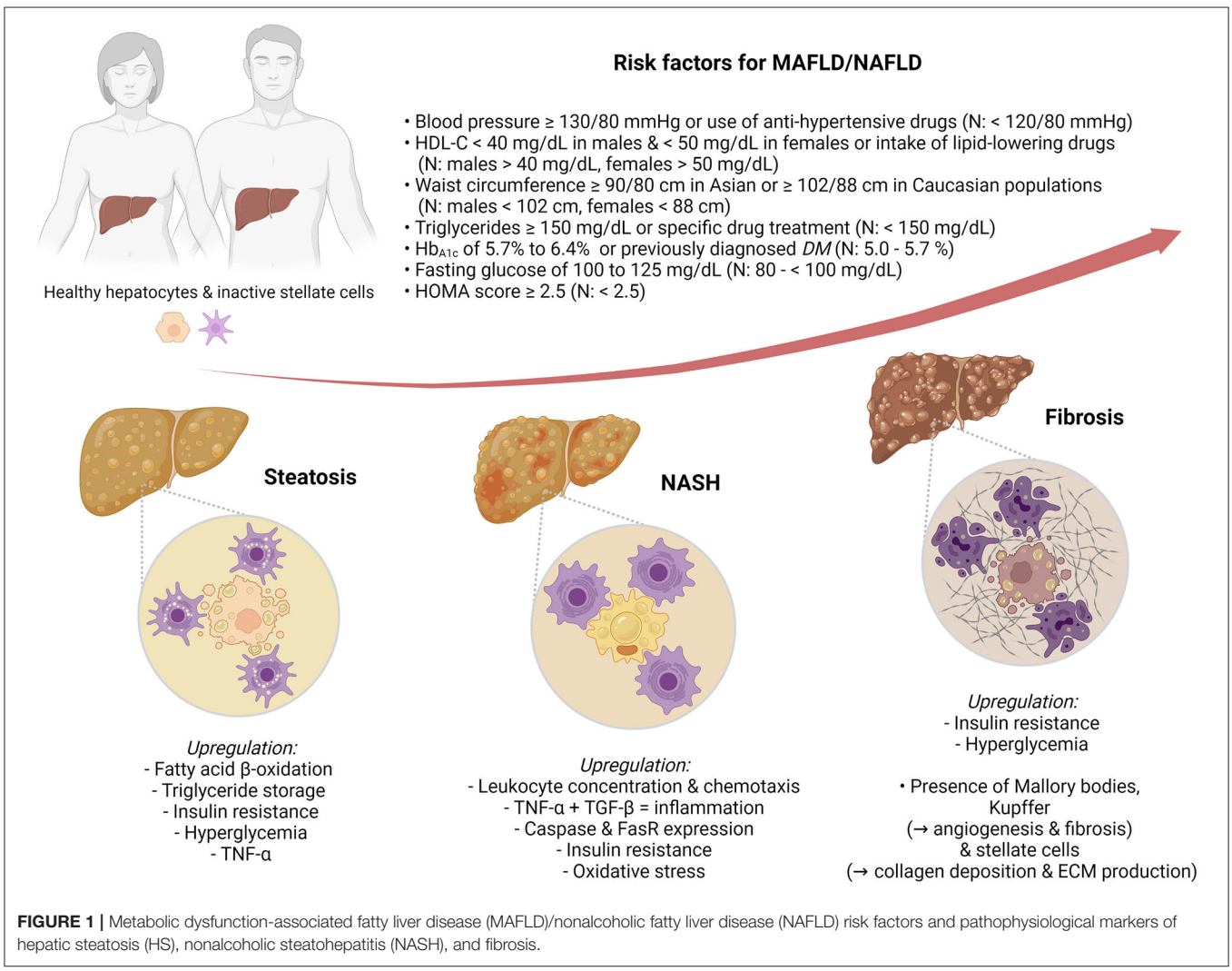

FIGURE 1 | Metabolic dysfunction-associated fatty liver disease (MAFLD)/nonalcoholic fatty liver disease (NAFLD) risk factors and pathophysiological markers of hepatic steatosis (HS), nonalcoholic steatohepatitis (NASH), and fibrosis.

TABLE 1 | Performance metrics and calculation formulas of biopsy-free scoring systems for metabolic dysfunction-associated fatty liver disease (MAFLD)/nonalcoholic fatty liver disease (NAFLD) staging.

Biopsy-free scoring systems	Application	NCV	PCV	Sensitivity	Specificity	NPV	PPV
Hepatic steatosis							
NAFLD ridge score[a] (35, 36)	MAFLD/NAFLD	0.24	0.44	**0.91**	**0.90**	0.95	0.70
NAFLD liver fat score[b] (36, 37)	MAFLD/NAFLD	< −0.64	> 0.64	0.86	0.71	ND	ND
Hepatic steatosis index[c] (38, 39)	MAFLD/NAFLD	< 30	> 36	**0.93**	**0.93**	0.84	0.86
Fatty liver index[d] (40–42)	MAFLD/NAFLD	< 30	> 60	0.87	0.86	ND	ND
Lipid accumulation product[e] (36, 43, 44)	MAFLD/NAFLD	ND	ND	0.78–0.85	0.78–0.85	ND	ND
Nonalcoholic Steatohepatitis							
CA index[f] (45)	NASH/Fibrosis	< 10.27	> 10.27	0.81	0.83	0.92	0.63
NAFIC score[g] (46)	NASH/Fibrosis	< 1.00	> 2.00	0.63	0.64	0.69	0.36
NASH diagnostics (47)	NASH	0.20	0.34	0.77	0.87	0.73	0.89
G-NASH model[h] (48)	NASH	ND	ND	0.73	0.32	0.59	0.54
ClinLipMet score[i] (49)	NASH	ND	ND	0.86	0.72	0.95	0.45
Fibrosis							
APRI[j] (50)	Fibrosis	< 0.60	> 1.50	0.74	0.67	0.72	0.70
Fibrosis-4 index[k] (29, 51)	Advanced fibrosis	< 1.30	> 1.30	0.84	0.68	0.95	0.70
Forns index[l] (30)	Advanced fibrosis	< 4.20	> 6.90	0.29	**0.95**	0.70	0.78
BARD score[m] (52)	Advanced fibrosis	0.1	> 3.25	0.88	0.88	0.96	0.68
NAFLD fibrosis score[n] (53)	Fibrosis	< −1.45	> 0.67	0.82	0.77	0.93	0.93
Hepamet fibrosis score (54)	Advanced fibrosis	< 0.12	> 0.47	0.74	**0.97**	0.92	0.76
Enhanced liver fibrosis test° (55)	Advanced fibrosis	< 7.70	> 9.80	0.74	**0.92**	0.92	0.75
Fibrometer[p] (56)	Advanced fibrosis	0.31	0.38	0.78	**0.95**	0.92	0.87
FibroMax (57)	NASH/Fibrosis	ND	ND	0.64–0.74	0.60–0.73	0.23–0.87	0.51–0.94
Other Biopsy-Free Scoring Systems							
BAAT score[q] (39, 58)	Fibrosis	0–1.00	> 2.00	0.71	0.8	0.86	0.61
Nice model (59, 60)	Advanced fibrosis	ND	0.14	0.84	0.86	0.98	0.44
OW liver test (61, 62)	NASH	< 0.54	> 0.54	0.83	**0.94**	0.90	0.89
NASH score (63)	NASH	ND	2.12	0.71	0.73	0.53	0.83
GlycoNASH test (64)	NASH	ND	ND	0.67	0.64	ND	ND
Liver biopsy (65)	All	-	-	**0.93**	**0.95**	-	-

NCV, negative cutoff value; PCV, positive cutoff value; NPV, negative predictive value; PPV, positive predictive value; ND, not determined; MAFLD, metabolic dysfunction associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis. Bold values denote figures of sensitivity and specify above 0.90.

Calculation formulas:

[a] $NRS: -0.614 + 0.007 \times ALT - 0.214 \times HDLC + 0.053 \times triglycerides + 0.144 \times HbA1c + 0.032 \times WBC + 0.132 \times hypertension.$

[b] $NLFS{:}1.18 \times MS + T2DM\ (2\ if\ yes;\ 0\ if\ no) + 0.15 \times fasting\ insulin\ (mU/L) + 0.04 \times AST(U/L)\ 0.94 \times (AST/ALT)\ 2.89.$

[c] $HSI: 8 \times (ALT/AST\ ratio) + BMI\ (+2,\ if\ female;\ +2,\ if\ T2DM).$

[d] $FLI: e^{0.953} \times Log_e{}^{(TG)} + 0.139 \times BMI + 0.718 \times Log_e{}^{(GGT)} + 0.053 \times WC - 15.745/[1 + e^{0.953} \times Log_e{}^{(TG)} + 0.139 \times BMI + 0.718 \times Log_e{}^{(GGT)} + 0.053 \times WC - 15.745] \times 100.$

[e] $LAP: (WBC\ 65) \times triglycerides\ if\ male;\ (WBC\ 58) \times triglycerides\ if\ female.$

[f] $CA: (0.994 \times type\ IV\ collagen\ 7S + 0.0255 \times AST).$

[g] $NAFIC{:}(ferritin \geq 200\ ng/mL\ [female]\ or \geq 300\ ng/mL\ [male]: 1\ point) + (fasting\ insulin \geq 10\ IU/mL : 1\ point) + (type\ IV\ collagen\ 7\ s \geq 5.0\ ng/mL : 2\ points).$

