NEW INSIGHTS INTO UNDERSTANDING AND MANAGING NAFLD

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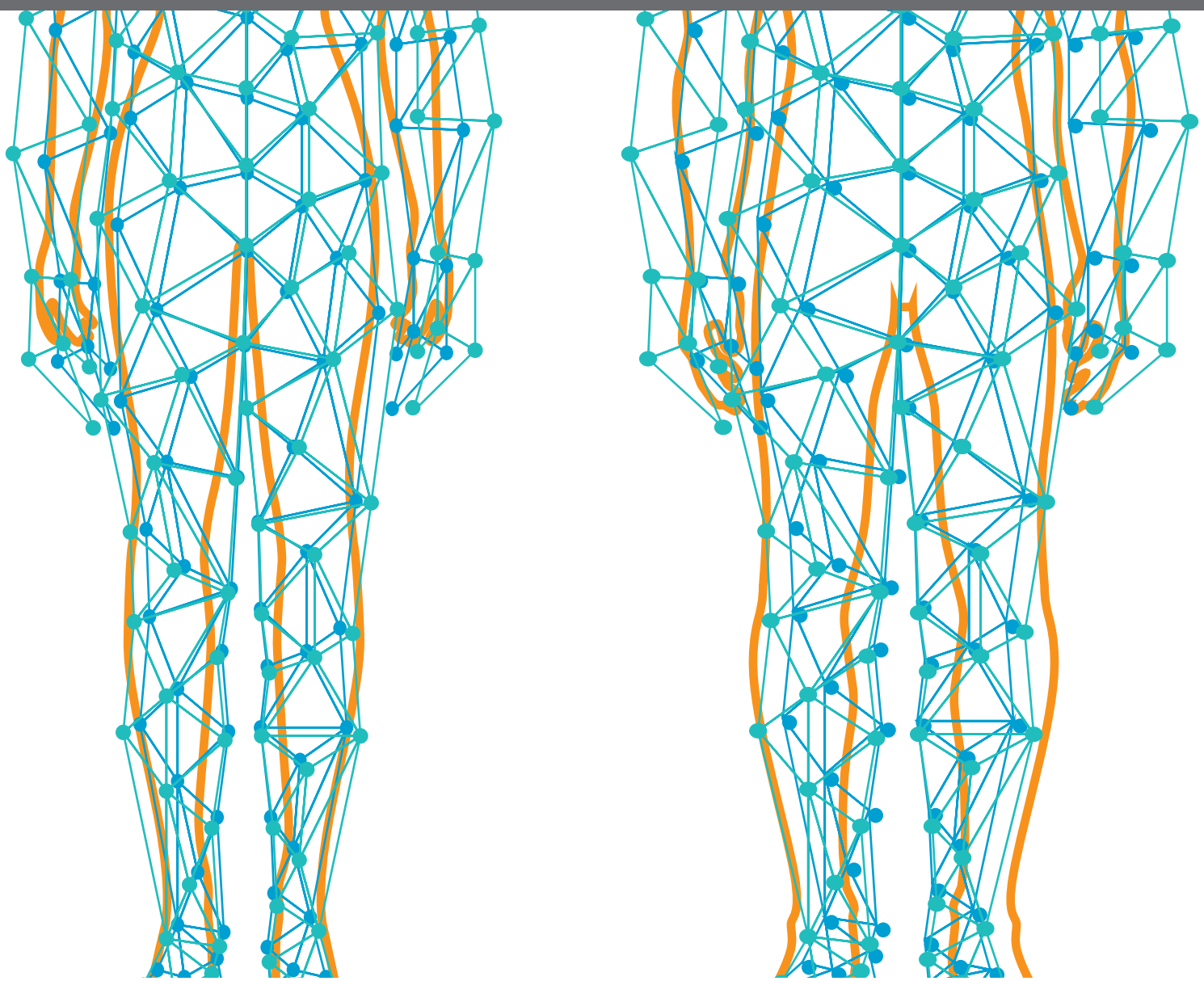

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NEW INSIGHTS INTO UNDERSTANDING AND MANAGING NAFLD

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EDITORIAL
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Editorial: New Insights Into Understanding and Managing NAFLD

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Keywords: NAFLD (non-alcoholic fatty liver disease), biomarkers, therapeutical approaches, liver pathophysiology, steatohepatitis

Editorial on the Research Topic

New Insights Into Understanding and Managing NAFLD

Non-alcoholic fatty liver disease (NAFLD) is a multisystem disease with complications related to the metabolic syndrome and with a diverse histopathological spectrum ranging from simple steatosis, also termed fatty liver (NAFL) without significant inflammation to steatohepatitis (NASH) with varying stages of fibrosis and, ultimately, cirrhosis, and hepatocellular carcinoma.

Since NAFLD is the most common chronic liver disease worldwide, causing a considerable health burden, there is an increasing number of research groups that are deeply interested in the identification of risk factors involved in the progression of the liver damage, the characterization of new biomarkers with utility for its non-invasive diagnosis and the recognition of novel molecular targets for its treatment.

This special issue contains 18 papers, including 10 original research articles and 8 reviews, reporting important data about novel perspectives regarding pathogenesis and management of NAFLD.

Although NAFLD has been conceived as a different entity from alcohol-related fatty liver disease (ALD), both diseases have an overlap in the pathophysiology, share genetic-epigenetic factors, and frequently coexist. In this sense, Idalsoaga et al. reviewed the overlapping pathophysiology of NAFLD and ALD, and also the effects of metabolic dysfunction and overweight in ALD (Idalsoaga et al.).

The identification of risk factors involved in NAFLD progression is important to develop preventive interventions. In this context, Charatcharoenwitthaya et al. described a detrimental effect of cigarette smoking on all-cause mortality in the study cohort (19,181 persons), with a similar but more robust association in women than in men with NAFLD (Charatcharoenwitthaya et al.). In another article, Rim et al. suggested that early screening strategies for people with abrupt chronological changes in serum triglycerides might be useful to predict NAFLD development (Rim et al.). Indeed, a better understanding of the different factors linked to NAFLD progression will also provide insight into preventive strategies to reduce the incidence of NAFLD-associated disorders. In this regard, Abdel-Razik et al. attempted to identify risk factors for portal vein thrombosis (PVT) in NAFLD patients, finding that among all PVT-associated factors studied only an increased central obesity and an elevated leptin/adiponectin ratio in these patients are independently associated with PVT development (Abdel-Razik et al.).

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Psychological disorders such as depression and anxiety are frequently present in patients with NAFLD. However, the link between mood disorders and NAFLD is still poorly understood. In this special issue, different studies have provided new insights about the association between NAFLD and mental disturbances that may have clinical implications for reducing the prevalence of comorbidities. In this sense, Choi et al. concluded that in a Korean group of study of more than 25,000 subjects, women with NAFLD show a higher tendency to suffer from depression and anxiety compared to non-NAFLD (Choi et al.). In the same way, the meta-analysis and systematic review performed by Xiao et al. suggested a strong association between depression and NAFLD. Indeed, they conclude that the worsening of NAFLD to NASH implies a higher risk of suffering depression (Xiao et al.). Moreover, it is still barely unstated how perceived social support and NAFLD development and progression alter the psychosocial profile of these patients, and which are relevant risk factors. In this special issue, Funuyet-Salas et al. demonstrated that low perceived social support, significant fibrosis, and female sex are independently associated with a higher-risk psychosocial profile in NAFLD. Therefore, a psychological intervention in NAFLD patients could be of great interest (Funuyet-Salas et al.).

The underlying mechanism for the setup and progression of NAFLD is complex and multifactorial, involving multiple parallel factors and signaling pathways. In this sense, microRNAs (miRNAs) function as critical post-transcriptional negative regulators involved not only in many biological processes but also in the development of many diseases such as NAFLD. López-Pastor et al. reviewed the latest advances in knowledge about the miRNAs involved in the development of NAFLD and related diseases, and examined how this knowledge could be used to identify new non-invasive biomarkers and new pharmacological interventions for this liver disease (López-Pastor et al.). Moreover, in an updated mini-review, Delli Bovi et al. summarized the current knowledge on the role of oxidative stress in the pathogenesis and progression of NAFLD, as well as on the preventive and therapeutic strategies available. The authors reviewed the participation of the intestinal microbiota in this process, and highlighted the use of probiotics as enhancers of antioxidant defenses, as well as focused on other antioxidant therapeutic interventions, particularly, the use of vitamin E (Delli Bovi et al.). Since several studies have reported that complement system, an innate immune system, plays an important role in the pathogenesis of NAFLD, in another review, the authors explored the role and molecular mechanism of complement component 3, a protein of the innate immune system, in NASH development as well as its implication in the diagnosis and treatment of this stage of the disease (Han and Zhang).

Likewise, clinical and preclinical studies have revealed that hypoxia may play an important role in the pathophysiology and progression of NAFLD, which have been included in a mini-review by Isaza et al. Interestingly, the article by Rey et al. described for the first time that intermittent hypoxia modulates free fatty acid (FFA) uptake by upregulating the hepatic expression of the FFA translocase CD36, and partly contributes to the NAFLD setup (Rey et al.).

The diagnosis of NAFLD is actually very difficult by non-invasive methods and usually requires a liver biopsy. For this reason, many researchers and clinicians are making strong efforts to find non-invasive and accurate methods to improve the diagnosis of this prevalent disease. Bearing this in mind, the study performed by Hokkanen et al. demonstrated that low-dose computed tomography (CT) scans could be a useful tool for liver fat quantification and NAFLD diagnosis. Their results suggest that low-dose CT is a feasible and well repeatable method but amount of liver fat contributes to repeatability. The assessment of liver fat content can be used as additional information in studies where a CT scan has been done for other medical reasons (Hokkanen et al.). In this search for non-invasive NAFLD biomarkers for diagnosis and prognosis of NAFLD, another article showed that serum leptin discriminates NAFLD, and adiponectin combined with specific lipids might be useful for the NASH stratification. Moreover, insulin-like growth factor 1 (IGF1), international normalized ratio (INR), and ferritin distinguish advanced fibrosis (Marques et al.). In another review submitted to this special issue, Garcia-Martinez et al. performed an exhaustive analysis on the literature regarding the extracellular vesicles (EV) link with hepatocytes stress which is one of the early steps leading to liver disease. Importantly, this review highlighted the potential use of EV as a new biomarker for NAFLD management (Garcia-Martinez et al.).

Due to the high prevalence of NAFLD and the absence of a specific treatment, many attempts are being done to find new therapeutic targets. In this sense, the discovery of dietary supplements is a great opportunity. Li and Zhao have analyzed the literature supporting the potential of carnitine, an amino acid-derived molecule, as a therapeutic agent for different liver diseases, particularly NAFLD. Regarding NAFLD, carnitine inhibits β-oxidation, improves mitochondrial dysfunction, and reduces insulin resistance. Therefore, carnitine supplementation may improve liver diseases including NAFLD (Li and Zhao). Yao et al. in their manuscript described the beneficial effects of myricetin, a polyphenolic flavonoid, in a murine dietary model of NASH, and even help to elucidate the underlying mechanisms: treatment with myricetin favors M2 polarity switch of liver macrophages, attenuating the activation of hepatic stellate cells and the consequent liver fibrosis (Yao et al.). Based on all this, they propose myricetin as a potential new therapeutic agent for the treatment of inflammation in NAFLD. In another article, authors explored the effect of crocetin, a bioactive ingredient of saffron, on this liver disease in a mouse model of NAFLD and revealed that crocetin ameliorates obesity-induced NAFLD by suppressing of oxidative stress and decreasing inflammation, demonstrating its therapeutic potential (Xu et al.).

In summary, this special issue will help to understand important aspects of NAFLD setup and progression. The articles published in this special issue show novel perspectives about pathogenesis and diagnosis of NAFLD as well as possible therapeutic approaches to ameliorate liver injury.

AUTHOR CONTRIBUTIONS

ÓE, DF, YO, JE, and AG-R wrote the manuscript. All authors critically revised the manuscript for important intellectual content.

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ORIGINAL RESEARCH
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Myricetin Modulates Macrophage Polarization and Mitigates Liver Inflammation and Fibrosis in a Murine Model of Nonalcoholic Steatohepatitis

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This study aimed to investigate the beneficial effects of myricetin in a diet-induced nonalcoholic steatohepatitis (NASH) model and the underlying mechanism. C57BL/6J mice were fed a standard chow or the choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) for 8 weeks with the treatment of myricetin (100 mg/kg) or vehicle by daily gavage. Hepatic inflammation, steatosis, fibrosis, and hepatic stellate cells (HSC) activation were assessed. We also analyzed M1 and M2 macrophages and its related markers in livers from NASH mice and in RAW264.7 macrophages stimulated by lipopolysaccharide (LPS) or interleukin 4 (IL-4) *in vitro*. Furthermore, we determined the effect of myricetin on the triggering receptor expressed on myeloid cells-1 (TREM-1), toll like receptor (TLR) 2 and 4, and myeloid differentiation factor 88 (MyD88) signaling both in livers from mice and in RAW264.7 cells stimulated by LPS. Our results revealed that myricetin remarkably ameliorated hepatic steatosis, inflammation, and inhibited hepatic macrophage infiltration in CDAHFD-fed mice. Myricetin-treated to CDAHFD-fed mice also inhibited liver fibrosis and HSC activation when compared with vehicle-treated to those mice. Moreover, myricetin inhibited M1 macrophage polarization and its relative markers in livers of NASH mice while induced M2 polarization. Similarly, *in vitro* study, myricetin inhibited the LPS-induced mRNA expression of M1 macrophages marker genes and induced IL-4-induced M2 macrophage marker genes in RAW264.7 macrophages. Mechanically, myricetin inhibited the expression of TREM-1 and TLR2/4-MyD88 signaling molecules in livers from NASH mice and in RAW264.7 macrophages stimulated by LPS *in vitro*. Additionally, myricetin inhibited the activation of nuclear factor (NF)-κB signaling and the phosphorylation of the signal transducer and activation of transcription 3 (STAT3) in LPS-stimulated RAW264.7 macrophages. Taken together, our data indicated that myricetin modulated the polarization of macrophages via inhibiting the TREM-1-TLR2/4-MyD88 signaling molecules in macrophages and therefore mitigated NASH and hepatic fibrosis in the CDAHFD-diet-induced NASH model in mice.

Keywords: NASH, hepatic fibrosis, myricetin, macrophage polarization, TREM-1, TLRs, MyD88

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has recently emerged as a significantly public health issue because of its high prevalence (1–3). NAFLD is characterized by a wide spectrum of liver phenotypes ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) (1–3). The most important clinical challenge in NASH is the progression to liver fibrogenesis, which may gradually develop to cirrhosis and eventually to hepatocellular carcinoma (HCC) (1–3). However, the molecular mechanisms underlying NAFLD onset and progression remain poorly understood, and there is currently no approved pharmacological therapy for NASH and fibrosis (1, 2). Therefore, a better understanding of the mechanisms of NASH development and progression is indispensable for identifying novel therapeutic strategies for this burgeoning hepatic disease.

Recently, it has become apparent that liver-resident macrophages and recruited macrophages play an important role in the development and progression of NASH and liver fibrosis (4–7). Macrophages are highly plastic cells that can shift to adapt to tissue microenvironment, which have different functional phenotypes with proinflammatory M1 macrophages and anti-inflammatory M2 macrophages (4, 7, 8). Moreover, M1-polarized macrophages exacerbate hepatic injury and inflammation through production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, while M2 polarity switch of macrophages could inhibit the activation of M1 macrophages through secreting anti-inflammatory cytokines, including IL-10 (4–9). Notably, there is growing evidence that M1 macrophages can promote disease development and progression in NASH (4, 7). In contrast, pharmacological alteration of polarization from M1 to M2 phenotype partially has ameliorated the pathogenesis of steatohepatitis and fibrosis (7–9). Those data suggest that the switch in macrophage phenotypes determines their role in liver inflammation and fibrosis, and thus regulating the polarization of macrophage by modulating the key macrophage transcription factors represents therapeutic targets for NASH and liver fibrosis (8–12).