[h] $G-NASH: 0.02 \times GP73\ (ng/ml) + 0.123 \times AST\ (U/L) + 0.1576 \times zinc\ (\mu mol/L) + 0.0227 \times total\ thyrosine\ (nmol/L) - 0.4525 \times SDPV\ (fL) + 2.0789 \times (BMI \geq 30\ kg/m2,\ yes = 1,\ no = 0).$

[i] $ClinLipMet: -0.305 + 0.562 \times PNPLA3\ genotype\ (CC - 1/GC - 2/GC - 3)\ 0.0092 \times fasting\ insulin\ (mU/L) + 0.0023 \times AST\ (IU/L) + 0.0019 \times (fasting\ insulin \times AST).$

[j] $APRI: \{AST\ (IU/1)/[upper\ normal\ value\ of\ 41\ (IU/l)]\}/platelets\ (\times 10^9/l) \times 100.$

[k] $FIB-4: age \times AST\ (IU/l)/platelets\ (\times 109/l) \times \sqrt{}ALT\ (IU/l).$

[l] $Forns: 7.811 - 3.131 \times ln(platelets) + 0.781 \times ln(GGT) + 3.467 \times ln(age) - cholesterol.$

[m] $BARD{:}(BMI > 28 = 1\ point) + (AAR > 0.8 = 2\ points) + (DM = 1\ point).$

[n] $NFS: 1.675 + [0.037 \times age] + [0.094 \times BMI\ (kg/m^2)] + [1.13 \times abnormal\ FGL\ or\ T2DM\ (yes = 1,\ no = 0)] + [0.99 \times AAR]\ [0.013 \times platelets\ (\times 10^9/l)]\ [0.66 \times albumin\ (g/dl)].$

° $ELF: 2.494 + 0.846\ ln(HA) + 0.735\ ln(PIIINP) + 0.391\ ln(TIMP1).$

[p] $Fibrometer: 0.4184\ glucose\ (mmol/L) + 0.0701\ AST\ (IU/L) + 0.0008\ ferritin\ (\mu g/L) - 0.0102\ platelet\ (G/L) - 0.0260\ ALT\ (UI/L) + 0.0459\ body\ weight\ (kg) + 0.0842\ age + 11.6226.$

[q] $BAAT: (BMI \geq 28 = 1\ point) + (age \geq 50\ years = 1\ point) + (ALT \geq 2N\ (1\ point)) + (triglycerides \geq 1.7\ mmol/L\ (1\ point)).$

NAFLD Ridge Score

This BFSS considers alanine aminotransferase (ALT), hemoglobin A_{1C}, high-density lipoprotein C, hypertension, leukocyte count, and triglycerides (35). The enzyme ALT level increases in serum as hepatocytes are damaged (36). Similarly, high levels of triglycerides, low levels of high-density lipoprotein C, hypertension, and increased hemoglobin A_{1C} level correlate with HS (70, 71). Moreover, increased intrahepatic leukocyte concentration is associated with the progression to NAFLD risk factors and stage-specific markers of NASH (72, 73).

Notably, this score has an area under the receiver-operating curve (AUROC) of 0.87 (74). Nevertheless, it is unreliable for distinguishing steatosis grades (36) and ends up classifying as indeterminate up to 30% of patients (35).

NAFLD Liver Fat Score

Developed in a Finnish population (37), this BFSS weighs aspartate aminotransferase (AST), AST/ALT ratio, fasting insulin, metabolic syndrome, and T2DM (75). Insulin levels correlate with HS grades, as insulin resistance is an important risk factor for the development of MAFLD/NAFLD (70). Moreover, AST levels increase as AST is released from injured hepatocytes, indicating liver dysfunction (36).

This BFSS can predict MAFLD/NAFLD and estimate the liver fat contents >5.56%, with an AUROC of 0.88 (36, 37). Moreover, it has shown a positive correlation with the incidence and mortality of cardiovascular disease, which are outcomes intricately related to metabolic syndrome and T2DM (76). Nonetheless, this score has a poor capacity for quantifying steatosis, as its AUROC for predicting >33% of steatosis significantly decreases at 0.72 (77).

HS Index

This index assesses MAFLD/NAFLD (78) on the basis of body mass index (BMI), AST/ALT ratio, and the presence of T2DM (38). AST/ALT ratio is used to assess the HS grade more accurately than any of its components individually (79). Similarly, both enzymes positively and almost linearly correlated with increased incidence of MAFLD/NAFLD and premature mortality risk (80). In addition, studies have reported that this test has an AUROC of 0.75 (78, 81). Moreover, this BFSS has a high correlation with HS grades diagnosed using ultrasonography, but this score has not yet been validated for NASH (38).

Fatty Liver Index

Created as an algorithm to detect fatty liver (40), this index is based on BMI, gamma glutamyl transferase (GGT), triglycerides, and waist circumference (82). Waist circumference correlates with visceral adiposity, an important predictor of metabolic syndrome (83). Similarly, the accumulation of triglycerides in hepatocytes produces hepatocyte ballooning and inflammation, both changes associated with MAFLD/NAFLD (84). High levels of GGT, in particular, are associated with increased incidence rates of hypertension and insulin resistance (85).

The BFSS has an AUROC of 0.82 for MAFLD/NAFLD detection (86). However, it was validated only in certain populations, such as Koreans (82), Chinese (87), and Northern Italians (40).

Lipid Accumulation Product

The BFSS is used to evaluate waist circumference and triglyceride levels (43). Distinctively, it has been adjusted for age, sex, and ethnicity (88). This score is only validated in a cohort in Northern Italy (89). Although it was originally developed as a reference for cardiometabolic risk, it was later validated as an HS index (36, 44).

Furthermore, it has an AUROC, 0.77 for NAFLD diagnosis and was more accurate in patients with hypertriglyceridemia (AUROC, 0.73) compared with patients with T2DM (AUROC, 0.67) (86). However, even if the BFSS can detect MAFLD/NAFLD clinically, its main limitation is in distinguishing patients with mild disease from those with more severe MAFLD/NAFLD (90).

NASH SCORING SYSTEMS

Nonalcoholic steatohepatitis consists of fatty liver in conjunction with inflammation and hepatocellular injury, with or without fibrosis (91). More than 20% of patients with NASH are expected to develop cirrhosis in their lifetimes (69). Consequently, this section delves into the BFSS proposed for its detection (92, 93).

CA Index

This index owes its name to its two parameters, type IV collagen 7S and AST. Specifically, type IV collagen 7S is an indirect marker of fibrogenesis (94) and AST reiterates its role in liver dysfunction (36). Currently, the BFSS is used to predict NASH and fibrosis, with AUROC of 0.85 and 0.91, respectively (95). Moreover, it identifies MAFLD/NAFLD without fibrosis and NASH-related fibrosis (94, 96). Unfortunately, the CA index was only validated in the Japanese population, similarly to the NAFIC score (97).

NAFIC Score

This score is based on ferritin, fasting insulin, and type IV collagen 7S levels (24, 98). Comparatively, the BFSS is used for evaluating ferritin levels, which increases in patients with NASH (99). Similarly, fasting insulin is considered as a correlation marker for HS (70), and type IV collagen 7S is used, as in the CA index (100).

The BFSS has an AUROC of 0.85 and 0.83 for NASH and fibrosis, respectively (46), both higher than the BARD [0.76 (101)] and NAFLD fibrosis score [0.77 (102)]. Nevertheless, such accuracy has been only validated in Japanese patients (46, 103).

NASH Diagnostics

This biomarker panel is used to diagnose obesity-related NASH based on adiponectin, cleaved cytokeratin 18 (CK-18) M30, and resistin levels (47). Adiponectin is inversely correlated with the risk of metabolic syndrome (104). Similarly, CK-18 M30 is proposed as a differentiator between NASH and MAFLD/NAFLD without inflammation (24, 105). Finally, resistin has been associated with obesity, insulin resistance, and T2DM (106, 107).

The BFFS has a reported AUROC value of 0.90 (47). However, it requires further validation in cohorts other than morbidly obese candidates for bariatric surgery (108). Similarly, a major limitation of its specificity is possibly due to all three of its parameters being increased in various liver diseases (106, 109), thus making them nonspecific markers of NASH (110, 111).

G-NASH Model

This novel BFSS is based on AST, BMI, CK-18 M30, Golgi protein 73, platelets, thyroxine, and zinc (48). Specifically, CK-18 M30 fragments increase in patients with MAFLD/NAFLD and T2DM (112), and correlate positively with high ALT, glucose, and

hemoglobin A_{1C} levels, systolic blood pressure, and triglyceride levels (113). Similarly, Golgi protein 73, which is only expressed in fibrotic and diseased liver tissue, is considered a promising marker of liver inflammation (114).

When grouped (48), these biomarkers identified NASH in patients with MAFLD/NAFLD who had normal ALT levels and those requiring liver biopsy, with an AUROC of 0.85 (48). Nonetheless, the BFSS lacks external validation in other populations and studies to determine its validity for screening patients at risk of developing NASH (48).

ClinLipMet Score

Although it was only tested in Finnish and Belgian Caucasian and morbidly obese populations (49), the BFSS identified patients with NASH, with an AUROC of 0.866 (115). It considers AST and fasting insulin levels; PNPLA3 genotype rs738409, a polymorphism closely associated with increased hepatic fat content (116); and amino acid and phospholipid levels (49).

The levels of Glu, Gly, and Ile amino acids increase during progression to NASH (117). By contrast, phospholipids lysophosphatidylcholine 16:0 and phosphoethanolamine 40:6 are used to determine alterations in cell membrane metabolism in patients with advanced MAFLD/NAFLD and a higher liver fibrosis stage (118, 119). Specifically, these two molecules significantly differentiate NASH from HS but fail to do so in patients with HS and controls (49).

HEPATIC FIBROSIS SCORING SYSTEMS

Chronic injury to liver myofibroblasts is known to induce fibrosis (120). In this regard, the risk of advanced fibrosis in patients with MAFLD/NAFLD is noteworthy (7.5%), along with other liver-related complications and eventually death (52, 121, 122). Correspondingly, the BFSS proposed for the diagnosis of liver fibrosis is scrutinized herein.

AST-to-Platelet Ratio Index

The BFSS is based on AST and platelets, both of which increase in the hepatic sinusoids of patients with MAFLD/NAFLD (123, 124). In addition, it detects advanced fibrosis in patients with chronic hepatitis C virus infection (125) and is later validated for the detection of MAFLD/NAFLD (126).

The AST-to-platelet ratio index (APRI) is considered a good predictor of advanced fibrosis in patients with MAFLD/NAFLD, having an AUROC of 0.71 and 0.79 in non-bariatric and bariatric patients, respectively (127). Notwithstanding, some authors have argued against its widespread use, mainly because of its low accuracy in staging fibrosis (128, 129).

Fibrosis-4 Index

This index had been validated for the assessment and detection of liver fibrosis based on age, ALT level, AST level, and platelet count (130, 131). Platelet count correlates with hepatocyte ballooning, fibrosis, and liver steatosis (123, 124).

Overall, the BFSS has an AUROC ranging from 0.80 to 0.86 (128). Specifically for non-bariatric and bariatric patients, it has an AUROC of 0.83 and 0.81, respectively, which are higher than those obtained for APRI (0.71 and 0.79, respectively) (127). Nonetheless, certain studies have argued that the inclusion of age might lead to a falsely worse score in the elderly population and thus increase the false-positive rate (132).

Forns Index

This index is based on platelet count, cholesterol level, GGT levels, and age (133, 134). The importance of this index relies on GGT, which has been associated with insulin resistance (85), and on cholesterol, which correlates negatively with the liver fibrosis stage, thus aiding in NASH diagnosis (30). In this regard, the BFSS is used as a predictor of advanced fibrosis in patients with chronic hepatitis C virus infections, with an AUROC of 0.79 (30, 105, 134, 135). Notwithstanding, information regarding its accuracy in MAFLD/NAFLD is limited (30).

BARD Score

The BARD score is based on BMI, AST/ALT ratio, and T2DM, all of which are markers of metabolic syndrome (61). Along with the NAFLD fibrosis and FIB-4 scores, the BFSS is validated for the detection of advanced fibrosis or cirrhosis, with an AUROC of 0.76 (101, 130). Even so, its low positive predictive value of 0.42 has limited its use in clinical practice (122). Nonetheless, its high reported negative predictive value of 0.96 makes the BARD score a reliable tool for ruling out advanced fibrosis (52).

NAFLD Fibrosis Score

The BFSS is currently used to predict advanced fibrosis (53), with an AUROC of 0.77 (102), and includes age, hyperglycemia, BMI, platelet count, albumin level, and AST/ALT ratio as parameters (136). Specifically, the albumin binding function and quantity are decreased in patients with long-standing MAFLD/NAFLD (137).

A high score (>0.68) significantly correlated with a 4-fold higher risk of death in patients with MAFLD/NAFLD (5). Nevertheless, this score has a limited value in predicting changes in fibrosis, even when it accurately predicts morbidity and mortality in all stages of fibrosis (138).

Hepamet Fibrosis Score

This novel BFSS is based on age; albumin, AST, and glucose levels; homeostatic metabolic assessment, which positively correlated with a higher stage of liver fibrosis and stiffness (139); insulin level; platelet count; sex; and T2DM (54, 140). It has a high accuracy for advanced fibrosis exclusion (30), with a reported AUROC value of 0.94 for advanced fibrosis prediction (30). Even so, this score had confounding results in patients with T2DM (141), a finding that created uncertainty because more than 70% of such patients concomitantly have MAFLD/NAFLD (142).

Enhanced Liver Fibrosis Test

This test is based on the levels of hyaluronic acid, type III procollagen peptide, and the tissue inhibitor of metalloproteinase 1 (143). Their concentrations and activities make this test useful for grading liver fibrosis (144, 145). In addition, studies have shown that the BFSS is an accurate tool for

detecting advanced fibrosis in patients with MAFLD/NAFLD (146), mainly owing to its AUROC of 0.85 for stage F2 and 0.90 for stage F3 with NASH (147). Recently, a meta-analysis revealed that this fibrosis test has a high sensitivity for advanced fibrosis, but a limited specificity in low-prevalence areas (148).

FibroMeter

On the basis of markers, such as age, ALT level, AST level, body weight, ferritin level, glucose level, and platelet counts (149). FibroMeter identifies fibrotic areas and fibrosis stage (150), with higher reproducibility when compared with other diagnostic tools (149). Quantitatively, FibroMeter has AUROC values of 0.94, 0.93, and 0.9 for significant fibrosis, advanced fibrosis, and cirrhosis, respectively (58, 149). Furthermore, its results for fibrotic areas have an AUROC of 0.94, which is more accurate in comparison with that of the NAFLD fibrosis score (0.88) and APRI (0.87) (7, 7, 149, 151, 152). Nonetheless, some authors argued that ethnicity-specific cutoff values would increase its validity (153).

FibroMax

FibroMax is a BFSS that combines five components into one algorithm (154). Among the components, ActiTest showed a significant accuracy in NASH diagnosis and MAFLD/NAFLD differentiation (155). It is considered as an accurate score for liver fibrosis (154, 156), with an AUROC of 0.68 for grade 2 and 3 steatosis, 0.59 for NASH, and 0.79 for fibrosis (157).

Furthermore, studies reported that FibroTest, another component of FibroMax, had higher accuracy in discriminating severe fibrosis stages and detecting cirrhosis than low to intermediate stages (158). FibroTest is not accurate for differentiating between the zonal distribution of fibrosis in MAFLD/NAFLD; thus, its effectiveness has been controversial (156).

Nonetheless, both components are affected by acute hemolysis, inflammation, and extrahepatic cholestasis (51). Similarly, in response to its low AUROC, they are considered unreliable alternatives for liver biopsy in MAFLD/NAFLD (157).

DISCUSSION

Numerous authors have proposed biopsy-free scoring systems as screening tools for fatty liver and risk-stratifying systems based on fibrosis (51, 144, 159) for the MAFLD/NAFLD spectrum (95). Nonetheless, they still emphasize the importance of liver biopsy as the diagnostic standard but urge for a clear identification of biopsy indications (conflicting clinical or serological data), an issue that can be addressed with noninvasive diagnostic tools, such as BFSS (160–162). Some BFSSs addressed in this review (G-NASH, ClinLipMet, and enhanced liver fibrosis test) measure components that are not readily available, seldom ordered, or expensive, such as the PNPLA3 genotype, CK-18 M30 fragments, Golgi protein 73, or the tissue inhibitor of metalloproteinase 1. Comparatively, other scores, such as the lipid accumulation product, fatty liver index, HS index, APRI, fibrosis-4 index, Forns index, and NAFLD fibrosis score rely on routinely ordered components, thus facilitating their use. Furthermore, as patients develop more metabolic abnormalities, they tend to yield higher scores (163, 164), making these BFSSs more reliable as the condition of the patient worsens. However, some scores have been validated only in limited populations, such as the CA index (97), fatty liver index (40, 82, 87), and NAFIC score (46, 103), whereas others are inaccurate for MAFLD/NAFLD staging [FibroMax (157)] or when associated with other comorbidities [Hepamet fibrosis score (142)]. These limitations must be addressed through validation in other populations (97), with attention to variables, such as BMI, comorbidities, and ethnicity (49, 125, 143, 165–167). Comparatively, other BFSSs have been shown to have high sensitivity, such as the NAFLD ridge score (35, 36) or HS index (38, 39), and specificity, such as the Hepamet fibrosis score (54), Forns index (30), and enhanced liver fibrosis test (55), making them accurate tests for screening and confirmation of disease, respectively. Certain BFSSs underperformed in validation studies, such as the BAAT score (168), Nice model (59, 60), OW liver test (61, 62), NASH score (63), CHeK model (165), or GlycoNASH test (64), making them unsuitable alternatives for MAFLD/NAFLD diagnosis; thus, they were consequently excluded from the scrutiny of this review. Comprehensive studies on the effects of age, BMI, obesity, and the prevalence rates in different populations (101, 140, 148, 169) are required to determine the role of current and future BFSSs in MAFLD/NAFLD diagnosis. Other non-invasive alternatives have been proposed recently, such as cell-free DNA, which has been found in extracellular vesicles in the serum of patients with fatty liver, and have yielded promising results (170). Moreover, novel considerations, such as the addition of enhanced liver fibrosis test to clinical practice guidelines (171, 172) will eventually play a larger role in the diagnosis and follow-up of patients. As more information is gathered, novel considerations will be implemented, aiding in a more precise understanding and accurate detection of MAFLD/NAFLD in the global population (173).