The triggering receptor expressed on myeloid cells (TREM)-1 is a kind of immunoglobulin superfamily activation receptors express on neutrophils and monocyte macrophages (13, 14). Upon activation, TREM-1 can trigger and augment inflammatory reaction, especially through synergism with toll-like receptors (TLRs) signaling in macrophages (13–16). Moreover, several studies have already revealed that TREM-1 play an important role in regulating the activation of Kupffer cell and is associated with macrophages polarization, which amplifies acute and chronic inflammatory responses in diseases (17–19). Interestingly, a recent report has demonstrated that overexpression of TREM-1 in the liver and M1 hepatic macrophages polarization were associated with obesity-induced insulin resistance (IR) (20). Additionally, in patients with metabolic syndrome, there was elevated levels of free fatty acids and lipopolysaccharide (LPS), which can stimulate TREM-1 expression and activate TLRs receptor cascade in lipid rafts (21). On the other hands, it is well known that TLRs-mediated signals are implicated in the pathogenesis of chronic liver diseases (22–24). Importantly, previous studies have revealed that TLR2/4-mediated myeloid differentiation factor 88 (MyD88) and nuclear factor-κB (NF-κB) signaling regulated macrophages polarization (20, 25, 26). Thus, we speculate that the TREM-1-TLR2/4-MyD88 signaling pathway may promote hepatic inflammation and fibrosis in NASH via modulation of macrophage polarization.

Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone; **Figure 1A**) is a polyphenol flavonoid that is widely found in most berries such as blueberries and strawberries, tea, vegetables, and various medicinal herbs (27–29). Myricetin is reported to possess many pharmacological properties, including antioxidant, anti-inflammatory, antifibrotic, anti-obesity, and anti-diabetic activities (27–34). Recent studies have also demonstrated that myricetin attenuated vascular endothelial dysfunction and hepatic injury in mice induced by high choline-fed (31) and mitigated liver fibrosis induced by CCl_4 in mice (29, 32). In particular, myricetin has proved to inhibit fatty acid biosynthesis and attenuate ethanol-induced lipid accumulation in liver cells (30). Furthermore, myricetin treatment also ameliorates hyperglycemia, IR, and steatosis in mouse models of obesity through antioxidant properties (33, 34). These findings indicate that supplementation of the diet with myricetin might be beneficial to NASH and liver fibrosis. Therefore, the purpose of this study is to investigate whether myricetin could attenuate NASH-related inflammation and fibrosis in a mouse model of NASH and to elucidate its underlying mechanisms; we particularly focus and assess the effects of myricetin on macrophages polarization.

MATERIALS AND METHODS

Reagents and Antibodies

Myricetin was purchased from Selleck Chemicals Co. Ltd. (Houston, TX, USA). Sodium carboxymethyl cellulose (CMC-Na) was obtained from Makclin Biochemical Co., Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) and lipopolysaccharide (LPS) were purchased from Sigma Chemical, Co. Ltd. (St. Louis, MO, USA). Myricetin was prepared as stocks in CMC-Na and diluted to various concentrations in medium.

The antibodies were obtained as following: rabbit anti-IL-12A, rabbit anti-IRF5, rabbit anti-CD163, rabbit anti-Ym-1/Ym-2, rat anti-TREM1, rabbit anti-NF-κB, rabbit anti-phosphor-NF-κB (Abcam, Cambridge, MA); rabbit anti-α-SMA, rabbit anti-F4/80, rabbit anti-Iκ-Bα, rabbit anti-phospho-Iκ-Bα, rabbit anti-JNK, rabbit anti-phospho-JNK, mouse anti-STAT3, rabbit anti-phospho-STAT3 (CST, Danvers, Massachusetts, USA); HRP-linked goat anti-rabbit IgG (Jackson Labs Technologies, Inc., Las Vegas, NV); rabbit anti-GAPDH and rabbit anti-β-actin (HuaAn Technologies, Inc., Hangzhou, China). HRP-linked goat anti-rat IgG (Byotime Institute Biotechnology, Shanghai, China), Cy3 conjugated goat anti-rabbit IgG H&L (Servicebio, Wuhan, China), Alexa Fluor 488 goat anti-rat IgG H&L (Invitrogen, USA).

Animals and Experimental Protocols

Six-week-old male C57BL/6J mice (20–23 g) were purchased from Shanghai Slack laboratory animal co., Ltd. (Shanghai, China). Mice were maintained in a temperature-controlled room

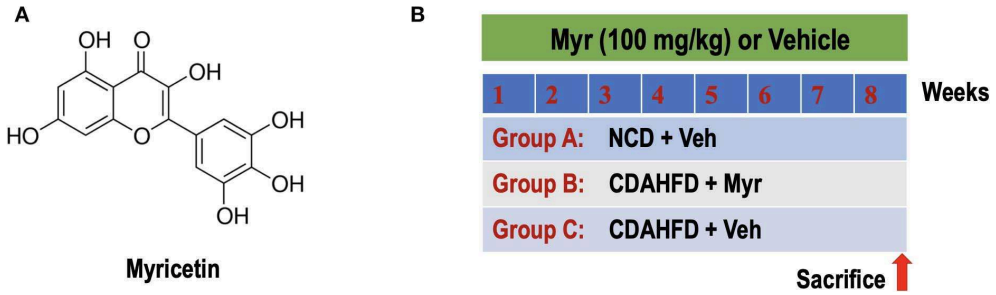

FIGURE 1 | Experimental study design. **(A)** The chemical structure of myricetin (3,3',4',5,5',7-hexahydroxyflavone). **(B)** Experimental protocol for assessment the preventive effect of myricetin (Myr) on the development of NASH and fibrosis in mice fed the choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD). Group A: control mice fed normal chow diet (NCD) and treated with the vehicle (Veh, 0.5% CMC-Na). Group B or C: CDAHFD-induced-NASH mice were randomly assigned to a treatment of Myr (100 mg/kg) or Veh by daily orally administration for 8 weeks.

(23 ± 3, $55 \pm 10\%$ humidity) with a 12-h light-dark cycle and had unrestricted to normal chow diet (NCD) or a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) consisting of 60% kcal fat and 0.1% methionine (Research Diets, Inc., New Brunswick, NJ, USA) (35, 36). The mice were randomly divided into three groups ($N = 8$ for each group). Group A was given NCD and treated with vehicle (0.5% CMC-Na). Group B or C: mice were fed CDAHFD with orally administration myricetin at 100 mg/kg per day or vehicle (**Figure 1B**). The doses of myricetin were chosen based on previous studies in mice (28, 32, 37). At the end of treatment period, mice were euthanized using ketamine/xylazine, blood samples were collected via cardiac puncture to detect biochemical biomarkers. Livers were removed for measurement weight, photographed, and processed for further histological and molecular assessment. All samples were stored at -80°C until use. All animal experiments were performed according to the guidelines of the care and use of laboratory animals of Fudan University and approved by the Animal Ethics Committee of Zhongshan hospital.

Culture and Treatment of RAW264.7 Macrophage Cells

RAW264.7 murine cells were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China) and cultured in undifferentiated RAW macrophages conditioned medium as previously described (38, 39). Briefly, the cells cultured in T25 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM L), penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37 and 5% CO_2.

In vitro experiments evaluating the effect of myricetin on the activation and polarization of macrophages, RAW264.7 cells were polarized by culturing 10^6 cells/well overnight in 6-well plates before replacing the conditioned-medium to induce M1- or M2-polarized macrophages as descripted previously (9, 11, 39). Briefly, cells were classically activated with 100 ng/mL LPS (M1 condition) or alternatively activated with M2 condition (20 ng/mL IL-4), respectively; control cells were cultured with DMEM alone (M0 condition). For selective experiments, cells were pretreated with myricetin (50 μM) or vehicle (0.5% DMSO) for 12 h, then cells were added the macrophages conditioned medium for another 12 h. Finally, cells were then washed and harvested by centrifugation for immunofluorescence analysis, RNA and protein analysis. All measurements were performed in triplicate wells. For cells experiment, a stock myricetin solution (10 mM) was prepared using DMSO as the solvent and stored at −20 until use. Myricetin concentration for cells treatment was based on our primary study and previous *in vitro* bioactivity work (29, 40, 41).

Cell Viability Assays

RAW264.7 cells viability was evaluated by the Cell Counting Kit-8 (CCK8)-based spectrophotometric methods (Beyotime Institute Biotechnology, Shanghai, China) according to the protocol provided by the manufacturer. Cells were seeded in 96-well flat-bottom plates at a density of 5×10^3 cells/well. After 6 h of culture, the medium was then changed to serum-free medium containing 0.5% DMSO (vehicle) or various concentrations of myricetin (0, 25, 50, and 100 μM) for 0, 12, 24, or 48 h at 37 and 5% CO_2. Following treatment, 10ul CCK8 solution was added in each cell and incubated for another 2 h at 37. Relative cytotoxicity was measured at 450 nm absorbance with Biotek EPOCH2 microplate reader (BioTek Instruments Inc., USA). Cell viability was defined relative to the vehicle-treated control, and each experiment was done three times independently to ensure reproducible results.

Serum Enzymes Assays

The serum alanine transaminase (ALT) and aspartate transaminase (AST) activity were analyzed using the kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) respectively following the manufacture's standard protocol.

Histopathology

Liver samples were collected from each mouse and fixed in 10% neutral buffered formalin and embedded in paraffin. Then these liver tissues were cut in 4-μm-thick sections and stained with hematoxylin and eosin (H&E), or Masson's trichrome according to standard procedures. Hepatic histopathological

examination was performed in a blinded manner by an experienced pathologist with the histological scoring system for NAFLD (35, 42). Briefly, hepatocellular steatosis and liver inflammation scores were classified into grades 0 to 3 with 0 being within normal limits and 3 being most severe; the staging of liver fibrosis was classified into stages 0 to 4. Individual scores were assigned for each parameter. Moreover, liver fibrosis was also evaluating using the NIH ImageJ free software (Bethesda, Maryland, USA) on Masson's trichrome-stained sections in a blinded manner (23, 38).

Oil Red O Staining

Lipid accumulation in the liver was evaluated using an Oil Red O (ORO) staining kit (Sigma Chemical, Co. Ltd., St. Louis, MO, USA) as described in the manufacturer's procedure. All images were obtained using an Observer A1 microscope (Carl Zeiss) at ×100 magnification. For quantification ORO-positive staining, 5 randomly non-overlapping ×100 fields per specimen were examined and determined for six animals in each group using the NIH ImageJ free software (Bethesda, Maryland, USA). Results are expressed as percentages of positive areas in the high-power field.

Immunohistochemistry Staining and Analysis of Histological Markers

Immunohistochemistry (IHC) staining was carried out as our previously described (23). Briefly, formalin-fixed tissues were embedded in paraffin, cut 4-μm-thick sections. Followed by dewaxing, hydration and antigen retrieval by heat, sections were then blocked and incubated overnight at 4 with primary antibodies as follows: anti-SMA (1:100), anti-F4/80 (1:100), anti-CD163 (1:100), anti-Ym-1/Ym-2 (1:100), anti-IL-12 (1:100), and anti-IRF5 (1:100), with each primary antibody diluted in TBS containing 2% bovine serum albumin (BSA). Sections then were subsequently washed 3 times and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody, followed by incubation for 5- to 10-min with 3, 3′-diaminobenzidine tetrachloride and visualization of specific staining by light microscopy. Images were acquired under high-power field with Nikon Eclipse Ti inverted microscope (Nikon, Amstelveen, The Netherlands).

Quantitative expression of immunostaining was carried out at a fixed threshold using NIH ImageJ software (Bethesda, Maryland, USA). For quantification α-SMA-positive areas in liver section, five random non-overlapping ×100 fields were examined and determined for six animals in each group (38, 43). For quantification the areas of hepatic macrophages ($F4/80^+$, $IL\text{-}12^+$, $IRF5^+$, $Ym\text{-}1/Ym\text{-}2^+$, and $CD163^+$ cells) in sections, six random non-overlapping selected fields of view per slide at ×200 magnifications were analyzed and expressed as the percentage of positive area in the high-power field; and five mice of each group were examined (44).

TUNEL Staining

Terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) staining was carried out to evaluate death hepatocytes in the liver. The paraffin-embedded liver tissue sections were stained using a DeadEnd™ Fluorometric TUNEL System based on the manufacturer's protocol (Promega, USA). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and hematoxylin solution and observed with Nikon Eclipse Ti inverted microscope (Nikon, Amstelveen, The Netherlands). The TUNEL-positive nuclei (blue) were quantified under ×200 magnification in 5 randomly non-overlapping fields and 5 animals of each group were assessed (16). The results were presented as the mean number of $TUNEL^+$ cells each field.

Immunofluorescence and Quantification

The dissected liver tissues from mice were fixed in 4% paraformaldehyde, washed with PBS (pH 7.4), embedded in optimum cutting temperature tissue compound (OCT compound, Sakura, Japan), and stored at −80 for 24 h (16). Then the sections (8μm in thickness) were prepared with a cryotome Cryostat (Leica, CM 1900, Germany). After antigen retrieval was performed, blocking was carried out in PBS with 3 % BSA. Slides were incubated with primary antibody against F4/80 (dilution, 1:50) at 4°C overnight, then incubated with TREM-1 antibody (dilution 1:50) at RT for 1 h in case of double-staining. Cy3 conjugated goat anti-rabbit IgG H&L and Alexa Fluor 488 goat anti-rat IgG H&L antibodies were incubated at 1:200 in PBS at RT for 1 h. After washing with TBS for 3 times, the cell nuclei were counterstained with DAPI-Fluoromount-G™ (Southern Biotech, USA). Finally, the stained tissues were analyzed by fluorescence microscopy (BX51, Olympus, Japan).

RAW264.7 cells were fixed with 4% paraformaldehyde for 15 min at RT followed by permeabilization using 0.2% TritonX-100 in PBS. Then nonspecific binding was blocked with 3% BSA for 1h at RT, followed by incubation with primary antibodies for IL12 (dilution 1/100) and IRF5 (dilution 1/100) at 4 overnight. After twice washing in PBS, cells were incubated with secondary antibody for 1 h at RT. DAPI was used for nuclear staining. The slides were washed twice with PBS, covered with DABCO (Sigma-Aldrich, St. Louis, MO), and imaged by fluorescence microscopy (IX51, Olympus, Japan). Percentage of the $IL\text{-}12^+$ and $IRF5^+$ staining area in 6 randomly selected fields using the NIH ImageJ free software (Bethesda, Maryland, USA).

Western Blotting

Western blotting was performed as our described in detain previously (23, 43). In brief, homogenized liver tissue or harvested cells were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Scientific). The protein concentration in each was determined via the Bradford method (Bio Rad, Sydney, Australia). Forty microgram protein was separated by electrophoresis on a proper concentration of SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin (BSA) in tris-buffered saline plus tween for 1 h and incubation with primary and secondary antibodies, the blots were visualized by ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech Inc., NJ, USA). The optical density of each band was quantified by NIH ImageJ free software (Bethesda, Maryland, USA) and normalized to GAPDH or β-actin as a loading control. The specific primary antibodies in our study were diluted as

follows: α-SMA (1:1000), TREM-1 (1:500), IL-12A (1:1000), IRF-5 (1:1000), NF-κB (1:1000), phospho-NF-κB (1:1000), Iκ-Bα (1:1000), phospho-Iκ-Bα (1:1000), STAT3 (1:1000), phospho-STAT3(1:1000), JNK (1:1000), phosphor-JNK (1:1000), anti-GAPDH (1:1000), and anti-β-actin (1:1000).

RNA Isolation and Quantitative RT-PCR Analysis

Total RNA was isolated from frozen snap-frozen mouse livers or RAW264.7 cells using the Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Single-stranded cDNA was synthesized using random hexamer primers and avian myeloblastosis virus reverse transcriptase commercial kit (Perfect Real Time, SYBR® PrimeScriP™TaKaRa, Japan). Quantitative RT-PCR reactions were carried out for assessment of mRNA expression on an ABI Prism 7500 sequence detection system (Applied Biosystems, Tokyo, Japan) as descripted previously (23, 43). Relative gene expression was normalized to β-actin as housekeeping gene, and fold change over the untreated control was calculated using the $2^{-\Delta\Delta}$Ct method (23). The primers sequences of the target genes were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and were provided in **Table S1**.