CONCLUDING REMARKS

Clinicians are urged to include BFSS for the diagnosis of early stages of MAFLD/NAFLD, particularly in patients with a high risk of liver fibrosis, even if these are still outperformed by biopsy in terms of accuracy. Increasing the awareness of the available BFSSs for staging is paramount to improving patient safety. The ever-growing MAFLD/NAFLD pandemic urges clinicians to seek alternatives for screening, early diagnosis, and follow-up, especially for those with contraindications for liver biopsy.

AUTHOR CONTRIBUTIONS

NS-A, CV-C, and PT-C contributed to the conceptualization of this manuscript and its graphic elements, wrote and revised the original draft, and contributed to the discussion, abstract, and final version of the manuscript. CV-C further contributed to the revision, completion, and content improvement of the manuscript. PT-C further oversaw the general progress of the

study, initial revision of the manuscript, structuring of the draft, and final revision of the manuscript, figures, and tables. All authors revised and agreed to the final version of the manuscript.

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MiR-200c-3p Regulates DUSP1/MAPK Pathway in the Nonalcoholic Fatty Liver After Laparoscopic Sleeve Gastrectomy

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Aim: Non-alcoholic fatty liver disease (NAFLD) is a health burden worldwide, which is closely related to obesity. The effect of sleeve gastrectomy (SG) on NAFLD is efficient, and the underlying mechanism remains unknown. Our study sought to investigate the mechanism of dual-specificity protein phosphatase 1 (DUSP1) expression regulation following the SG procedure in NAFLD patients and C57BL/6J mice *via* miR-200c-3p.

Methods: The serum was extracted from NAFLD patients who underwent laparoscopic sleeve gastrectomy (LSG) and volunteers. Next, the correlation between miR-200c-3p and DUSP1 was identified *in vitro*. NAFLD mice were modelled by high-fat diets (HFD). The hepatic tissue expression levels of miR-200c-3p, DUSP1, phospho-extracellular regulated protein kinases1/2 (p-ERK1/2), phospho -p38 mitogen-activated protein kinases (p-p38), and phospho-c-Jun N-terminal kinases (p-JNK) induced by SG procedure were evaluated.

Results: The SG procedure contributed to significant weight loss, reduced lipids in NAFLD patients and mice. The increased expression level of miR-200c-3p and reduced expression of DUSP1 were observed in NAFLD patients and mice ($p<0.05$). The reduced expression levels of miR-200c-3p and increased expression of DUSP1 were observed in patients and mice with NAFLD who underwent SG procedure. DUSP1 is a potential target of miR-200c-3p.

Conclusions: A novel mechanism was identified in which miR-200c-3p regulates the MAPK-dependent signals that are linked to the promotion of hepatosteatosis *via* DUSP1 after sleeve gastrectomy. The findings suggested that miR-200c-3p should be further explored as a potential target for the treatments of NAFLD.

Keywords: NAFLD, sleeve gastrectomy, miR-200c-3p, DUSP1, MAPKs

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INTRODUCTION

NAFLD is the most common cause of liver disease worldwide, considered the hepatic manifestation of metabolic syndrome associated with obesity, insulin resistance (IR), dyslipidemia, diabetes, and heredity (1). NAFLD encompasses a spectrum of diseases includes nonalcoholic hepatic steatosis, nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma(HCC) (2, 3).

Currently, nearly 1 billion people worldwide are affected by NAFLD due to the dramatic increase in obesity, with the incidence of NAFLD in the Asian population reaching about 27% (4). The most recent national data showed that 16.4% of Chinese adults had obesity (BMI 28.0 kg/m² or higher) and another 34.3% were overweight (24.0–27.9 kg/m²) (5).

Overweight and obesity were the sixth leading risk factor for death and disability (6). Obesity is an independent risk factor for NAFLD, and weight loss is the only safe and effective treatment for NAFLD (7, 8). A recent randomized controlled study found that a low-calorie diet sustained for 3 months could significantly reduce body weight (4.5%) and improve liver enzymes, nevertheless, without significant changes in liver adipose degree (9). Physical exercise is recommended as a routine treatment for NAFLD (10). Metformin, sodium-glucose transporter 2 (SGLT2) inhibitors, lipase inhibitors, and Glucagon-like peptide 1 (GLP 1) receptor stimulants all have been reported weight reduction effects (11–13). However, it is difficult for most obese patients to achieve and maintain an ideal state of body mass after lifestyle intervention and medical treatment, and surgical treatment should be considered.

Bariatric surgery is the most effective way to lose weight in morbid obesity. In patients with biopsy-proven NAFLD/NASH, bariatric procedures are similarly effective in improving liver function (1). Nevertheless, fatal hyper-ammonemic encephalopathy encompassing genetic and non-genetic causes was reported after laparoscopic Roux-Y gastric bypass (LRYGB) (14, 15). Absorption-restricted surgery may cause the displacement of intestinal flora and the activation of the inflammatory system, which causes endotoxin damage to the liver, and now there are some cases of liver function deterioration (16–18). No exacerbation of NAFLD after laparoscopic sleeve gastrectomy (LSG) has been reported. LSG is a bariatric surgical technique that can result in considerable weight loss with negligible complications (19).

DUSP1, also known as MKP 1(mitogen-activated protein kinase phosphatase 1, MKP 1), localizes to the nucleus, can be activated by stress or misuse induction, and can selectively inactivate the MAPK signaling pathway (20). DUSP1 was downregulated 2.04 FC (fold change, FC) between Definite NASH and Not NAFLD, 1.94 FC between Definite NASH and Borderline (21). MAPK signaling pathway is involved in the two "hits" of NAFLD.

MicroRNAs are small, non-coding RNAs that are essential post-transcriptional regulators of gene expression. By binding to the 3' untranslated region (3'UTR) of their target genes, they globally repress gene expression (22). The gene encoding miRNA is located in the nucleus, and the miRNA is transcribed into pre-miRNA with the action of RNA polymerase pol II. Dorsha endonuclease further transformed it into an intermediate pre-miRNA with a stem-loop structure of about 60 bases, which is then transported into the cytoplasm. After the action of Dicer endonuclease, the intermediate pre-miRNA formed an incomplete pairing miRNA-miRNA double-stranded complex. It also plays a role in gene expression regulation through the formation of a nucleic acid-protein complex (miRNP) (23–25). A previous study reported that miR-200c-3p was up-regulated in NAFLD of rats (26). However, there was no in-depth investigation on miR-200c-3p before and after bariatric surgery.

This study aims to investigate the mechanism of dual-specificity protein phosphatase 1 (DUSP1) expression regulation following the sleeve gastrectomy (SG) procedure in NAFLD patients and C57BL/6J mice *via* miR-200c-3p. In our study, we demonstrated that miR-200c-3p regulates DUSP1 expression in the HepG2 cell line, and its expression was increased in NAFLD patients and mice. Our results suggested that the SG procedure could significantly ameliorate the NAFLD compared with food restriction, miR-200c-3p expression level was decreased after the SG procedure. Decreased expression of miR-200c-3p increased hepatic DUSP1, decreased MAPK activity, which plays a protective role in the development and progression of NAFLD.

MATERIALS AND METHODS

Ethics Statement

All the procedures within this study were performed following the Helsinki declaration of 1975, 1983 revision. All human and animal studies were approved by the committee of our center (YN2020-028-04). All methods were carried out following the relevant guidelines and regulations.

Human Subjects Experimentation

The study was a retrospectively observational design, which consisted of 7 NAFLD patients with BMI> 27.5 kg/m^2 who underwent LSG and five healthy volunteers with no NAFLD admitted to our center from September 2018 to September 2019. Inclusion criteria: (a) the age of patients and volunteers was at least 18 to 60 years old (including both ends) at the time of signing the informed consent; (b) meeting the LSG surgical indications and successfully performing LSG surgery; (c) being able to follow the case manager's requirements for a regular diet and out-patient visit follow-up;(d) being approved by the ethics committee; (e) the diagnostic criteria for NAFLD and the criteria for ultrasound evaluation were following the guidelines for the prevention and treatment of NAFLD (27). Exclusion criteria: (a) patients underwent LSG procedure but not complicated with NAFLD; (b) failure to follow up regularly after surgery; (c) alcoholic liver disease, patients who drink alcohol equivalent to the amount of ethanol >140g per week for men or >70g for women; (d) viral hepatitis, drug-induced liver disease, total parenteral nutrition, hepatolenticular degeneration, autoimmune liver disease and other specific diseases that can lead to NAFLD; (e) liver function damages caused by other causes.

The clinical biochemical and physical indicators of the patients were followed up, the expression of miR-200c-3p in peripheral sera was determined by quantitative real-time transcription-polymerase chain reaction (qRT-PCR) and the expression of DUSP1 in the blood was determined by enzyme-linked immunosorbent assay (Elisa). BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Total serum cholesterol and triglycerides, hepatic enzyme, and

other routine laboratory tests were measured as previously. Patients were requested to withhold alcohol and caffeine for at least 12 h before the collecting of blood samples. The blood samples were collected one day before the surgery, the 1st month, 3rd month, and 6th month after surgery, respectively.

Elisa

ELISA kit (SEC902Hu, USCN KIT INC) was pre-coated with an antibody specific to the DUSP1 antibody. Standards or samples were then added to the appropriate microplate wells with a biotin-conjugated antibody specific to DUSP1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Followed by incubation at room temperature. After tetramethylbenzidine (TMB) substrate solution was added, only those wells that contain DUSP1, biotin-conjugated antibody, and enzyme-conjugated. After the termination of the reaction, the uncoupled conjugate was washed away. Avidin will exhibit a color change to blue and turn yellow after the addition of an acidic stop solution. The density of yellow and the content of DUSP1 in the samples were in proportion to the bottom of the kit. Then, the concentration of DUSP1 in the samples was then determined by comparing the optical density (OD) of the samples to the standard curve.

Animal Model of NAFLD

Seven-week-old male C57BL/6J mice (n=30) (approx 22gm body weight) were obtained from Liaoning Changsheng biotechnology company limited and maintained on a standard chow diet ad libitum and a standard 12 h:12 h light/dark cycle until eight weeks of age. Mice at this age were then given a high-fat diet (Research Diets D12451, 45 kcal% saturated fat, n=22), and regular chow (5% fat, 53% carbohydrate, and 23% protein) was given to control rats (n = 8) for 12 weeks. One mouse was randomly selected from the control and NAFLD groups respectively, and the hepatic tissue was harvested and stained with Hematoxylin and eosin (HE) and oil red O methods. After confirming the successful modeling of NAFLD, the NAFLD group was then randomly divided into three groups, followed by the NAFLD+SG group (n=7), NAFLD+Food restriction group (FR, n=7) and NAFLD+Sham surgery group (n=7). The mice in the CON group were maintained on standard chow for 18 weeks. The weight and food intake of the mice in the different groups were documented weekly.

Serum Biochemical Assays

At the end of the experiment, all mice were sacrificed, peripheral blood was collected from the vein of the inner canthus. Triglycerides (TG), total cholesterol (TC), alanine aminotransferase (ALT), aspartate aminotransferase (AST), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterin (LDL-c) were assayed.

SG Surgery

Four percent of isoflurane was used to induce anesthesia of operated mice, and 2% isoflurane was used to maintain anesthesia. Seventy to eighty percent of the lateral stomach was excised, leaving a tubular gastric remnant in continuity with the esophagus superiorly and the pylorus and duodenum inferiorly. The NAFLD+SHAM group involved analogous isolation of the stomach followed by manually applying pressure with blunt forceps along a vertical line between the esophageal sphincter and the pylorus. After the surgery, mice were maintained on a liquid diet (ENSURE) during the 7-day recovery period. The HFD diet in the NAFLD+SG group was weighed once a day to calculate the average daily intake of each mouse. The NAFLD+FR group was kept the same designated diet as the NAFLD+SG group after the 7-day recovery period. Both NAFLD+SG and NAFLD+SHAM groups received gentamicin for seven days after surgery.

RNA Preparation and Quantitative Real-Time PCR (qRT-PCR)

Human peripheral blood serum was centrifuged at 3000 RPM (Revolution Per Minute) for 5 minutes and refrigerated at -80°C, total RNA was extracted using TRIpure (RP1001, BioTeke, Beijing) following the manufacturer's instructions. Mice hepatic tissue and HepG2 cells were lysed, and total RNA was extracted using TRIpure (BioTeke, Beijing). For microRNA analysis, miRNA-specific cDNA was generated with Super M-MLV reverse transcriptase (BioTeke, Beijing). Primer sequences were synthesized in GenScript Biotechnology (**Table 1**) and followed by qRT-PCR using SYBR Green master mix (Solarbio, Beijing) in Exicycler 96 (BIONEER, Korea). Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method.

MRNA Extraction and Quantitative Analysis

RNA was isolated using TRIZOL (RP1001, BioTeke, Beijing) from the HepG2 cells and hepatic tissue of C57BL/6J. RT-PCR was performed using BeyoRT II M-MLV reverse transcriptase (Beyotime, Shanghai, China) and a custom-made DUSP1 primer (GenScript Biotechnology, **Table 1**). The data were normalized to β-actin mRNA. RT-PCR was conducted in a reaction volume of 20 μl.

Western Blotting

The hepatic tissue was rapidly removed after the mice's sacrifices, immediately frozen in liquid nitrogen. The hepatic tissue of mice and HepG2 cells were lysed in Whole-Cell Lysis

TABLE 1 | Primer sequences for RT-qPCR.

NAME*	Sequence(5'→ 3')
U6 F	CGCAAGGATGACACGCAAAT
U6 R	GCAGGGTCCGAGGTATTC
mmu-miR-200c-3p F	GCCGGGTAATGATGGAGT
mmu-miR-200c-3p R	GCAGGGTCCGAGGTATTC
hsa-miR-200c-3p F	GCCGGGTAATGATGGAGT
hsa-miR-200c-3p R	GCAGGGTCCGAGGTATTC
DUSP1 mRNA F	GTGCCTATCACGCTTCTCG
DUSP1 mRNA R	CCTCCACAGGGATGCTCTT
β-actin F	CTGTGCCCATCTACGAGGGCTAT
β-actin R	TTTGATGTCACGCACGATTTCC

Abbreviations: F, forward primer; R, reverse primer.

Assay (Solarbio, China) for 5 min on ice and centrifuged (12 000 rpm, 10 min, and 4°C). Protein concentrations were measured using a bicinchoninic acid assay kit protein assay kit (Wanleibio, China) according to the manufacturers' instructions. The diluted protein samples were mixed with loading buffer (5×; Wanleibio, China). Then the samples were boiled for 5 min at 95˚C. Proteins (40ug/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to the PVDF membrane (80V, 1.5h). The membranes were blocked by a solution of 5% non-fat dried milk or albumin from bovine serum (BSA) in Tris-buffered saline with Tween (TBST) for 1h at room temperature. The membranes were then incubated at 4 overnight with primary antibodies for DUSP1 antibody (the dilution ratios 1:1000, Abclonal, China), β-actin antibody (the dilution ratios 1:400, Wanleibio, China), p-ERK1/2(the dilution ratios 1:400, Wanleibio, China), p-p38 (the dilution ratios 1:500, Wanleibio, China), and p-JNK(the dilution ratios 1:500, Wanleibio, China). After washing with the TBST, membranes were incubated with IgG-horseradish peroxidase-conjugated sheep anti-rabbit secondary antibodies (the dilution ratios 1:5000, Wanleibio, China) for 45 min at 37˚C. Membranes were then washed six times with TBST and the chemiluminescent signals were detected with enhanced chemiluminescent (ECL) luminous fluid (Wanleibio, China) using the Gel-Pro-Analyzer System (WD-9413B, China).

Bioinformatics Analysis

The bioinformatics analysis for identifying target genes and microRNAs was done using StarBase and TargetScan software tools.

Transcriptional Activity by Luciferase Reporter Assays

Briefly, the 293T cells purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology were cultured in DMEM (Dulbecco's modified eagle medium, Gibico) containing 10% fetal calf serum at 37°C under 95% humidity and 5% CO_2. Subsequently, the pmirGLO-DUSP1-3'UTR-wt (Genscript, Nanjing) or pmirGLO-DUSP1-3'UTR-mut vector (Genscript, Nanjing), along with the miR-200c mimic or the mimic-control, were transfected with Lipofectamine ® 2000 into 293T cells. Following 48 h, the cells were then lysed using passive lysis buffer, and the luciferase activity was measured using a Dual-Luciferase Reporter Gene Assay Kit (KeyGEN BioTECH, China). Renilla luciferase activity was used for the normalization of the firefly luciferase activity. The luciferase enzyme activity was presented as fold-change relative to the vehicle control.

Cell Culture and Transfection

The HepG2 cells were seeded in DMEM (Dulbecco's modified eagle medium, Gibico) medium containing 10% fetal bovine serum for 24 h before transfection and transfected with miR-200c mimics (Jintuosi, China), miR-200c inhibitor (Jintuosi, China), and NC (Jintuosi, China) for 44h. The groups were as follows: A: Non-transfected group (HepG2); B: Negative mimic control group (HepG2+mimic-NC); C: Transfected group with mimic (HepG2+ miR-200c-3p mimic); D: H_2O_2 intervention in the mimic group (HepG2+miR-200c-3p mimic+H_2O_2); E: Negative control group (HepG2+inhibitor NC); F: Inhibitor transfection group (HepG2+miR-200c-3p inhibitor); G: H_2O_2 intervention group (HepG2+ miR-200c-3p inhibitor+H_2O_2). The cells were cultured in an incubator at 37°C with 5% CO_2 until the cells adhered to the wall. Transfection could be performed when cell density was 70%. Preparations were as follows: Solution 1: Optimized solution 100μ L + LIPO 2000 6μ L, fully mixed and placed at room temperature for 5 min; Solution 2: Optimized solution 100μ L + fragment 100pmol, the same as solution 1. After mixing the two solutions thoroughly and evenly, let them stand for 20 minutes at room temperature. After the above solution was slowly added to the cells, gently shaken continuously, and then the cells were cultured in an electrothermal constant temperature incubator (37°C, 5%CO_2). The Group D and G cells were treated with H_2O_2 (100 uM) for four hours. Cells in different groups were harvested after 48 h of transfections for the subsequent detections.