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. All statistical analyses were carried out using GraphPad Prism 8.0 software (La Jolla, CA, USA). Differences between multiple groups were compared by one-way analysis of variance (ANOVA) with *post hoc* test (Tukey's correction for multiple tests). For comparison between two groups, the unpaired two-tailed Student's *t*-tests were used. In case of non-normality in distribution Wilcoxon-Mann-Whitney *U*-tests or Kruskal-Wallis tests were used to compare quantitative data, as appropriate. For all tests, $P < 0.05$ were considered statistical significance and the level of significance was shown by asterisks ($^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; and $^{*}P < 0.05$).

RESULTS

Myricetin Alleviated Hepatic Steatosis, Hepatocytes Injury and Death, and Inflammation in a Diet-Induced Murine Model of NASH

To assess the effect of myricetin on diet-induced NASH, mice were fed separately a CDAHFD diet with vehicle or myricetin for 8 weeks. As shown in **Table 1**, there were remarkably differences in body weight (BW) and liver/body weight ratio between CDAHFD-fed mice and NCD-fed mice. However, compared with the vehicle-treated to NASH mice, myricetin-treated to NASH mice did not alter either BW or liver/body weight ratio. Also, there was no differences in liver weight between myricetin-treated NASH mice and vehicle-treated NASH mice **(Table 1)**. Moreover, we found CDAHFD-fed induced an increase in the activity of ALT and AST, which are surrogate markers used to indicate hepatocytes injury and death. However, myricetin-treated to CDAHFD-fed mice showed a statistically relevant reduction the levels of serum ALT and AST when compared with vehicle-treated to those mice (**Table 1**).

TABLE 1 | The impact of myricetin treatment on body weight, liver weight, and serum enzymes at 8 weeks in mice fed NCD or CDAHFD.

	NCD (N = 8)	CDAHFD + Veh (N = 8)	CDAHFD + Myr (N = 8)
Body weight (g)	26.95 ± 0.65	20.31 ± 0.42[a]	21.65 ± 0.56[a]
Liver weight (g)	1.35 ± 0.06	1.34 ± 0.04	1.36 ± 0.09
Liver/body weight ratio (%)	4.98 ± 0.12	6.31 ± 0.15[a]	6.74 ± 0.32[a]
Serum ALT (U/L)	10.1 ± 2.2	281.1 ± 7.7[a]	188.6 ± 14.9[a,c]
Serum AST (U/L)	18.5 ± 2.4	137.7 ± 10.8[a]	88.7 ± 9.4[a,b]

Mice were fed a normal chow diet (NCD) or the choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) for 8 weeks and concomitantly treated with myricetin (100 mg/kg) or vehicle by daily gavage. Values are expressed as the mean ± SEM. ALT, alanine transaminase; AST, aspartate transaminase; Myr, myricetin; Veh, vehicle; N, sample size. [a]P < 0.001 vs. NCD; [b]P < 0.01, [c]P < 0.001 vs. CDAHFD + Veh.

Histological assessment exhibited that CDAHFD-fed mice highly induced lipid accumulation, hepatocyte death, and liver inflammation, with markedly enhanced macrophages infiltration that limited to the area surrounding the centrilobular veins of the liver (**Figure 2**). However, these morphological alterations were remarkably attenuated in NASH mice treated with myricetin. These observations were further confirmed by the NAFLD activity score from H&E-stained sections, which were lower in the myricetin-treated NASH mice than that in the vehicle-treated NASH mice (**Figures 2A,B**). As shown by ORO-staining, lipid accumulation in hepatocytes was also decreased in NASH mice with myricetin treatment when compared with the animals with vehicle treatment (**Figures 2C,E**). Moreover, hepatic macrophages were assessed by IHC staining for F4/80. As shown in **Figure 2D**, the basal amounts of hepatic macrophages were observed in NCD-fed mice, the number of hepatic macrophages and the foci containing macrophages were remarkably enhanced in mice fed with CDAHFD for 8 weeks. But treatment with myricetin obviously reduced the staining signaling of $F4/80^+$ macrophages in CDAHFD-fed mice compared to vehicle-treated to those mice. These observation was further confirmed by quantification of the $F4/80^+$ staining area, indicating that CDAHFD-fed for 8 weeks facilitated macrophages recruitment into the livers, and that the increased number of $F4/80^+$ cells were remarkably decrease in myricetin-treated to NASH mice as compared with that in vehicle-treated to NASH mice (**Figure 2F**). In addition, we used TUNEL assay to evaluate the effect of myricetin on hepatocytes apoptosis in the liver from mice after feeding with CDAHFD for 8 weeks. As expected, the number of $TUNEL^+$ cells were significantly increased in CDAHFD-fed mice compared that in NCD-fed mice; however, CDAHFD-fed mice receiving myricetin administration could lower the elevated number of $TUNEL^+$ cells when compared to those animals receiving vehicle administration (14.16 ± 0.88/field vs. 25.24 ± 0.83/field; $P < 0.0001$; **Figures 2G,H**).

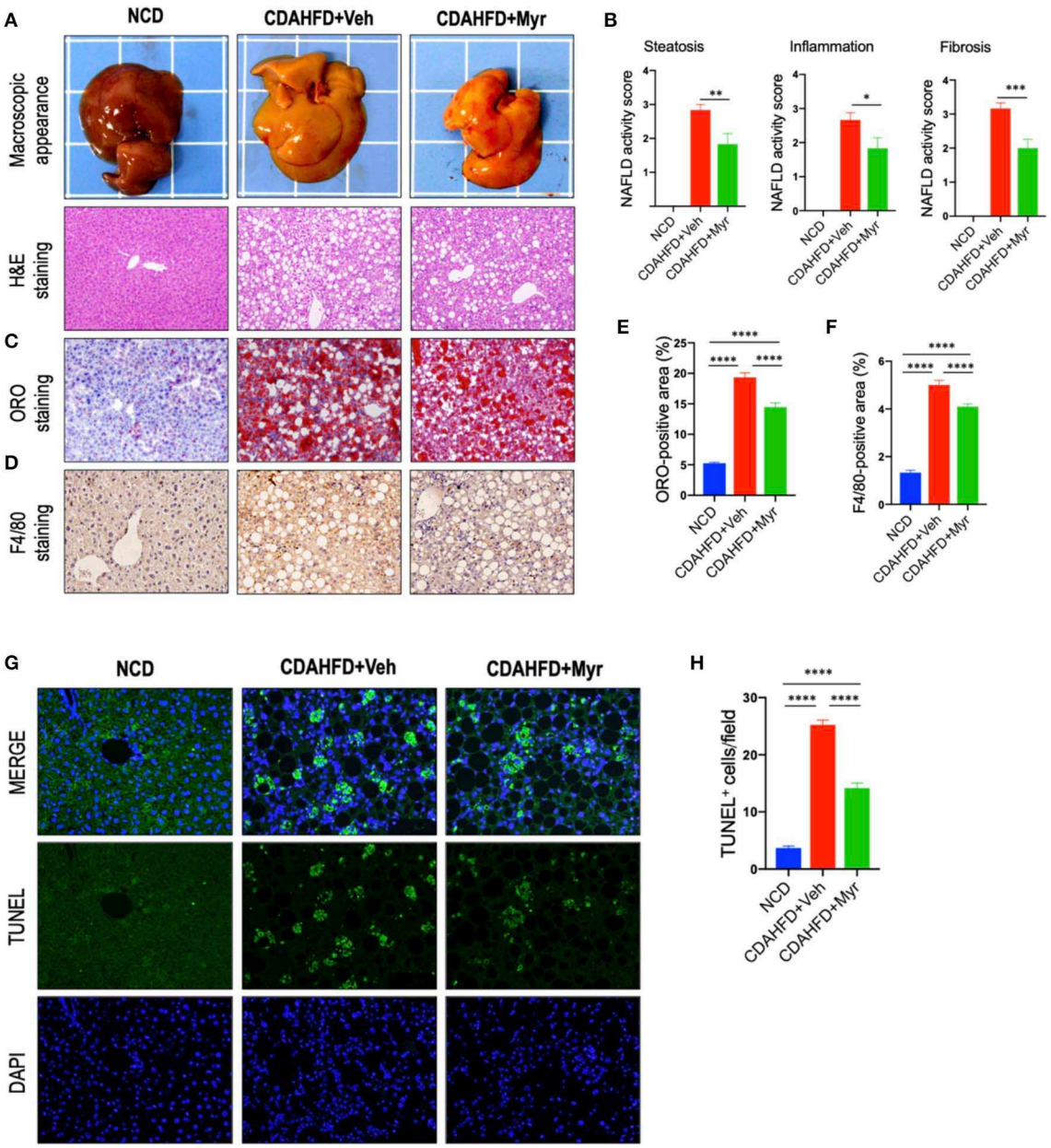

FIGURE 2 | Myricetin (Myr) alleviated hepatic steatosis, hepatocytes injury, and death, and inflammation in a diet-induced murine model of NASH. **(A)** Representative macroscopic appearance of livers and H&E staining of the liver sections. Original magnification: ×100. **(B)** Histological analysis using NAFLD activity score (NAS). $N = 6$ for each group. **(C)** Oil Red O (ORO) staining of liver sections from mice. Original magnification: ×100. **(D)** Representative images of Immunohistochemical (IHC) staining of F4/80 in liver sections. Original magnification: ×200. **(E)** Quantification of ORO-positive staining. **(F)** Quantification of F4/80$^+$ area staining in liver sections. Results mean of six fields and $n = 5$/group. **(G)** TUNEL staining for apoptotic cells of the liver sections from each group mice. Original magnification, ×200. **(H)** Quantification of the TUNEL$^+$ cell number per high-power field (×200). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Taken together, our results demonstrated that myricetin alleviated hepatic steatosis, hepatocytes injury and death, and inflammation in a diet-induced murine model of NASH.

Myricetin Inhibited Liver Fibrosis and HSC Activation in NASH Mice

As shown in **Figure 3A**, mice fed of CDAHFD for 8 weeks led to obviously collagen accumulation, with deposition of extracellular matrix and formation of thin portal-to-portal fibrous septa. In contrast, CDAHFD-fed mice with myricetin administration showed thinner septa and more preserved intact hepatocytes than those animals with vehicle administration (**Figure 3A**). These findings were further confirmed by the percentage of fibrotic areas from each section, which was remarkably decreased in CDAHFD-fed, myricetin-treated mice vs. vehicle-treated mice on the same diet (5.88% vs. 10.56%,

$P < 0.0001$; **Figure 3C**). Similarly, the mean fibrosis score was significantly reduced in myricetin-treated NASH mice than that in vehicle-treated NASH mice (1.83 ± 0.31 vs. 2.83 ± 0.17, $P = 0.009$; **Figure 2B**). Consistent with these findings, we noted that the mRNA expression of profibrogenic markers, including collagen 1α1 (Col1α1), connective tissue growth factor (CTGF), matrix metalloproteinase-9 (MMP-9), and tissue inhibitor of metalloproteinase-1 (TIMP-1), were markedly increased in NASH livers than that in NCD-fed livers; however, myricetin administration remarkably abrogated the effect of CDAHFD and downregulated the expression of these profibrogenic markers (**Figure 3F**).

In order to investigate that effect of myricetin on HSC activation in the liver; we here examined the activated HSC marker α-SMA expression by IHC staining in liver sections. Our IHC showed that there was remarkably increased α-SMA immunostaining in the fibrotic septa in livers from CDAHFD-fed mice, while little staining of α-SMA in livers from NCD-fed mice; however, there was a relatively weak intensity in livers from myricetin-treated NASH mice when compared that from vehicle-treated NASH mice (**Figure 3B**). Consistent with this observation, semi-quantitative analysis demonstrated that the α-SMA-positive area was significantly lower in the liver from myricetin-treated NASH mice than those from vehicle-treated animals ($2.82 \pm 0.15\%$ vs. $5.15 \pm 0.18\%$, $P < 0.0001$; **Figure 3D**). These findings were further validated by western blotting and quantitative RT-PCR analysis that revealed lower expression α-SMA gene and protein in NASH mice with myricetin treatment compared with those mice with vehicle treatment (**Figures 3E,F**).

Collectively, these results indicated that myricetin strikingly attenuated NASH-related fibrosis and the activation of HSCs in a murine NASH model induced by CDAHFD.

Myricetin Treatment Suppressed M1 Polarity Switch in the Liver Macrophages in NASH Mice

To determine whether myricetin limits hepatic inflammation and fibrosis by switching macrophages polarization and modulating their function, we assessed the effect of myricetin on M1 polarization and activation in the pathogenesis of NASH with fibrosis. Here, we selected IL-12A and interferon regulatory factor 5 (IRF5) as molecular markers of M1 polarization (5, 45). We found that CDAHFD-fed remarkably enhanced M1-polarized macrophages, as indicated by IHC staining of IL-12A and IRF5 (**Figures 4A,B**) and by analysis of the IL-12^+ area and IRF-5^+ areas in liver sections (**Figures 4C,D**); however, myricetin-treated NASH mice inhibited M1 macrophage phenotype as determined by M1-polarized markers. Of note, these positive macrophages were limited into hepatic sinusoids and fibrotic septa in NASH mice. These results were further confirmed by quantitative RT-PCR for M1-related markers and indicated that CDAHFD-fed mice were related to the increased proinflammatory cytokine markers in livers, including TNF-α, IL-1β, IL-6, and MCP-1 mRNA, as compared with the NCD-fed mice. However, compared with vehicle-treated to NASH diet mice, myricetin-treated to NASH diet mice reduced the levels of those M1 markers genetic expression (**Figure 4E**). Taken together, these results suggested that myricetin administration remarkably inhibited the M1-poliarized macrophages and reduced the expression of inflammatory properties in livers of NASH mice.

Myricetin Treatment Enhanced M2 Polarity Switch in the Liver Macrophages in NASH Mice

We also determined the effect of myricetin on M2-polarized macrophages in NASH mice with fibrosis. We applied chitinase-3-like 3 and 4 (also known as Ym-1 and Ym-2 in mice, respectively) and CD163 as molecular markers of M2-polarized macrophage (5, 45); and we noted that M2 macrophages in the liver were slightly increase in NASH mice induced by CDAHFD for 8 weeks as assessed by the Ym-1/Ym-2 and CD163 immunostaining (**Figures 5A,B**); however, NASH mice with myricetin treatment enhanced the density of Ym-1/Ym-2 and CD163 staining when compared with the NASH mice with vehicle treatment (**Figures 5A,B**). These results were further validated by percentages of Ym-$1/2^+$ and $CD163^+$ staining area (**Figures 5C,D**), suggesting myricetin-treated induced M2-polarized macrophages in livers from mice fed with CDAHFD. As a further corroboration, the M2 skewing was supported by the quantitative RT-PCR for selective M2-polarized markers such as CD163, IL-10, and Ym-1; and our results demonstrated that myricetin increased expression of CD163 and IL-10 mRNA, but there was no difference in Ym-1 gene expression between the myricetin-treated NASH mice and vehicle-treated NASH mice (**Figure 5E**).

Together, these results showed that myricetin treatment induced hepatic M2 macrophage polarization in CDAHFD-induced NASH and immunosuppressive genes (IL10 and CD163).

Myricetin Treatment Suppressed M1-Polarized and Induced M2-Polarizd Macrophages *in vitro*

To investigate the effect of myricetin on macrophages polarization, we examined M1 and M2 markers by myricetin treatment in RAW264.7 cell induced by LPS or IL-4, respectively. As the RAW264.7 cell line can be reliably polarized to M1 phenotype *in vitro* by LPS stimulation (9, 11, 38), using the model of M1 macrophages, we found that administration with myricetin to the cells remarkably inhibited M1 polarity switch in macrophages as indicating in immunostaining with anti-IL12A and anti-IRF5 (**Figures 6A–C**). Next, we determined the expression of M1 macrophages markers such as IL12 and IRF5 by western blotting, our results revealed that myricetin administration markedly lowered the protein expression of those markers when compared with vehicle administration (**Figure 6D**). Moreover, our quantitative RT-PCR results also confirmed that myricetin treatment led to inhibiting M1 polarity switch in macrophages as depicted in mRNA expression of M1 markers (TNF-α, IL-1β, IL-6, and NOS2) (**Figure 6E**).