Haematoxylin-Eosin Staining

Haematoxylin-eosin staining (HE) was conducted according to standard frozen section protocols. Briefly, after the sections were embedded, ten μm longitudinal sections were rinsed three times in distilled water for 5 mins respectively, and then stained with hematoxylin solution for 5 mins followed by 3 secs in 1% acid ethanol (1% HCl in 70% ethanol), then the sections were washed with tap water for 10 mins. After that, the sections were rinsed in distilled water for 2 mins. Then the sections were stained with eosin solution for 3mins and followed by dehydration with graded alcohol. Next, routine dehydration, clearing, and mounting were performed: 95% ethanol (I) for 5 mins, 95% ethanol (II) for 5 mins, xylene (I) for 10 mins, and xylene (II) for 10 mins. Finally, the sections were mounted by neutral resin, which was then observed and photographed using an Olympus BX53 fluorescence microscope (Tokyo, Japan).

Lipid Deposition Analysis by Oil Red O Staining

Histological visualization of lipid deposition in the mice's hepatic tissue was carried out using oil red O staining. Briefly, the mice liver sections were first incubated in distilled water for 2 mins, then 60% propylene glycol for 2 mins, and then in oil red O solution for 5 mins. The sections were soaked in the 60% propylene glycol until the interstitial tissue was colorless. After being rinsed for 2 mins with distilled water, the slides were incubated at 37°C with Hematoxylin for 1 min, then mounted with gelatin mounting medium.

Statistical Analysis

The continuous variables were shown as the mean and standard deviation; the categorical variables were shown as the number and percentage. Each experiment was performed in at least triplicate. Paired t Student tests were used to compare baseline data with pre-and postoperative ones. One-way analysis of variance (ANOVA) was used to compare the means of multiple samples in a group design. Wilcoxon tests were used for non-normal distributions. Statistical analysis was performed using SPSS version 23.0 (IBM Corporation, New Orchard Road

Armonk, NY 10504. Produced in the United States of America). The graphics were performed using Graph Pad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). A p value<0.05 was considered statistically significant.

RESULTS

LSG Induced Weight Loss and Amelioration of NAFLD

There was no death or serious complication during the follow-up period. According to the general hepatic ultrasound results, only 1 patient showed no improvement during the follow-up period. Changes in BMI were plotted over time from surgery in obese patients combined with NAFLD (**Figures 1A**–**C**). ALT, AST, TG showed a downward trend (**Figures 1D**–**F**) and TC, HDL-C, LDL-C showed an upward trend after the operation (**Figures 1G**–**I**).

LSG Downregulated miR-200c-3p and Upregulated DUSP1

The expression level of miR-200c-3p in the NAFLD group was upregulated and DUSP1 downregulated compared with healthy volunteers (**Figures 2A, E**). The expression level of miR-200c-3p was downregulated and DUSP1 was upregulated at the 3rd month postoperation with statistical significance (**Figures 2C, G**), the trend was more obvious at the 6th month (**Figures 2D, H**).

MiR-200c-3p Bonded With DUSP1 3'UTR to Inactive DUSP1 Expression

We observed 3'UTR of DUSP1 mRNA showing a complementary site for the seed region of miR-200c-3p using miRNA target prediction programs (StarBase and TargetScann, **Figures 3A, B**). Western Blotting and RT-qPCR were used to confirm the role of miR-200c-3p in regulating DUSP1 expression in the HepG2 cell line. The miR-200c-3p expressions in the different groups were determined with RT-qPCR (**Figure 3C**). The DUSP1 mRNA and protein expression levels were lower in the miR-200c-3p mimic group and higher in the inhibitor group than in the CON group (**Figure 3D**).

H_2O_2 (100uM) was used in the miR-200c-3p mimic and inhibitor groups for stimulating oxidative damage. The results showed that the DUSP1 mRNA and protein expression levels

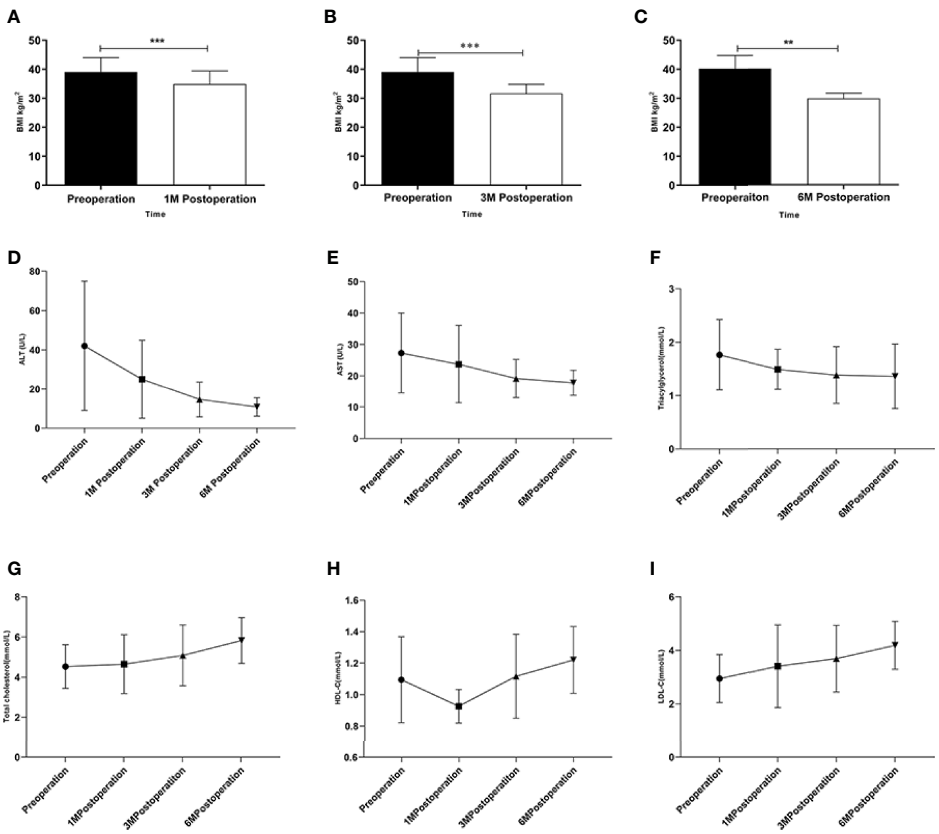

FIGURE 1 | Changes in obese patients combined with NAFLD underwent LSG. **(A–C)** Changes in BMI after the LSG procedure during the follow-up at four time points; **(D–I)** Changes in ALT、AST、TC、TG、HDL-C and LDL-C after LSG procedure during the follow-up at four time points. **p < 0.01; ***p < 0.001. Measurement data were expressed as mean ± standard deviation and compared by paired t-test or one-way ANOVA.

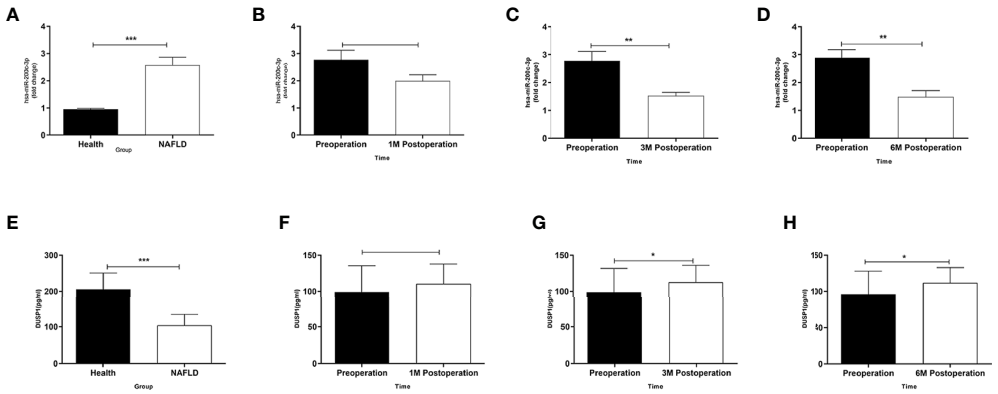

FIGURE 2 | Different expressions of miR-200c-3p and DUSP1 in serum of patients. **(A)** MiR-200c-3p expressions in serums of NAFLD patients and healthy volunteers determined by RT-PCR (normalized to U6). **(B–D)** Changes in miR-200c-3p in serum of obese patients combined with NAFLD after LSG procedure during follow-up at four time points. **(E)** DUSP1 expressions in serums of NAFLD patients and healthy volunteers determined by ELISA. **(F–H)** Changes in DUSP1 in serum of obese patients combined with NAFLD after LSG procedure during follow-up at four time points. *p < 0.05; **p < 0.01; ***p < 0.001. Measurement data were expressed as mean ± standard deviation and compared by paired t-test.

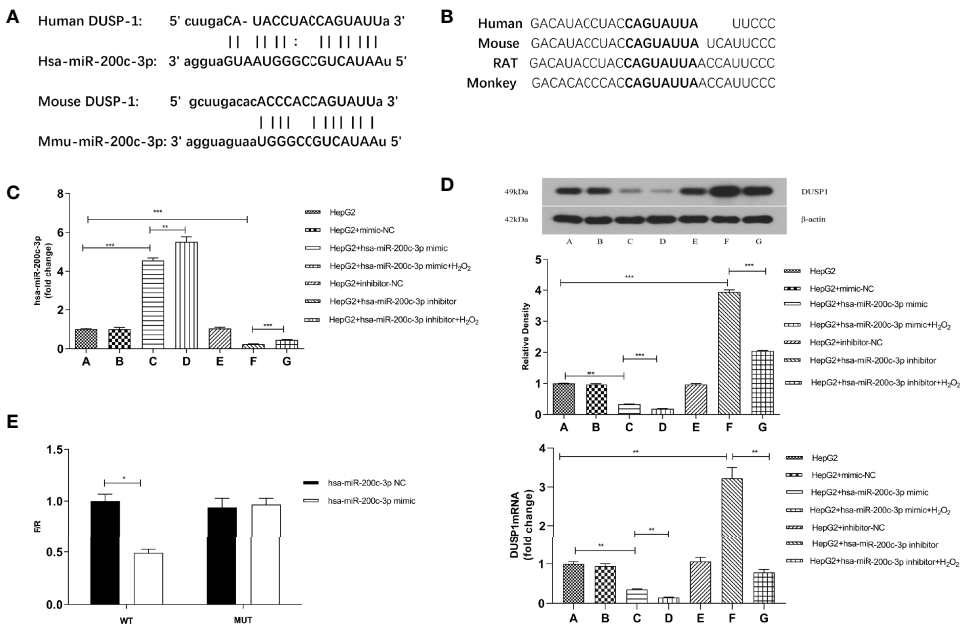

FIGURE 3 | DUSP1 expression was directly regulated by miR-200c-3p. **(A)** Bioinformatics prediction of the binding sites between miR-200c-3p and DUSP1. **(B)** The predicted 8-mer binding region (black bold sequence) of miR-200c-3p. **(C)** miR-200c-3p expressions in different groups of HepG2 cell line. **(D)** The DUSP1 mRNA (normalized β-actin) and protein expressions (normalized to β-actin) in different groups of HepG2 cell line. **(E)** The luciferase activity of WT-DUSP1-3'UTR and MUT-DUSP1-3'UTR detected by dual-luciferase reporter assay. *p < 0.05; **p < 0.01; ***p < 0.001. Measurement data were expressed as mean ± standard deviation and compared by paired t-test.

were lower in the H_2O_2 groups and the miR-200c-3p were upregulated (**Figure 3D**).

The predicted region, containing the wildtype or mutant seed sequence of miR-200c-3p in the 3'UTR of DUSP1, was cloned into the luciferase reporter plasmid. The 293T cells transfected with the miR-200c-3p mimic and the DUSP1-3'UTR had a lower luciferase intensity, but the mutant reporter-transfected group did not, thus indicating that DUSP1 was a direct target of miR-200c-3p (**Figure 3E**).

SG Induced Weight Loss, Amelioration of Lipid Panel and Liver Functions, Downregulated miR-200c-3p and Upregulated DUSP1 mRNA and Protein Expression in C57BL/6J Mice

The bodyweight of C57BL/6J mice in different groups was measured weekly following SG procedures (**Figure 4A**). At the 6th week postoperatively, the bodyweight in the NAFLD+SG group was significantly decreased compared with the NAFLD +FR group and NAFLD+ SHAM group (**Figure 4A**). Consistent with the changes in body weight, lipid panel, and hepatic enzymology analysis also demonstrated that TC、TG、LDL-C、ALT、AST of the NAFLD+SG group were greatly lowered compared with the NAFLD+SHAM and NAFLD+FR group (**Figures 4B–F**). The HDL-C of the NAFLD+SG group was statistically increased compared with NAFLD+SHAM and NAFLD+FR group (**Figure 4G**). Our results demonstrated that miR-200c-3p were distinctly increased in the NAFLD+SHAM group, compared with the CON group (**Figure 4H**). The SG procedure decreased the miR-200c-3p expression (**Figure 4H**). On the contrary, the expression level of DUSP1 mRNA and protein were upregulated in the NAFLD+SG group compared with NAFLD+SHAM and NAFLD+FR group (**Figure 4I**).

SG Causes the DUSP1 Mediated Amelioration of NAFLD

HE staining and oil red O staining results were described and diagnosed by two pathologists without special morphometric analysis. Results of the oil red-O dyed-tissues and HE staining showed that the fat deposition in the NAFLD group was greatly enhanced compared with the CON group, but the SG procedure significantly repressed lipid deposition in the NAFLD+SG group

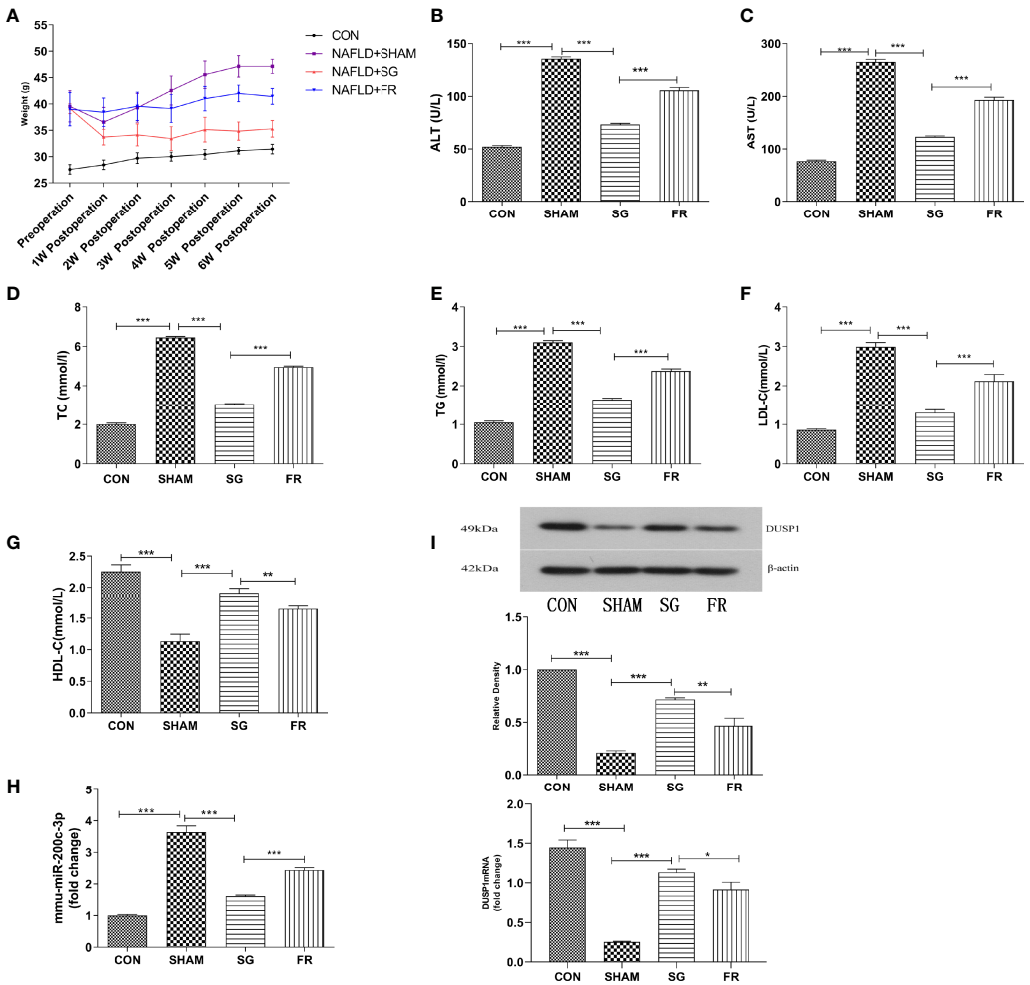

FIGURE 4 | Weight loss, amelioration of lipid panel and liver functions, downregulated miR-200c-3p, and upregulated DUSP1 mRNA and protein expression in C57BL/6J mice. **(A)** Changes in body weight of C57BL/6J mice in different groups. **(B–G)** Different expressions of ALT、AST、TC、TG、LDL-C、HDL-C in different groups of C57BL/6J mice after SG procedure. **(H)** Different expressions of mmu-miR-200c-3p in different groups of C57BL/6J mice. **(I)** Different expressions of DUSP1 mRNA and protein in different groups of C57BL/6J mice. CON, Control group; SHAM, NAFLD+SHAM group; SG, NAFLD+SG group; FR, NAFLD+F R group. *p < 0.05; **p < 0.01; ***p < 0.001. Measurement data were expressed as mean ± standard deviation and compared by one-way ANOVA.

compared with NAFLD+SHAM and NAFLD+FR group (**Figure 5A**). DUSP1 specifically dephosphorylates the members of the MAPK kinase family, including ERK1/2, p38, and JNK. At the sixth week postoperatively, we demonstrated that p-ERK1/2, p-p38 and p-JNK were significantly downregulated in the NAFLD+SG group compared with the NAFLD+SHAM and NAFLD+FR group (**Figure 5B**).