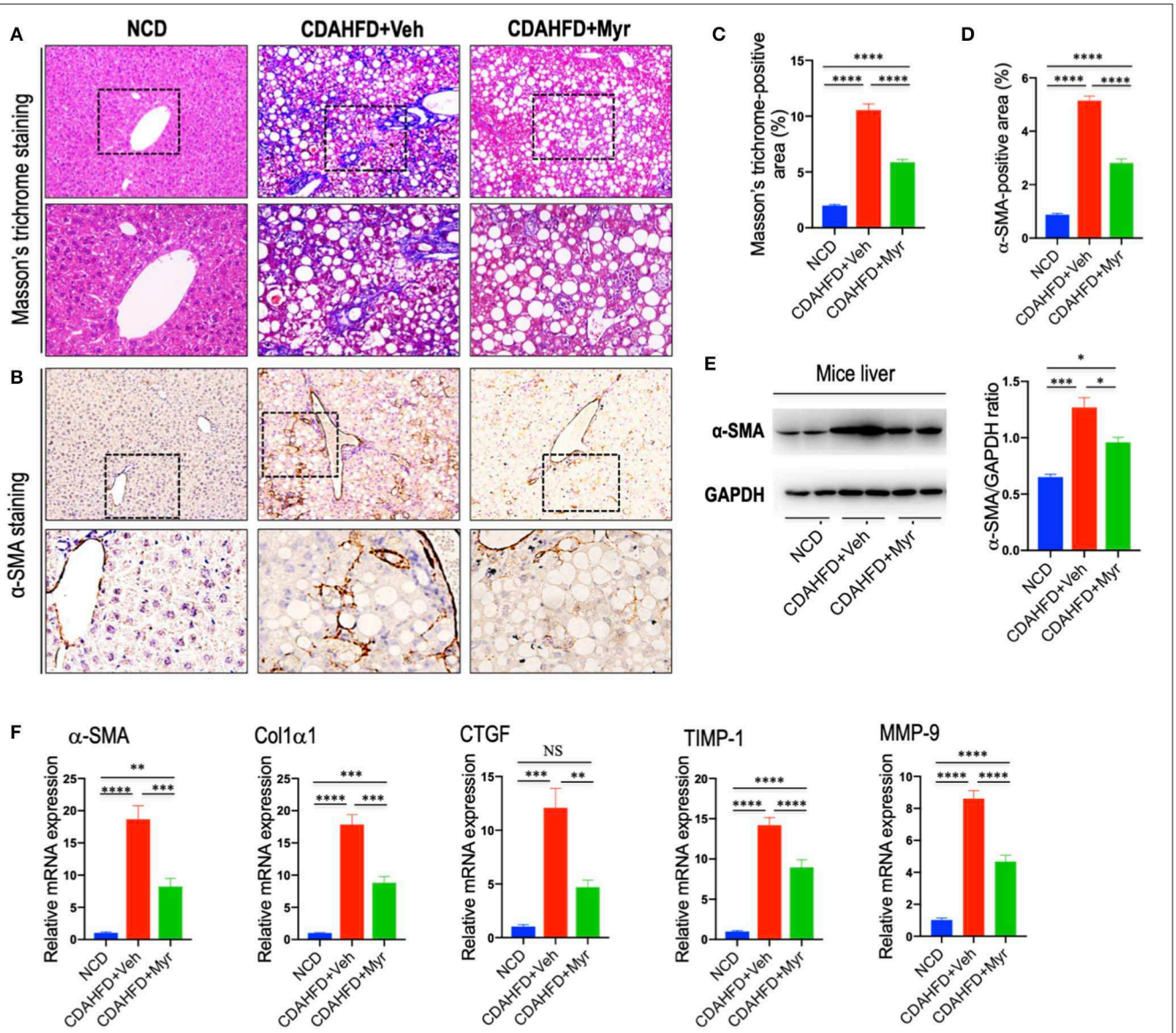

FIGURE 3 | Myricetin inhibited liver fibrosis and HSC activation in NASH mice. **(A)** Masson's trichrome staining of liver sections in mice from fed an NCD or CDAHFD with myricetin (Myr) or Vehicle (Veh). Upper panel (original magnification, ×100) and lower panel (Original magnification, ×200). **(B)** Immunohistochemical (IHC) images of liver sections stained for α-smooth muscle actin (α-SMA). Bottom row contains images enlarged from the boxed area in the corresponding panel in the top row. Original magnification, ×100 (upper panel) and ×200 (lower panel). **(C)** Quantitative analysis of Masson's trichrome positive area. **(D)** Quantification of the α-SMA-positive area. **(E)** Western blotting analysis of α-SMA protein in livers; results were normalized relative to expression of GAPDH. **(F)** Transcript levels of fibrotic markers (α-SMA, Col1α1, CTGF, TIMP-1, and MMP-9) were measured by quantitative RT-PCR in whole liver samples ($n = 5$). Results were normalized to β-actin mRNA and expressed as folds change compared to NCD-fed mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; "NS" indicates not significant.

Additionally, at this dosage, myricetin had not affect cell viability of macrophages *in vitro* (**Figure S1**).

On the other hand, to investigate M2 macrophages phenotypes switching by myricetin in RAW267.4 cells, the culture of RAW267.4 cells were administrated with vehicle, myricetin (50 μM), or IL-4 (20 ng/ml) for 12 h. We found that the mRNA expression of M2 marker (Ym-1, Arg1, CD163, and IL-10) was highly enhanced by administration with myricetin when compared with administration with vehicle (**Figure 6F**).

Collectively, these findings indicated that myricetin administration could suppress the M1-polarized macrophages and induce M2 polarity upon stimulation *in vitro*.

Myricetin Treatment Inhibits the TREM-1-TLR2/4-MyD88 Signaling in the Liver of CDAHFD-Treated Mice and in LPS-Stimulated RAW264.7 Cells

To further explore whether the decreased M1 macrophages by myricetin was due to inhibition TREM-1-mediated signaling, we

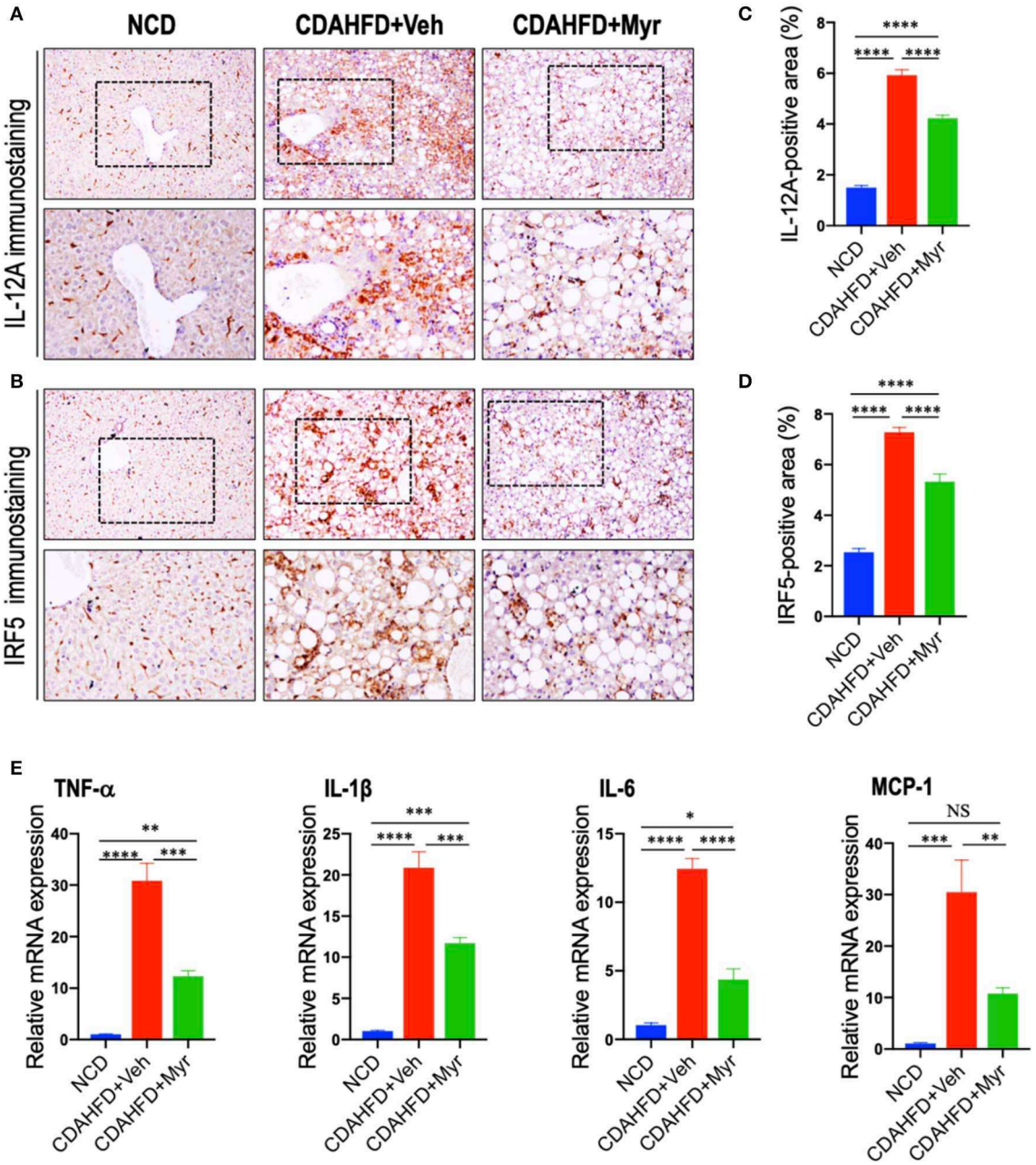

FIGURE 4 | Myricetin treatment suppressed M1 polarity switch in the liver macrophages in NASH mice. **(A,B)** Representative immunostaining of IL-12A and interferon regulatory factor 5 (IRF5) in liver sections. Bottom row contains images enlarged from the boxed area in the corresponding panel in the top row. Original magnification: ×100 (upper panel) and ×200 (lower panel). **(C,D)** Quantification of the IL-12A$^+$ and IRF5$^+$ staining area in the liver from each group. **(E)** Hepatic mRNA expression of M1-polarized markers (TNF-α, IL-1β, IL-6, and MCP-1) was determined by quantitative RT-PCR, and results are shown as fold change compared with NCD-fed mice and β-actin served as loading control. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; "NS" indicates not significant.

firstly examined the TREM-1 expression and subcellular location in the liver. As shown in **Figure 7A**, immunofluorescence double staining of TREM-1 and F4/80 in liver sections from mice revealed that TREM-1$^+$ and F4/80$^+$ cells almost overlapped; and the staining signal of TREM-1 in NASH and fibrotic liver is stronger than NCD-treated liver. However, compared with vehicle-treated NASH mice, the double-staining signaling of the F4/80$^+$ TREM-1$^+$ cells were remarkably decreased. The number of double-positive TREM-1$^+$ F4/80$^+$ cells increased remarkably in NASH mice compared with normal control, but this increase was inhibited by myricetin treatment in NASH mice (**Figure 7B**). We further determined the gene and protein

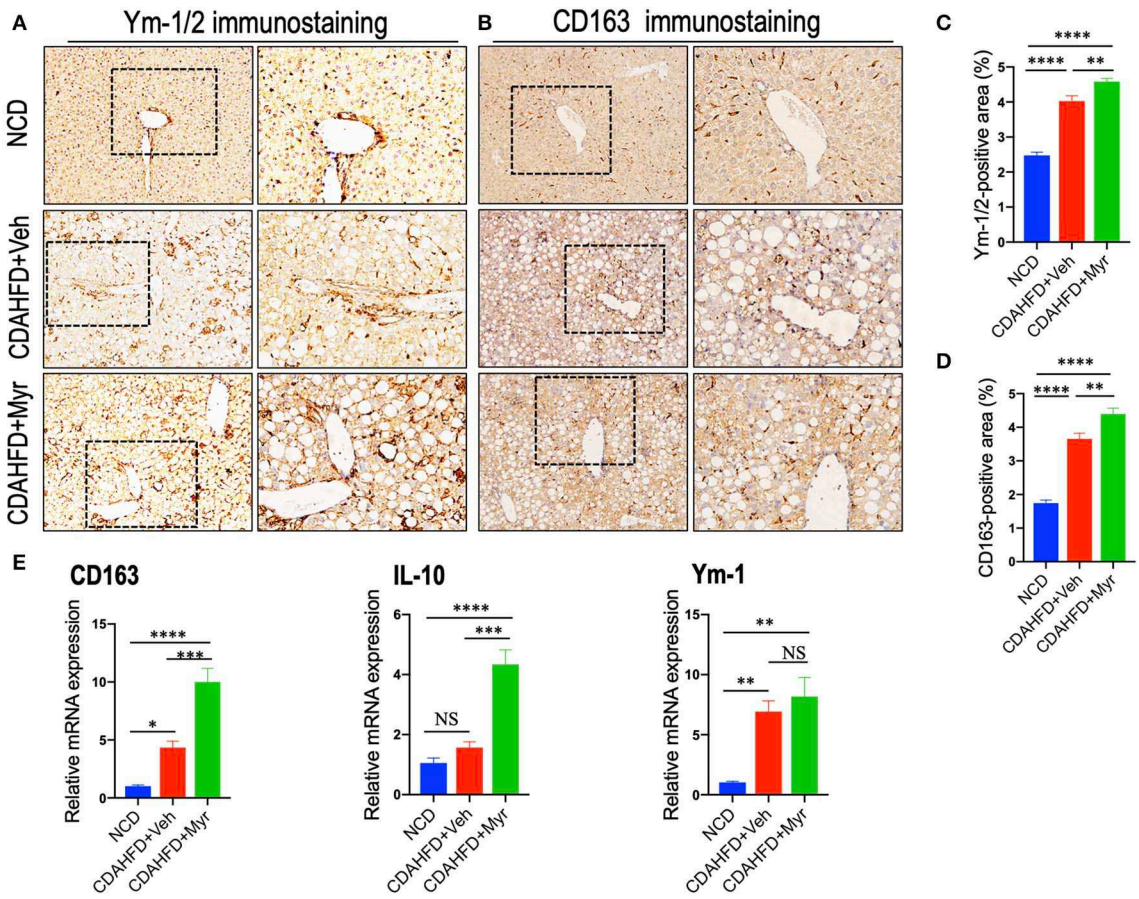

FIGURE 5 | Myricetin (Myr) treatment enhanced M2 polarity switch in the liver macrophages in NASH mice. **(A,B)** Representative immunostaining of Ym-1/2 and CD163 in liver sections. Bottom row contains images enlarged from the boxed area in the corresponding panel in the top row. Original magnification: ×100 (left panel) and ×200 (right panel). **(C,D)** Quantification of the Ym-1/2$^+$ and CD163$^+$ staining area in livers from each group. **(E)** Hepatic mRNA expression of M2-polarized markers (CD163, IL-10, and Ym-1) was determined by quantitative RT-PCR, and the results are shown as folds change compared with NCD-fed mice and β-actin served as loading control ($n = 5$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; "NS" indicates not significant.

expression of TREM-1 in the liver from mice by quantitative RT-PCR and western blotting, respectively. Our results demonstrated that there was a remarkable increase in TREM-1 at both the mRNA and protein levels in livers from NASH as compared with those in livers from NCD-fed controls; however, myricetin-treated NASH mice decreased the TREM-1 expression both in gene and protein levels when compared with vehicle-treated NASH mice (**Figures 7C,D**). Additionally, we also determined the mRNA expression of TLR2, TLR4, and the adapter MyD88 in the liver; we also found that CDAHFD-fed induced those genes expression as compared with NCD-fed mice. However, the upregulated expression of those genes in NASH livers was significantly inhibited by myricetin treatment as compared with vehicle treatment (**Figure 7D**).

To better characterize whether myricetin inhibits macrophage polarization to M1 via regulating TREM-1-mediated signaling on macrophages, we investigated the expression of TREM-1, TLR2, TLR4, and MyD88 in RAW264.7 cells that incubated with myricetin prior to induction M1-polarized macrophages using LPS *in vitro*. We found that LPS remarkably enhanced the expression of TREM-1in RAW264.7 cells at gene level, whereas these elevated expression of TREM-1 was markedly abrogated by administration of 50 μM myricetin. Moreover, myricetin also significantly reduced the mRNA expression of TLR2, TLR4, and MyD88 in LPS-stimulated RAW264.7 cells relative to DMSO-treated cells (**Figure 7E**).

Taken together, these findings indicated that myricetin treatment inhibited TREM-1-TLR2/4-MyD88 signaling both *in vivo* and *in vitro* study, which might be, at least in part, involved in regulating liver macrophages polarization.

Myricetin Inhibited the Activation of NF-κB Signaling and Reduced the Phosphorylation of STAT3 and JNK in LPS-Stimulated RAW264.7 Macrophages

Since the transcription factor NF-κB plays a critical role in inflammatory response, and we explored the effects of myricetin on the activation of NF-κB pathways by assessing the phosphorylation of I-κBα and NF-κB in LPS-stimulated RAW

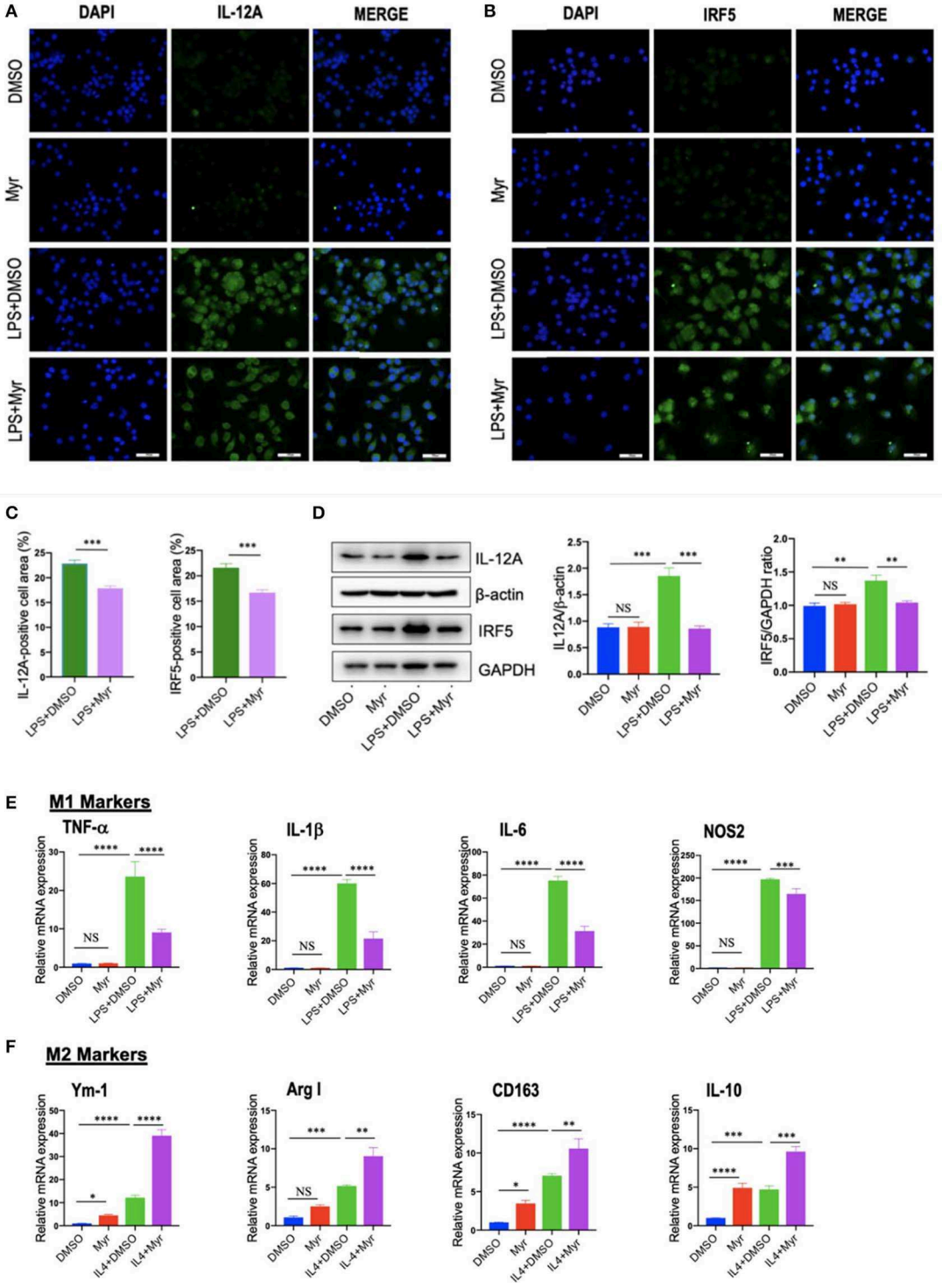

FIGURE 6 | Myricetin (Myr) treatment suppressed M1 and induced M2 polarization of macrophages *in vitro*. **(A,B)** Representative fluorescence microscopic images of RAW264.7 cells with anti-IL12 and anti-IRF5 whole-mount staining. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Undifferentiated RAW macrophages conditioned medium (M0), M1-differentiated macrophages conditioned medium, Myr (50 μM) or DMSO treated M1-differentiated macrophages conditioned medium. Bars represent mean ± SEM of at least three independent experiments. Scale bar = 50 μm. **(C)** Percentage of the IL-12$^+$ and IRF5$^+$ staining area in 6 randomly selected fields. **(D)** Western blotting and quantification of M1-marker IL12 and IRF5 protein expression in macrophages RAW264.7 cells, with results normalized relative to the expression of β-actin or GAPDH, respectively ($n = 3$). **(E)** Quantification gene expression analysis of M1 markers TNF-α, IL-1β, IL-6, and NOS2. The mRNA levels were normalized to β-actin mRNA levels and presented as folds change vs. DMSO-treated control. **(F)** Quantification gene expression analysis of M2 markers Ym-1, Arg1, CD163, and IL-10. The mRNA level was normalized to β-actin mRNA level and presented as folds change vs. DMSO-treated control. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; "NS" indicates not significant.

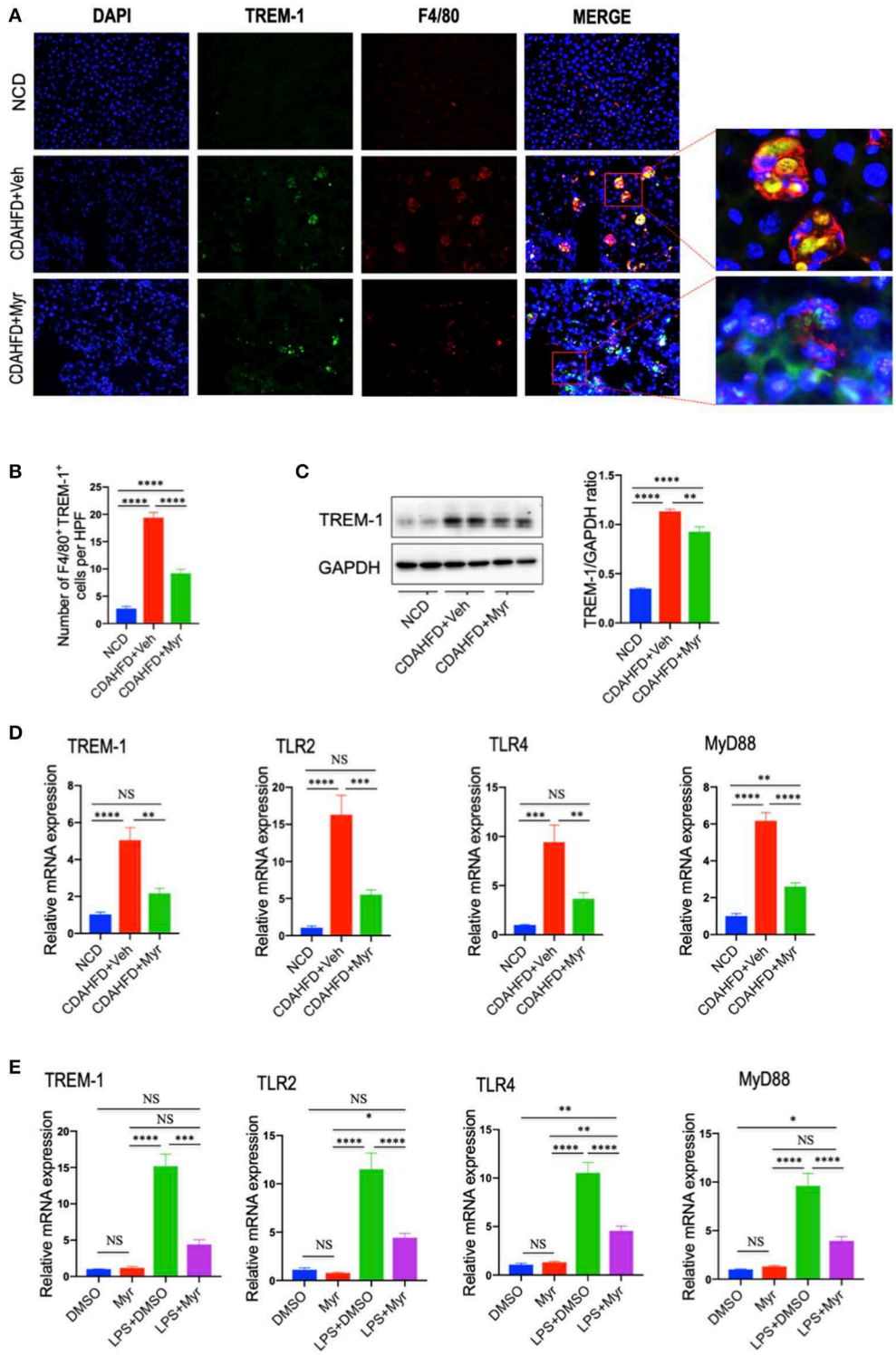

FIGURE 7 | Myricetin treatment inhibits the TREM-1-TLR2/4-MyD88 signaling in the liver of CDAHFD-treated mice and in LPS-stimulated RAM267.4 cells. **(A)** Immunofluorescent double staining in liver sections from each group. Liver sections were double stained for TREM-1 (green) and F4/80 (Red) macrophages. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Original magnification: ×200; Scale bar = 50 μm. **(B)** Quantification of TREM-1 and F4/80 double-positive cells. **(C)** Western blotting and quantification of TREM-1 expression in lysed liver tissues, with results relative to the expression of GAPDH ($n = 3$). **(D)** Hepatic mRNA expression of TREM-1, TLR2, TLR4, and MyD88 was measured by quantitative RT-PCR. Results are shown as fold change compared with NCD-fed mice and β-actin served as loading control ($n = 5$). **(E)** The mRNA expression of TREM-1, TLR2, TLR4, and MyD88 in RAW264.7 cells were measured by quantitative RT-PCR. The mRNA levels were normalized to β-actin mRNA levels and presented as fold stimulation (mean ± SEM) vs. DMSO. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; "NS" indicates not significant.

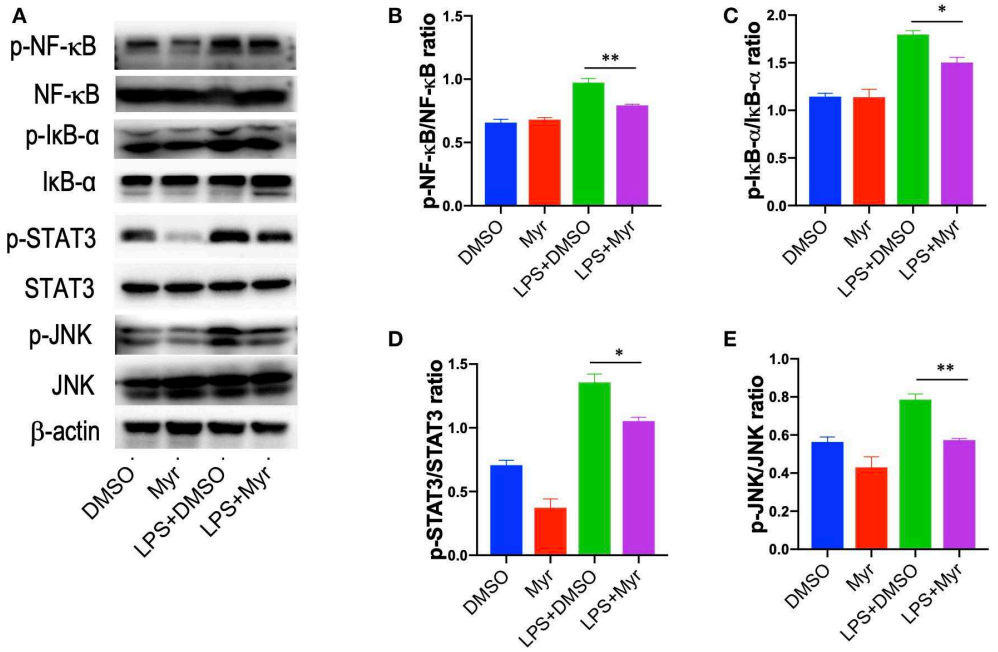

FIGURE 8 | Myricetin inhibited the activation of NF-κB signaling and reduced the phosphorylation of STAT3 and JNK in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with myricetin (50 μM) for 12 h and then incubated for 12 h with LPS (100 ng/mL). **(A)** Western blotting analysis of the p-NF-κB p65, NF-κB p65, p-Iκ-Bα, Iκ-Bα, p-STAT3, STAT3, p-JNK, and JNK levels in LPS-stimulated RAW264.7 macrophages; and β-actin protein levels served as control. **(B,C)** Quantification of p-NF-κB p65/ NF-κB p65 ratio and p-Iκ-Bα/ Iκ-Bα ratio. **(D,E)** Quantification of p-STAT3/STAT3 ratio and p-JNK/JNK ratio. *$P < 0.05$, **$P < 0.01$.

264.7 cells. Our results revealed that treatment of RAW264.7 cells with LPS induced an obviously increase of the phosphorylation of I-κBα and NF-κB, while application of myricetin inhibited the phosphorylated expression I-κBα and NF-κB (**Figures 8A–C**). Additionally, STAT3 and JNK signaling are progressively elevated in livers of NASH (7, 46), in this study, we found that p-STAT3 and p-JNK were also increased in LPS-stimulated RAW264.7 cells, however, myricetin pre-treated to LPS-stimulated RAW264.7 cells led to lower the increased expression of the p-STAT3 and p-JNK as compared with vehicle-treated to the cells (**Figures 8A,C,D**). Collectively, these finding indicated that myricetin inhibited LPS-induced TREM-1-TLR2/4-MyD88-mediated inflammatory response via inhibiting of NF-κB activation and reducing the phosphorylation of STAT3 and JNK expression.

DISCUSSION

Currently, there is no pharmacological agents that has been convincingly efficient against NASH and fibrogenesis (4, 47). In this study, we have provided both *in vivo* and *in vitro* evidence regarding the potent role of the myricetin in reducing the severity of steatosis, inflammation, hepatocyte cell injury and death, and fibrosis in CDAHFD-derived NASH model. Importantly, our results provide an evolving insight into the anti-inflammatory and antifibrotic effects of myricetin against NASH, which owning to its regulation the polarization of macrophages in the livers. Additionally, our data also have strongly reinforced the notion that liver macrophages polarization are associated with the development of NASH and fibrogenesis, and strategies inhibiting M1 polarity switch of macrophages may protect against exacerbated NASH and fibrosis (7, 9, 48).