DISCUSSION

Currently, weight loss is the only approved safe and effective treatment for NAFLD (28, 29). Bariatric surgery is superior to lifestyle modifications for treating patients with morbid obesity (30). However, controversy still exists concerning the impacts of bariatric surgery in patients with NAFLD (31). LSG can result in a considerable weight loss with negligible complications (19); nevertheless, the mechanism of SG in NAFLD remains unclear.

Ultrasonography is the most commonly used imaging examination method for the evaluation of NAFLD (32). Previous literature reported that only 6.3% still had some grade of steatosis one year after LSG (33). Results of general ultrasonography demonstrated that only one patient (14.3%) showed no improvement during the follow-up period in our study, which may be due to the insufficient follow-up time. The clinical follow-up data demonstrated that LSG could alleviate NAFLD.

The expression level of miR-200c-3p was significantly increased in the hepatic tissue of NAFLD rats (26, 34). Lin et al. (35) reported that miR-200c-3p showed an increasing trend in diabetic DB/DB mice liver tissue using miRNA array. Ramachan-Dran et al. (36) reported that miR-200c-3p promotes the progression of hepatitis C fibrosis by regulating the cellular Src (cellular sarcoma gene, cSrc) kinase signal cascade reaction through fas-associated phosphatase 1 (FAP1). In the present study, we demonstrated that the expression of miR-200c-3p in the serological specimens of patients with NAFLD was markedly upregulated in NAFLD patients, and then distinctly decreased following the LSG procedure, with the remission of NAFLD (**Figures 2A–H**).

DUSP1, a nucleus localized MKP, is a major negative regulator of the MAPK pathway and participated in maintaining homeostasis of glucose metabolism and energy balance in peripheral tissues (37, 38). Microarray analysis showed that the DUSP1 of hepatic tissue was downregulated in NAFLD compared with volunteers without NAFLD (21). In cardiomyocytes treated with high glucose, the inhibition of miR-200c-3p led to the overexpression of DUSP1 and the low expression of p-ERK1/2, p-P38, and p-JNK, thus attenuating the cardiac hypertrophy (39). Our study demonstrates that LSG

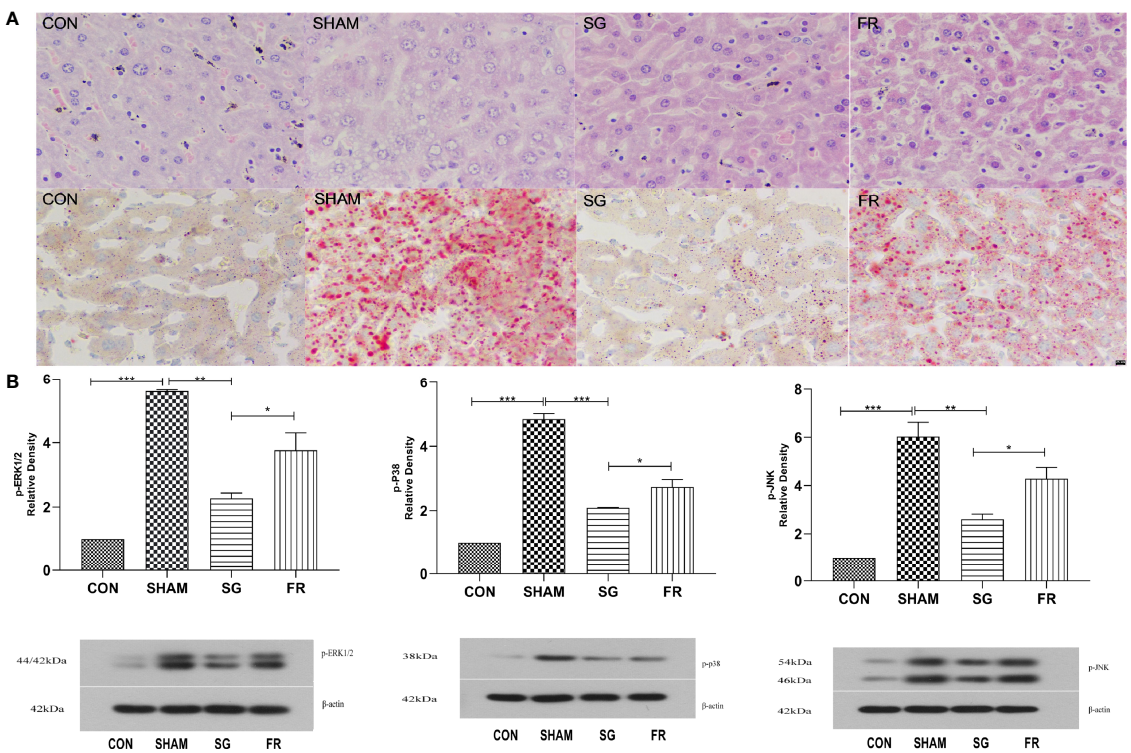

FIGURE 5 | Changes of liver pathology and MAPKs in C57BL/6J mice. **(A)** Lipid accumulations in different groups of C57BL/6J mice after SG procedure determined with HE staining and Oil Red O staining (× 400). **(B)** Expressions of p-ERK1/2, p-p38, and p-JNK in different groups of C57BL/6J mice. *p < 0.05; **p < 0.01; ***p < 0.001. Measurement data were expressed as mean ± standard deviation and compared by paired t-test.

could downregulate the serum expression of miR-200c-3p and reduce the degradation of DUSP1 (**Figures 2A–H**) at the 3rd month postoperation in patients with NAFLD.

As a result of the *in vivo* studies being complex and accompanied by other factors, we extended these observations from NAFLD patients to HepG2 and 293T cell lines *in vitro*. The DUSP1 mRNA and protein expression levels were lower in the miR-200c-3p mimic group than in the CON group (**Figure 3D**). Besides, the DUSP1 mRNA and protein expression levels were higher in the miR-200c-3p inhibitor group than in the CON group (**Figure 3D**). H_2O_2 can induce large amounts of reactive oxygen species (ROS), which can initiate the oxidative stress response, 0and was widely used to construct cell models of oxidative stress (40). It has been reported that H_2O_2 can induce the DUSP1 expression in the breast cell line MCF-7 (41). However, in our research, HepG2 cells were induced by 100uM H_2O_2 for 4 hours, the DUSP1 mRNA and protein expression levels were downregulated in the H_2O_2 groups and the expression levels of miR-200c-3p were upregulated (**Figure 3D**), suggesting that there may be other mechanisms to regulate DUSP1 or miR-200c-3p expression *via* H_2O_2 in the HepG2 cell line. Taken together, the results showed that miR-200c-3p regulated DUSP1 expression at the mRNA level. The following luciferase experiment verified that miR-200c-3p inhibited the expression of DUSP1 by binding the seed sequence of the 3'UTR region (**Figure 3E**).

To further demonstrate our hypothesis, we further verified the results in animal models. The NAFLD+FR groups were included in the animal model to investigate whether a reduction in food intake was sufficient to induce weight loss and metabolic remodeling. Compared with the NAFLD+FR group, the NAFLD+SG group demonstrated greater post-surgical effects on weight loss, liver function enzymology assay, lipid panel (**Figures 4A–G**), and pathological phenotypes of NAFLD (**Figure 5A**). In addition, the expression level of miR-200c-3p decreased while DUSP1 increased in the NAFLD+SG group (**Figures 4H, I**).

Last but not the least, the present study validated that SG inactivated the MAPK signaling pathway in NAFLD mice. It has been widely known that activation of ERK can lead to cell proliferation, whereas activation of JNK and p38 causes cell death. The MAPK pathway was an established regulator of hepatic metabolism (42). The interventions of MAPK phosphorylations can protect the liver from inflammations, and inhibitions of the MAPK signaling pathway can improve liver fibrosis (43, 44). The expression levels of p-ERK1/2, p-p38, and p-JNK were significantly downregulated in the NAFLD+SG group compared with the NAFLD +SHAM group and NAFLD+FR group (**Figure 5B**), which was secondary to the upregulation of DUSP1 after SG.

Limitations

Our study is limited by retrospectively observational design and the short time of following-up, which was based on small sample size. In the cell experiments, only the regulation and direct binding mechanisms between miR-200c-3p and DUSP1 were verified, validation of the MAPK pathway is lacking.

CONCLUSION

In conclusion, we reported the inhibition of miR-200c-3p responsible for the overexpression of DUSP1 expression and inactivation of downstream MAPK pathway during NAFLD resolution induced by SG. The study provides meaningful insight into the molecular processes of NAFLD following SG, and that miR-200c-3p may be a therapeutic target in NAFLD pathogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Dalian Municipal Central Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of Dalian Municipal Central Hospital.

AUTHOR CONTRIBUTIONS

T-tZ was responsible for the collection of samples, statistical analysis, interpretation of data and writing of the manuscript. YW and X-wZ was responsible for the study concept and design, collection of samples, data collection, analysis of dietary records, interpretation of data, editing the manuscript, and study supervision. K-Yy provided help with statistical analysis. X-qM was responsible for the collection of samples. G-hZ was responsible for the interpretation of data and edited the manuscript. All authors approved the final version of the article.

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