Myricetin is one of the common plant-derived flavonoids, which exhibits a wide range of pharmacological effects including anti-inflammatory, antioxidant, anti-obesity, and antitumor activities (28–34, 37, 49). Here, using a murine model of diet-induced NASH, we demonstrated myricetin-treated to NASH mice significantly inhibited the pro-inflammatory cytokines (such as TNF-α, IL-1β, IL-6, and MCP-1) expression (**Figure 4E**). In line with decreased hepatic inflammation, plasma levels of ALT and AST were markedly decreased in myricetin-treated NASH mice (**Table 1**). Moreover, myricetin-treated to CDAHFD-fed mice markedly reduced hepatocyte apoptosis compared with vehicle-treated to CDAHFD-fed mice (**Figures 2G,H**). Meanwhile, our results also revealed that liver injury and hepatocytes death triggered the activation of Kupffer cell (**Figure 2D**), leading to inflammatory cytokine and chemokine production during NASH development. The perpetuation of inflammation and hepatocytes death further led to the pathogenesis of NASH and liver fibrosis (4, 5). However, myricetin treatment could inhibited this pathologic process. Indeed, our results confirmed that myricetin-treated to NASH mice effectively inhibited the activation of HSCs and hepatic fibrosis compared with vehicle-treated mice on the same diet (**Figure 3**). Supported our data, myricetin attenuated liver fibrosis-induced by CCl_4 in mice (29, 32)

and ameliorated high-fat diet-induced obesity and IR through enhancing antioxidant capacity (34). However, the exact molecular mechanisms by which myricetin exerts its beneficial effects on liver inflammation and fibrosis in NASH has been largely unknown.

Here, we used an established model of NASH to determine the mechanism by which myricetin improved liver disease. We highlighted the effects of myricetin on the activation and polarization of macrophages. Interestingly, we found that myricetin-treated to CDAHFD-fed mice suppressed M1-polarized macrophages in livers as shown by IHC staining of IRF5 and IL-12A and reduced mRNA expression of proinflammatory M1 marker (TNF-α, IL-1β, IL-6, and MCP-1) (**Figure 4**). Moreover, using murine macrophage RAW267.4 further confirmed that myricetin significantly inhibited LPS-induced M1-polarized macrophages *in vitro* (**Figures 6A–E**). It well documented that excessive or unresolved M1-polarized macrophages could incur chronic inflammation and tissue injury (39). Therefore, myricetin administration inhibited M1-polarized macrophages, which might be the mechanism of protection from NASH development and liver fibrogenesis exerted by myricetin.

On the other hand, we also assessed the effect of myricetin on M2 polarity switch of macrophages in NASH and fibrosis in mice. Our IHC results showed that M2 macrophage markers, Ym-1/2$^+$ and CD163$^+$, were both remarkably increased at 8-week CDAHFD-fed when compared with NCD-fed controls; however, myricetin treatment further induced the numbers of Ym-1/2$^+$ cells and CD163$^+$ cells. We also found hepatic mRNA expression of M2 markers (IL-10, CD163, and Ym-1) in NASH mice were induced as compared to that in NCD-fed control mice (**Figure 5**); however, myricetin-treated to NASH mice further increased hepatic mRNA expression of CD163 and IL-10 markers when compared with vehicle-treated to those animals, but there was no difference in Ym-1 gene expression (**Figure 5**). *In vitro* experiment, using IL-4 induced M2 polarization, we found myricetin induced M2-polarized macrophage genes (Ym-1, Arg1, CD163, and IL-10) similarly IL-4-induced macrophages (**Figure 6F**). Noticeably, several previous studies have also demonstrated that M2 macrophages activation (Ym-1$^+$, CD206$^+$, or CD163$^+$) was induced and had the potential effect on inflammatory response and fibrogenesis both in chronic liver diseases and in animal models (47, 50–52). However, it's worth to note that M2 macrophages significantly decreased at the later stage of NASH and fibrosis (51). Although M2 polarity of macrophages is often thought to having anti-inflammatory or reparative properties, excessive or unrestricted M2-polarized macrophage can also result in immune dysregulation and liver fibrosis (38, 39, 52). Therefore, our results provided a new insight for understanding the anti-inflammatory and antifibrotic effect of myricetin in NASH, which owning to its attenuation of liver macrophages infiltration and suppression of the M1 polarity of liver macrophages.

Importantly, our results further revealed that the molecular mechanism of modulation of macrophages polarization in livers by myricetin was surmised to be direct inhibition of the TREM-1-TLR2/4-MyD88 signaling pathways (**Figure 7**). Our immunofluorescent double staining result indicated that the gene and protein expression of TREM-1 on macrophages was upregulated in the liver of NASH mice together with quantitative RT-PCR and Western blotting. However, myricetin treatment abrogated the increase levels of TREM-1 gene and protein expression (**Figures 7A–D**). Activation of TREM-1 has been shown to trigger and aggravate inflammation, especially through synergism with TLRs signaling (13–16); therefore, we further assessed the effects of myricetin on the mRNA expression of TLR2, TLR4, and its adapter protein MyD88. As expected, our result confirmed that myricetin treatment significantly inhibited TLR2/4-MyD88 signaling expression in livers from NASH mice. Thus, TREM-1-mediated inflammatory response at least in part was associated with hepatic macrophages polarization in NASH. Indeed, previous data have also revealed that TREM-1 pathway plays an important role in macrophage-mediated inflammatory response (18–21). Interestingly, a recent study suggested that TREM-1 receptor also mediated in reversing M2 polarization induced by hypoxia (18). Although the specific ligands of TREM-1 have not been identified yet, it has been revealed that TREM-1 activation amplifies the TLR2/4-mediated proinflammatory signals, allowing the secretion of proinflammatory chemokines and cytokine (14, 17). Moreover, recent studies have demonstrated that TREM-1 mediated signaling modulation of M1 macrophage activation promoted the inflammatory response in alcoholic liver disease and obesity-induced fatty liver disease (18, 20). To further delineate how myricetin switches macrophage polarity in NASH development, we examined TREM-1-mdiated inflammatory responses *in vitro* of LPS-induced macrophage activation, consistent with M1 macrophage polarization, the expression of TREM-1 was upregulated on LPS-induced macrophages. However, treatment of myricetin attenuated M1 polarity switch of macrophages and that was associated with the reduce of TREM-1 signaling expression on macrophages (**Figure 7**). Given that TLR2/4 signaling pathway also contribute to proinflammatory macrophages activation (53, 54), we deduced that this inhibitory effect of myricetin may be also involved in blocking M1 macrophages polarization. Taken together, our data highlighted an important role for the TREM-1-TLR2/4 signaling pathways in regulating M1 macrophage polarization in CDAHFD-fed induced NASH and liver fibrosis, indicating that inhibition of TREM-1 signaling might be an effective therapeutic target for NASH and liver fibrosis.

Of note, our results further revealed that myricetin led to inhibiting LPS-induced TREM-1-mediated inflammatory response via downregulation of NF-κB activation and reducing the phosphorylation of STAT3 and JNK expression as shown by our western blotting (**Figure 8**). These findings were consistent with previous studies exhibiting that myricetin attenuated inflammatory response in LPS-induced macrophages *in vitro* and in streptozotocin-induced diabetic nephropathy by inhibiting NF-κB signaling pathways (28, 37).

In conclusion, administration of myricetin attenuated hepatocyte injury and death, inflammation, and fibrogenesis in the CDAHFD-diet-induced NASH model through regulating polarization of macrophages in livers via TREM-1-TLR2/4-MyD88 signaling pathways. These results suggested that

myricetin could be considered a potential therapeutic agent for NASH and liver fibrosis.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Ethics Committee of Zhongshan hospital.

AUTHOR CONTRIBUTIONS

QY, SL, and CT conceived the study and wrote the manuscript. XL, QY, FW, and CT contributed to the work designing, performing, analyzing, and interpreting data from all the experiments. QY, SL, FW, and CT participated in the design, acquisition, analysis, and interpretation of data. CT, XL, and SL carried out the animal model and all the *in vivo* animal experiments. CT, QY, and SL interpreted the data and finalized the article. All authors have critically revised and approved the final manuscript and agreed 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.2020.00071/full#supplementary-material

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frontiers in Medicine

BRIEF RESEARCH REPORT
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Intrahepatic Expression of Fatty Acid Translocase CD36 Is Increased in Obstructive Sleep Apnea

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Nocturnal intermittent hypoxia (IH) featuring obstructive sleep apnea (OSA) dysregulates hepatic lipid metabolism and might contribute to the development of non-alcoholic fatty liver disease (NAFLD) observed in OSA patients. However, further research is required to better understanding the molecular mechanisms underlying IH-induced hepatic lipid accumulation. Therefore, the aim of the present study was to determine the effects of OSA on hepatic CD36 expression and the impact of IH by using a mouse model of OSA. Histological analysis, lipid content and CD36 expression were assessed in livers from subjects who underwent liver biopsy and polygraphic study during sleep, and in livers from mice submitted to chronic IH mimicking OSA. Among those who presented OSA features, NAFLD were significantly more frequent than in control subjects with normal respiratory function (77.8 vs. 36.4%, respectively), and showed more severe liver disease. Interestingly, CD36 expression was significantly overexpressed within the liver of OSA patients with respect to controls, and a significant positive correlation was observed between hepatic levels of CD36 and the values of two well-known respiratory parameters that characterized OSA: apnea/hypopnea index (AHI) and oxygen desaturation index (ODI). Moreover, hepatic lipid accumulation as well as induction of hepatic lipogenic genes, and CD36 mRNA and protein expression were significantly higher in livers from mice exposed to IH conditions for 8 weeks than in their corresponding littermates. This study provides novel evidence that IH featuring OSA could contribute to NAFLD setup partly by upregulating hepatic CD36 expression.

Keywords: obstructive sleep apnea, intermittent hypoxia, CD36, steatosis, NAFLD

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by metabolic dysfunction and accumulation of lipid deposits in the livers of patients in whom alcohol abuse is not the causal agent of disease onset (1). NAFLD encompasses a wide range of histologic findings from simple steatosis to non-alcoholic steatohepatitis (NASH) with fibrosis and, ultimately, liver cirrhosis, and hepatocellular carcinoma (2). The number of individuals affected by some clinical form of this chronic liver disease is steadily increasing because NAFLD is highly associated with obesity and type 2 diabetes, being considered the hepatic manifestation of the metabolic syndrome (3).

It is well-known that the liver maintains bodily lipid homeostasis by regulating hepatic free fatty acid (FFA) uptake, lipid synthesis, lipid oxidation, and lipid export; however, an imbalance between these metabolic pathways can lead to an excessive lipid accumulation within the liver (4), being an increased *de novo* lipogenesis and largely an enhanced uptake of FFAs released from insulin resistant-adipocytes the main sources of these lipid accumulates (5). The uptake of FFAs into hepatocytes is mainly dependent on the fatty acid translocase CD36 which, under physiological conditions, is weakly expressed in the liver and its expression increases by a number of different stimuli, such as insulin and lipid metabolites, facilitating the process of FFA uptake (6). Some experimental studies have demonstrated that CD36 plays an important role in NAFLD setup in rodents (7, 8) and, reinforcing this notion, it has been observed that fatty liver attenuates in mice fed high fat diet (HFD) upon either systemic or hepatocyte-specific deletion of CD36 (9, 10). Moreover, a growing clinical evidence suggests that this FFA transporter could play a relevant role in NAFLD pathogenesis in humans as well. In particular, Greco et al. showed that hepatic CD36 mRNA levels correlated with liver fat content in morbidly obese patients (11). In addition, different clinical studies have convincingly shown that the amount of both CD36 mRNA and protein was higher in the livers of biopsy-proven NAFLD patients than in subjects with histologically normal liver (12–14).

An increasing number of clinical studies point out to a potential link between obstructive sleep apnea (OSA), a respiratory disorder featured by nocturnal intermittent hypoxia (IH) and sleep fragmentation, and NAFLD (15–18). To highlight, both OSA and NAFLD are especially prevalent among obese individuals and, more interestingly, the severity of nocturnal IH positively correlates with histological features of NASH in OSA patients (19). Although the underlying molecular mechanisms are not fully understood, it has been reported that IH exacerbated fatty liver in obese mice by inducing hepatic lipid biosynthesis (20) likely due to the upregulation of the HIF1α/SREBP1c signaling pathway (21), and that promoted liver inflammation and fibrosis in mice fed with a HFD (22). However, further research is required to unveil the pathophysiological interplays between IH and lipid accumulation. In that regard, whether IH is able to regulate CD36 gene expression in hepatocytes still remains to be elucidated.

Therefore, the primary objective of this study was to determine the impact of IH on CD36 expression as well as on lipid content in livers from OSA patients with biopsy-proven NAFLD and in livers from mice exposed to IH.

MATERIALS AND METHODS

Patients

This study was performed in agreement with the Declaration of Helsinki, and with local and national laws. The Human Ethics Committee of the Hospital Universitario Santa Cristina (HUSC, Madrid, Spain) approved all procedures (PI-688A). This cross-sectional study included 20 patients with gallstones to whom a programmed laparoscopic cholecystectomy was performed in the HUSC. All participants gave a written consent for a perioperative liver biopsy and a postoperative respiratory polygraphy as part of an experimental protocol designed to evaluate the relationship between sleep disturbances and liver disease. All subjects included drank <20 g/day of alcohol, had no previous respiratory disorders, were not having potentially hepatotoxic drugs, had no analytical evidence of iron overload, and were seronegative for autoantibodies, for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus.

Sleep Study

The polygraphic studies were performed at night in the Sleep Laboratory of the HUSC (Madrid, Spain). For interpretation, the recommendations of the American Academy of Sleep Medicine (AASM) for the diagnosis of OSA were followed. The apnea/hypopnea index (AHI) was used as diagnostic criteria for severity of OSA: AHI < 5, no OSA; AHI 5–14, mild OSA; AHI 15–29, moderate OSA; AHI ≥30, severe OSA. In addition, nocturnal hypoxemia parameters including oxygen desaturation index (ODI), cumulative sleep time percentage with oxyhemoglobin saturation (SpO_2) < 90% (Tc90) and minimum SpO_2 were analyzed.

Animal Care and Intermittent Hypoxia Protocol

Twelve-weeks-old C57BL/6J mice were purchased from Charles River Laboratories (Saint Germain sur L'Arbresle, France) and divided into two groups of 10 mice. The control mice (C mice) were placed in conditions of normoxia while the experimental group (IH mice) was subjected to IH conditions. Every minute, IH mice received air containing an oxygen fraction of 5% for 20 s, followed by 40 s of room air, during 6 h per day, 5 days a week for a total of 8 weeks. Control mice were only exposed to room air (23). At the end of the experiment, mice were anesthetized, sacrificed and livers were harvested. All experimental procedures were approved by the Ethical Committee of the University of Barcelona (174/18−10268).

Histopathology Assessment

Liver sections (5 μm) were embedded in paraffin and cut using a Microm microtome (Midland, ON, Canada). After cutting, sections were stained with hematoxylin (1.09235.0500, PanReac AppliChem, Barcelona, Spain) and eosin (71211, Thermo Fisher Scientific, Inc., Madrid, Spain) and with Masson's Trichrome Solution (Masson Trichome Kit with Aniline Blue 04-010802, Milan, Italy). Once stained, the severity of steatosis was quantified by a single-blind hepatopathologist. Specifically, Kleiner's histological scoring system was employed to evaluate the degree of steatosis, lobular inflammation, hepatocellular ballooning, and the stage of fibrosis (24). The following percentages of steatotic hepatocytes were used in the histological assessment: 0–5% hepatocytes, grade 0; 5–33%, grade 1; 33–66%, grade 2; and >66%, grade 3. Histologic diagnosis of liver biopsies was classified into two groups: simple steatosis without hepatocellular ballooning nor lobular inflammation, also termed NAFL, and NASH. Minimal criteria for NASH included the combined presence of grade 1 steatosis, lobular inflammation and hepatocellular ballooning with or without fibrosis. NAFLD

activity score was also calculated for each liver biopsy (24). To this end, three different lobular areas were analyzed in each sample. Representative images were taken using a Nikon Eclipse E400 optical microscope (Nikon, Tokyo, Japan) and the NIS Elements Imaging Software (Melville, NY, USA).

Assessment of Lipid Accumulation

Liver tissue was embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe, Netherlands). Sections (10 μm) were then cut using a Leica CM1510S cryostat (Leica Microsistemas S.L.U, Barcelona, Spain), stained using an Oil Red O biological stain (Sigma-Aldrich, St. Louis, MO, USA) working solution (60% ORO/isopropanol w:v), and counterstained with hematoxylin (1.09235.0500, PanReac AppliChem). Three different lobular areas were analyzed in each sample and photographed using a Nikon Eclipse E400 optical microscope (Nikon) and the NIS Elements Imaging Software (Melville). Intensity of red stain was quantified using ImageJ Biological Image Analysis (NIH) and reported as the average value in arbitrary units (a.u.).

Quantitative Analysis of Hepatic Triglycerides

Triglycerides (TGs) were extracted as described previously (25). Briefly, liver biopsy samples (15–20 μg) were homogenized in distilled water. Chloroform (−20°C) and methanol (−20°C) were added to each sample. Samples were centrifuged and the triglyceride-containing layer was collected. Once purified, TGs were suspended in isopropanol and analyzed using a colorimetric kit (SpinReact, Girona, Spain). Absorbance values were obtained using a Dynex Spectra MR Microplate spectrophotometer/computer software (Chantilly, VA, USA) and graphically expressed as mg/dl.

Protein Extraction and Western Blot Analysis

Liver biopsy samples (15–20 μg) were homogenized in an extraction buffer containing the following: 10 mM ethylene-diamine-tetraacetic acid (EDTA), 50 mM Hepes, 50 mM sodium pyrophosphate, 0.1 mM NaF, 10 mM Na_3VO_4, 1% Triton X-100 and protease inhibitors. Protein extracts were stored at −80°C after centrifugation. A small aliquot of sample was used for protein quantification (Bradford method). The samples were then prepared to be loaded into 8% SDS-PAGE gels. After running, proteins were further transferred to Inmunoblot nitrocellulose membranes (BioRad Inc., Madrid, Spain), blocked with 5% non-fat dry milk and incubated overnight with primary antibodies: CD36/SR-B3 (1:1000, NB400-144, Novus Biotechne, Abingdon, United Kingdom) and anti-βactin (1:5000, A-5441, Sigma Aldrich). Then, the corresponding secondary antibodies were added (Santa Cruz Biotechnology Inc., Heidelberg, Germany). Using the Bio-Rad Clarity™ Western ECL Substrate (BioRad Inc.), the immunoreactive bands were visualized by the ImageQuant LASD 4000 digital imaging system (GE Healthcare Europe, Barcelona, Spain). Densitometric analysis of the band was performed using ImageJ Biological Image Analysis (NIH), normalized against the loading control (βactin), and graphically expressed as fold change relative to control condition (1).

Quantitative Real-Time PCR (RT-qPCR)

RNA was extracted from liver samples using the TRIzol® reagent (Vitro, Sevilla, Spain). Samples were then reverse transcribed using the Reverse Transcription System kit (Promega Inc., Madison, WI, USA). A BioRad T100™ Thermal Cycler was used to carry out the reverse transcription. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed to assess gene expression using a StepOnePlus™ Real Time PCR System Sequence Detector (Thermo Fisher Scientific Inc.). Samples were prepared using a SYBER Green qPCR Kit (Promega Inc.) and d(N)6 random primers were purchased from Metabion (Planegg, Germany). Primer sequences are detailed in **Supplementary Table 1**. Each sample was run in duplicated, normalized in comparison to *36B4* gene expression, and graphically expressed as fold change relative to control condition (1).

CD36 Immunohistochemistry

Paraffin-embedded liver biopsy sections (5 μm) were deparaffinized and rehydrated. Sections were then placed in antigen retrieval buffer (10 mM sodium citrate, pH 6–7), boiled for 20 min at 95°C and incubated with a blocking solution for 1 h before being immunostained with the CD36 antibody (1:200, NB400-144, Novus) for 16 h in a moisture chamber. The EnVision™ FLEX Mini Kit, High pH (Link) (Agilent, Santa Clara, CA, USA) was used for visualization according to the manufacturer's instructions. Three different lobular areas were analyzed in each sample and images were captured using a Nikon Eclipse E400 optical microscope (Nikon) and the NIS Elements Imaging Software (Melville). Intensity of CD36 stain was quantified using the FIJI software (NIH) and reported as the average value in arbitrary units (a.u.).

Statistical Analysis

Categorical variables were presented as percentage and were compared by the Pearson χ^2 test. Continuous data were shown as standard deviation (*SD*) or standard error of mean (SEM), and were compared using the unpaired *t*-test or Mann-Whitney *U*-test, as indicated. The Spearman's *r*-test was used to evaluate correlations. All statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) and SPSS statistical software version 24.0 (IBM SPSS Statistics, Armonk, NY), with a $p < 0.05$ considered statistically significant.

RESULTS

Characteristics of the Study Patients

Twenty patients undergoing programmed cholecystectomy had both a liver biopsy and a sleep study. Overall, the mean age of the study population was 46.5 years, 14 (70%) were female and 9 (45%) had a diagnosis of OSA by polygraphy (AHI > 5). Patient characteristics from the cohort included in this study are presented in **Table 1**.

TABLE 1 | Demographic, metabolic, biochemical, respiratory, and histological characteristics of the study population.

Feature	Patients without OSA ($n = 11$)	Patients with OSA ($n = 9$)	p-value
Age (years)	39.9 ± 9.6	54.6 ± 10.6	0.004
BMI (kg/m^2)	26.5 ± 6.7	28.6 ± 5.1	0.201
Waist circunference (cm)	91.8 ± 14.4	105.5 ± 11.6	0.034
Glucose (mg/dL)	92.2 ± 9.3	96.2 ± 11.9	0.359
Insulin (μU/L)	7.2 ± 2.9	9.2 ± 4.5	0.245
HOMA-IR	1.6 ± 0.7	2.1 ± 1	0.192
Triglycerides (mg/dL)	111.2 ± 54.2	136.4 ± 68.9	0.467
Total Cholesterol (mg/dL)	207 ± 32.3	210 ± 29.5	0.832
HDL-cholesterol (mg/dL)	53.5 ± 12.8	46.9 ± 9.5	0.212
ALT (IU/L)	24.6 ± 17.4	27.2 ± 19.3	0.489
AST (IU/L)	22.1 ± 9.3	21.3 ± 5.6	0.808
γGT (IU/L)	33.4 ± 24.4	28.4 ± 14.5	0.601
Alkaline phosphatase (IU/L)	70.3 ± 22.7	61 ± 13.6	0.359
Bilirubin (mg/dL)	0.7 ± 0.36	0.63 ± 0.32	0.780
Average oxygen saturation (%)	94 ± 1.7	93.3 ± 1.4	0.225
Minimum oxygen saturation (%)	88.2 ± 4.2	83.7 ± 5.9	0.073
AHI (events/hour)	0.9 ± 1	10 ± 9.2	0.002
ODI (events/hour)	0.6± 0.6	9.2 ± 9.5	<0.001
Tc90 (%)	7.5 ± 16.9	6 ± 8.8	0.053
NAS Score (%)			
0		22.2%	
1	18.2%	22.2%	
2	18.2%	22.2%	
3		11.2%	
4		22.2%	
Steatosis (%)			
Grade 0	63.6%	22.2%	
Grade 1	27.3%	22.2%	
Grade 2	9.1%	44.5%	
Grade 3		11.1%	
Lobular Inflammation (%)			
Grade 0	91%	77.8%	
Grade 1	9%	22.2%	
Grade 2			
Ballooning (%)			
Grade 0	100%	77.8%	
Grade 1		22.2%	
Grade 2			
Fibrosis (%)			
Stage 0	100%	100%	

Data are shown as mean ± standard deviation.
OSA, obstructive sleep apnea; BMI, body mass index; HOMA-IR, homeostatic model assessment-insulin resistance; HDL, high density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γGT, gamma-glutamiltranspeptidase; AHI, apnea-hipopnea index; ODI, oxygen desatutaion index; Tc90, sleep time with oxygen saturation <90%.

OSA patients were significantly older than those without OSA ($p = 0.004$) and women predominated in both groups. In order to evaluate the presence of obesity in the entire study population, body mass index (BMI) was calculated and waist circumference was measured in each participant. Regarding BMI, the study population showed an overweight status and no significant differences were found among the different patient groups studied ($p = 0.201$), whereas waist perimeter was significantly higher in patients with OSA than in those without ($p = 0.034$).

As expected, OSA patients had a significantly higher rate of oxygen desaturation per hour of sleep (ODI) and percentage of sleep time with oxygen saturations lower than 90% (Tc90) with respect to patients without OSA (**Table 1**).

Regarding metabolic parameters, basal glucose levels did not significantly differ between groups, while insulin levels and the degree of insulin resistance assessed by HOMA-IR index were higher in OSA patients than in those without OSA, but these differences were not statistically significant (**Table 1**).

Although no significant differences were observed in liver enzymes between the two groups, there was a higher prevalence of NAFLD and evidence of more severe disease among patients with OSA (**Table 1** and **Figure 1A**). Surprisingly, 36.4% of patients without OSA exhibited NAFLD, all of them featuring simple steatosis. In the OSA group, however, 55.6% of them had simple steatosis and 22.2% showed histological features of steatohepatitis (NASH) (**Table 1** and **Figure 1A**). Hepatic fibrosis was not observed. All other variables did not significantly differ between groups.

Expression of CD36 Is Increased Within the Liver of OSA Patients

Next, we wanted to investigate whether OSA might alter CD36 expression in the liver. Interestingly, hepatic mRNA levels of CD36 were significantly higher in patients with OSA when compared with control patients (**Figure 1B**), and its expression significant positively correlated with both AHI and ODI values in the entire study population (**Figure 1C**), but not with Tc90 (**Supplementary Figure 1A**). In parallel, an increase of CD36 protein expression was also observed in the livers from OSA patients compared with those from the control group detected by immunostaining (**Figure 1D**).

Intermittent Hypoxia (IH) Triggers Hepatic Steatosis in Mice

In order to investigate whether intermittent hypoxia (IH), one of the main features of OSA, contributes to liver steatosis and to the increase of CD36 expression observed in OSA patients, a mouse experimental model of OSA was used. After 2 months of IH exposure, histological examinations of liver tissue revealed that 60% of the mice submitted to IH exhibited signs of mild hepatic steatosis while control mice displayed normal liver features (**Figures 2A,B**). Liver fibrosis was not detected in any of the groups (**Supplementary Figure 2**).

Next, we investigated the amount of lipids performing an Oil Red O staining on sections of liver biopsy samples from

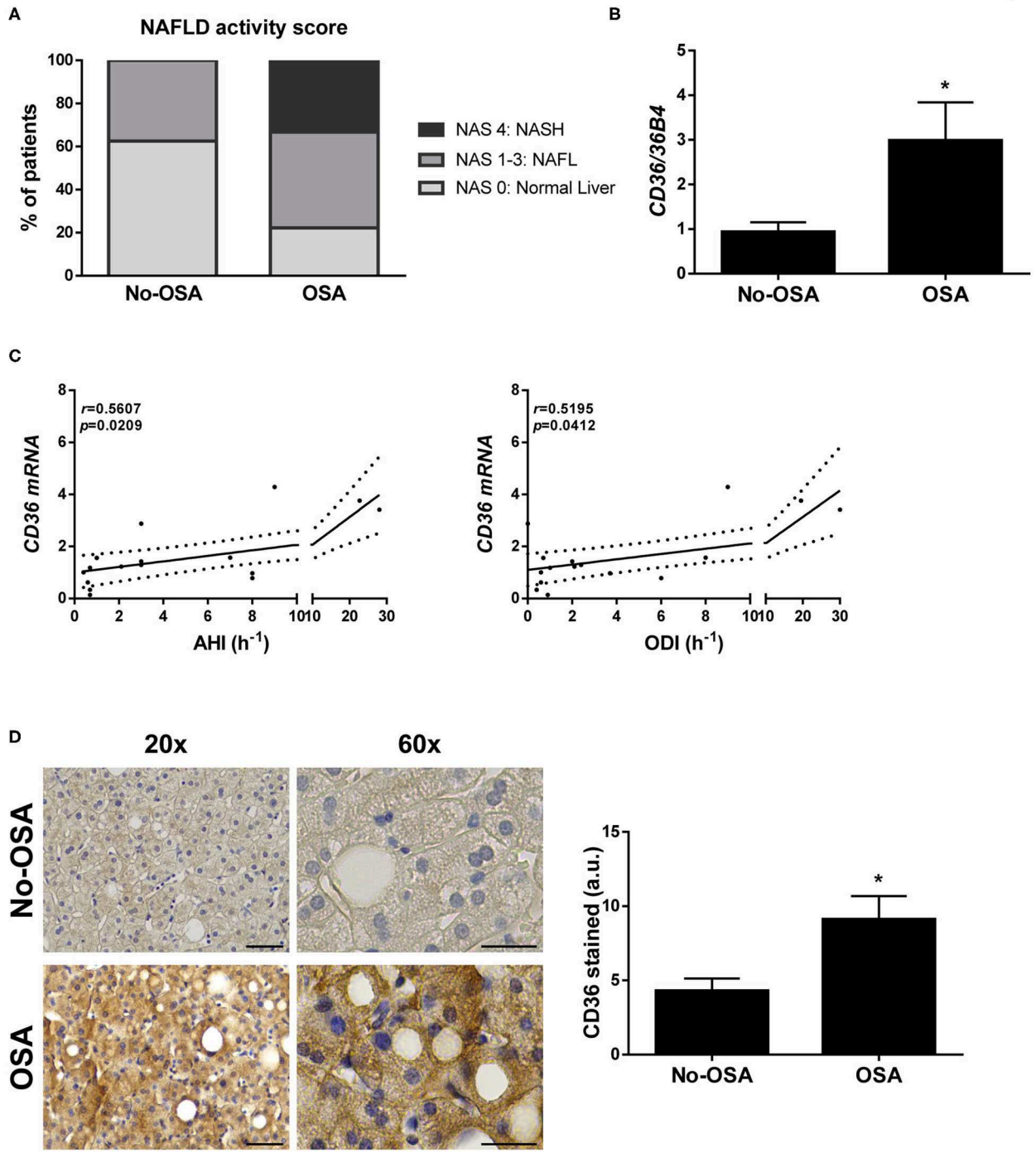

FIGURE 1 | Prevalence of NAFLD and hepatic CD36 expression is higher in patients diagnosed with OSA. **(A)** NAFLD activity score. **(B)** *CD36* mRNA levels. **(C)** Correlation in the study population of matched mRNA values for CD36 with the indicated respiratory parameters, evaluated by Spearman's *r*-test. **(D)** Representative 20X and 60X images of CD36 immunostaining, and quantification of CD36-expressing cells. Scale bar 100 and 50 μm, respectively. Study population: control group (No-OSA) ($n = 11$) and OSA patients ($n = 9$). *$p < 0.05$, OSA vs. Control, compared using the Mann-Whitney *U*-test.

all mice. The results indicated that there was a significant increase in average red-stain intensity (directly proportional to lipid content) among the IH mice when compared to control mice (**Figure 2C**). To further evaluate hepatic lipid content, triglycerides were extracted and quantified from liver biopsies of both IH and C mice. Triglyceride levels of the IH mice were greater than those observed in the C mice (**Figure 2D**).

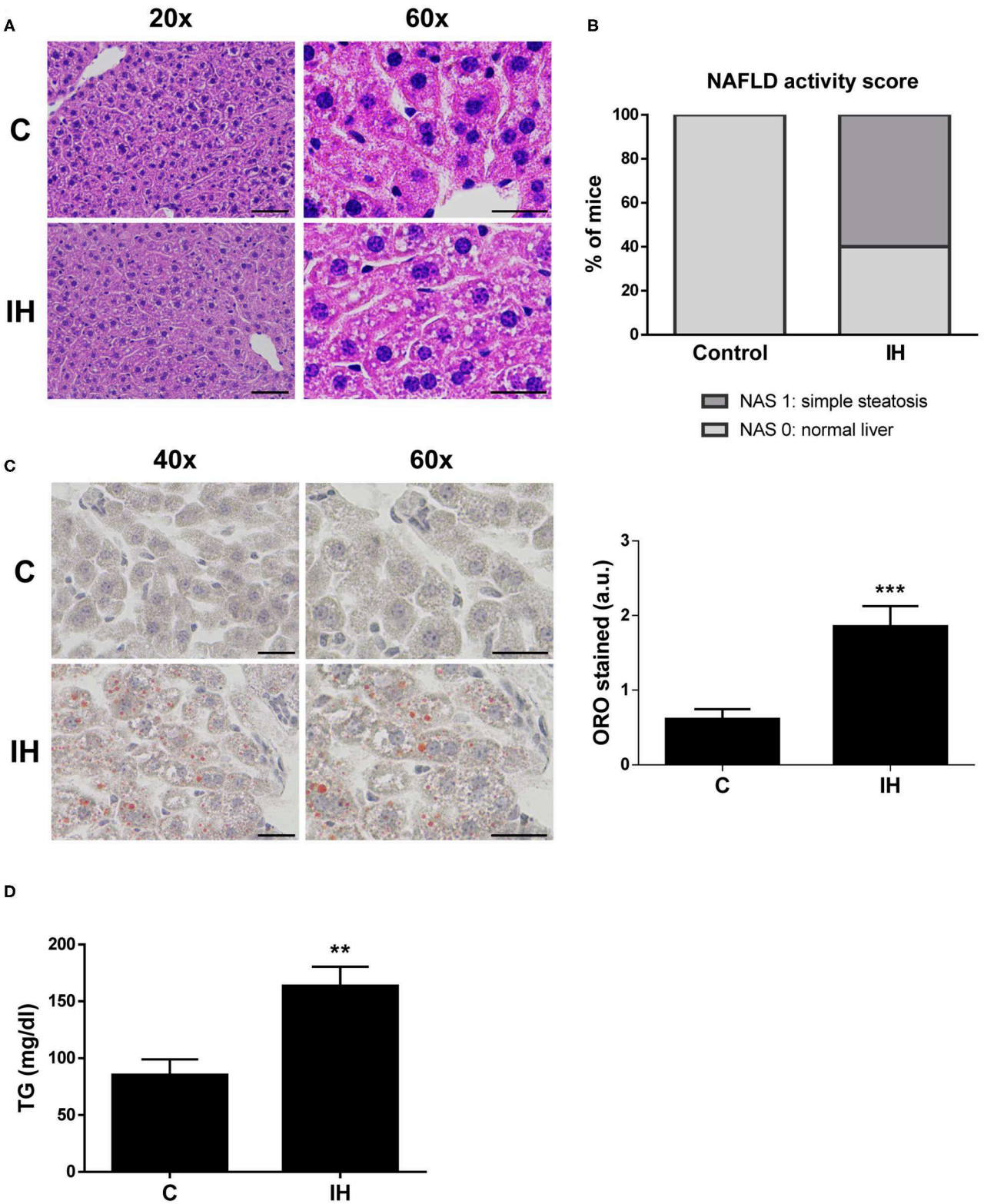

FIGURE 2 | Intermittent Hypoxia (IH) is associated with increased hepatic lipid content in mice. **(A)** Representative 20X and 60X images of liver sections stained with hematoxylin and eosin (H&E). Scale bar 100 and 50 μm, respectively. **(B)** NAFLD activity score. **(C)** *(left panel)* Representative 40X and 60X images of Oil Red O stained liver sections. Scale bar 50 μm. *(right panel)* Quantification of red-stain intensity. **(D)** Analysis of hepatic triglyceride (TG) levels. Experimental groups: mice maintained in normoxic conditions (Control, C) and mice exposed to intermittent hypoxia (IH) ($n = 10$ mice in each group). $^{**}p < 0.01$ and $^{***}p < 0.005$, IH vs. C, compared using the unpaired *t*-test.

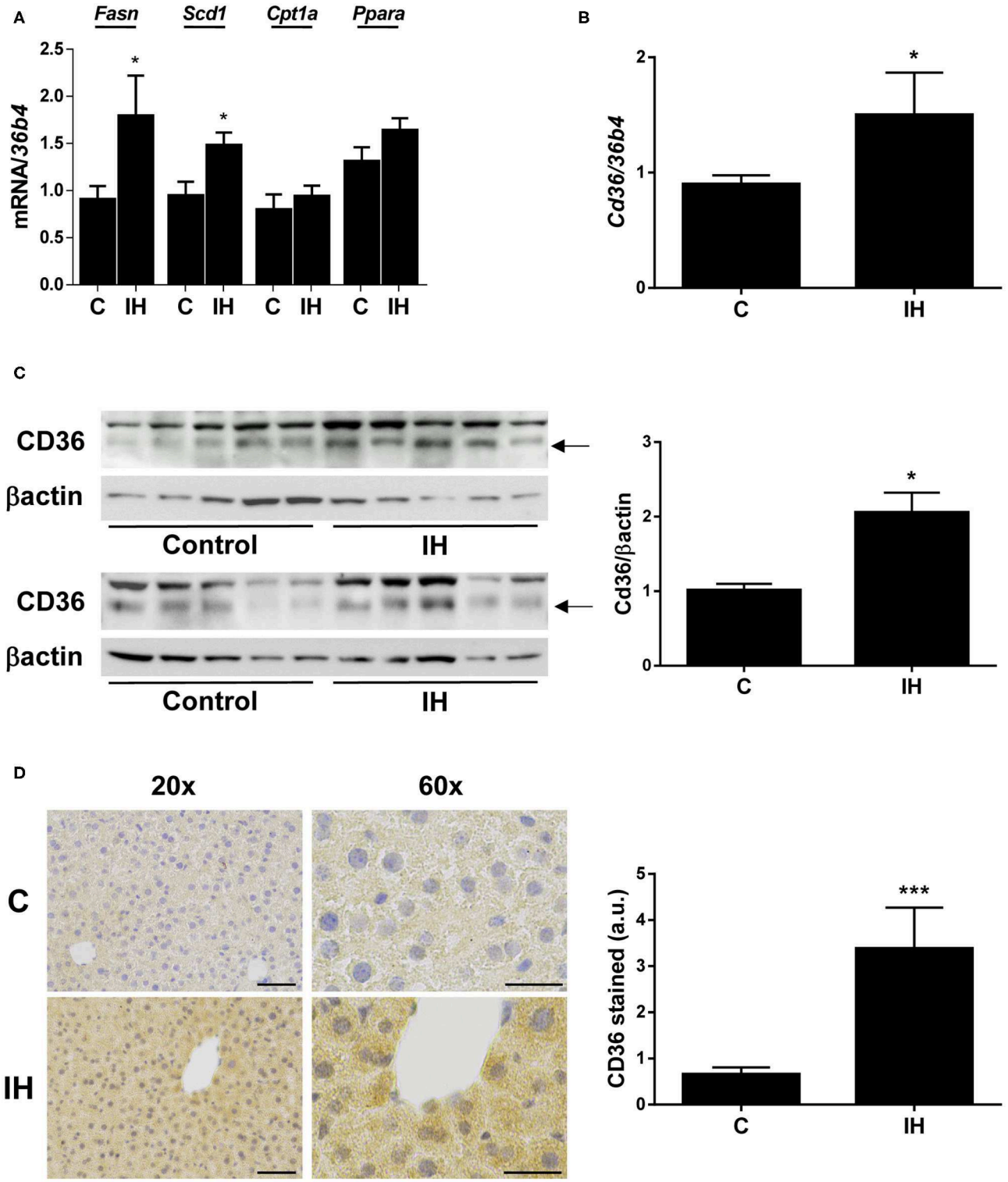

FIGURE 3 | Intermittent Hypoxia (IH) induces hepatic CD36 expression in mice. **(A)** mRNA levels of *Fasn, Scd1, Cpt1a*, and *Ppara.* **(B)** *Cd36* mRNA levels. **(C)** *(left panel)* Representative blots with the indicated antibodies. *(right panel)* Quantification of all blots with respect to loading control, βactin. **(D)** *(left panel)* Representative 20X and 60X images from CD36 immunochemistry. Scale bar 100 and 50 μm, respectively. *(right panel)* Quantification of CD36-stain intensity. Experimental groups: mice maintained in normoxic conditions (Control, C) and mice exposed to intermittent hypoxia (IH) ($n = 10$ mice in each group). $^{*}p < 0.05$ and $^{***}p < 0.005$, IH vs. C, compared using the unpaired *t*-test.

Intermittent Hypoxia (IH) Induces Hepatic CD36 Expression

tHEN, we analyzed the hepatic expression of genes involved in the regulation of lipid metabolism. We observed a significant increase in the expression of genes implicated in lipid synthesis, such as *Fasn* (fatty acid synthase) and *Scd1* (stearoyl-CoA desaturase 1), among livers from mice exposed to IH (**Figure 3A**); however, no differences were observed in the expression of genes implicated in β-oxidation, such as *Cpt1a* (carnitine palmitoyltransferase I) and *Ppara* (peroxisome proliferator activated receptor alpha) (**Figure 3A**).

With respect to CD36, its hepatic mRNA expression was significantly increased in mice submitted to IH compared to those maintained in normoxic conditions (**Figure 3B**), which was also found in OSA patients. In parallel, its protein expression determined by both Western blot (**Figure 3C**) and immunohistochemistry (**Figure 3D**) was elevated in the livers of IH mice.

DISCUSSION

Distinct clinical studies have reported that OSA is significantly associated with NAFLD severity (26) and there is an increasing experimental evidence that chronic IH, the best characterized OSA manifestation, is a major trigger for oxidative stress and inflammatory liver injury leading to NAFLD progression (17). In agreement with these previous studies, we found that 22.2% of OSA patients had NASH whereas none of those without OSA had histological features of NASH, supporting the assumption that OSA is a risk factor for progression from simple steatosis to NASH (27). Interestingly, our findings showed that there were no differences regarding BMI between the two study groups, but waist circumference was significantly higher in patients with OSA, suggesting that is abdominal obesity, but not overall obesity, what actually has a clinical impact on the features of metabolic syndrome, including OSA and NAFLD.

To the best of our knowledge, this is the first study revealing that CD36 expression is significantly elevated in livers from patients with OSA. Moreover, both AHI and ODI positively correlated with hepatic *CD36* mRNA levels, indicating a potential role for nocturnal IH in the upregulation of this FFA transporter. An intriguing question regarding our findings showed herein is whether age might influence the hepatic CD36 expression pattern observed in OSA patients because they were significantly older (54.6 ± 10.6 years) than those without OSA (39.9 ± 9.6 years). Indeed, we have reported that hepatic CD36 expression increased with aging in mice and humans (13), but the age-dependent increases in hepatic CD36 expression were observed comparing young (20–38 years old) with aged individuals (50–83 years), thus we believe that age differences seen in our study population are not sufficient to explain the hepatic CD36 upregulation observed in OSA patients. Supporting this assumption, no correlation was found between *CD36* mRNA expression and age in our study population (**Supplementary Figure 1B**).

In agreement with our findings in OSA patients, the majority of mice exposed to IH displayed simple steatosis as well as a higher hepatic TG content and CD36 expression than in control mice breathing normal oxygen concentrations. Collectively, our results indicate that IH may contribute to hepatosteatosis setup, partly by the upregulation of hepatic CD36 expression, but the underlying molecular mechanisms still remain to be elucidated.

It is well-known that cellular adaptive responses to hypoxia are tightly regulated by hypoxia-inducible transcription factors (HIFs), being HIF1α and HIF2α the best characterized (28). In that regard, Li et al. demonstrated that IH exacerbated hepatosteatosis in mice in parallel with an upregulation of key genes for hepatic lipid biosynthesis, such as *Srebp1* and *Scd1* (20) and that this effect is mediated through HIF1α (29). In line with these results, our study also found increased mRNA levels of relevant genes for *de novo* lipogenesis, such as *Fasn* and *Scd1,* along with an upregulation of *Cd36* gene expression in livers of mice exposed to IH, suggesting that HIF1α might regulate *Cd36* gene expression as well. There is convincing evidence, however, indicating that the regulation of CD36 expression is not largely linked to the HIF1α/SREBP1c signaling pathway in hepatocytes. Notably, it has been recently reported that hepatocyte-specific *Srebp1* downregulation did not affect expression of genes involved in FFA uptake as *Cd36* in mouse livers (30). In addition, we have just demonstrated that both CD36 expression and triglyceride content increased in mouse and human liver cells under hypoxic conditions and that silencing *HIF2A* gene markedly suppressed both *CD36* gene upregulation and lipid accumulation in hepatocytes (14). The novelty of our present study is that both CD36 expression and the degree of steatosis are increased in livers from animal models of IH and in patients with OSA featured by nocturnal IH, supporting the notion that CD36 could be a key factor driving hepatosteatosis in OSA patients.

In conclusion, the results of the present study demonstrate that CD36 expression is increased within the liver of patients with OSA and in mice exposed to IH, the clinical hallmark featuring OSA. Moreover, our results point out that the excessive lipid accumulation observed in livers of mice under IH conditions is likely due to the upregulation of CD36, which is involved in FFA uptake into hepatocytes, along with that of genes implicated in *de novo* lipogenesis, thus leading to the onset of hepatosteatosis, the earliest phase of NAFLD. Collectively, our findings shed light on the molecular mechanisms underlying IH-induced hepatosteatosis helping to understand better the NAFLD pathogenesis and identifying CD36 as a potential target for new pharmacological therapies to NAFLD patients.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Ethics Committee of the Hospital Universitario Santa Cristina. The patients/participants provided

their written informed consent to participate in this study. The animal study was reviewed and approved by Ethical Committee of the University of Barcelona.

AUTHOR CONTRIBUTIONS

CG-M, IA, and ÁG-R designed the study. EP-M, PL, and CG-M carried out and analyzed the clinical study. ER, BB, PM, and SI carried out the experimental study. RF, CG-M, IA, and ÁG-R analyzed and discussed data. IA and ÁG-R wrote the manuscript. All authors were involved in editing the paper and had final approval of the submitted and published versions.

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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.2020.00450/full#supplementary-material

Supplementary Table 1 | Primer sequences for RT-qPCR.

Supplementary Figure 1 | Correlation in the study population of matched mRNA values for CD36 with Tc90 values **(A)** and age **(B)**, evaluated by Spearman's *r*-test. Study population: control group (No-OSA) ($n = 11$) and OSA patients ($n = 9$).

Supplementary Figure 2 | Representative images of liver sections stained with Masson's trichrome solution. Experimental groups: mice raised in normoxic conditions (Control, C) and mice exposed to intermittent hypoxia (IH) ($n = 10$ mice in each group).

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REVIEW
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Insights Into Extracellular Vesicles as Biomarker of NAFLD Pathogenesis

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Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease around the world estimated to affect up to one-third of the adult population and is expected to continue rising in the coming years. Nonalcoholic fatty liver disease is considered as the hepatic manifestation of the metabolic syndrome because it is strongly associated with obesity, insulin resistance, type 2 diabetes mellitus, and cardiovascular complications. Despite its high prevalence, factors leading to NAFLD progression from simple steatosis to nonalcoholic steatohepatitis, cirrhosis, and, ultimately hepatocellular carcinoma remain poorly understood. To date, no treatment has proven efficacy, and also no reliable method is currently available for diagnosis or staging of NAFLD beyond the highly invasive liver biopsy. Recently, extracellular vesicles (EVs) have emerged as potential candidate biomarkers for the diagnosis of NAFLD. Extracellular vesicles are circulating, cell-derived vesicles containing proteins and nucleic acids, among other components, that interact with and trigger a plethora of responses in neighbor or distant target cells. Several mechanisms implicated in NAFLD progression, such as inflammation, fibrosis, and angiogenesis, all related to metabolic syndrome–associated lipotoxicity, trigger EV production and release by liver cells. As hepatocytes represent ∼80% of the liver volume, in this review we will focus on hepatocyte-derived EVs as drivers of the interactome between different liver cell types in NAFLD pathogenesis, as well as in their role as noninvasive biomarkers for NAFLD diagnosis and progression. Based on that, we will highlight the research that is currently available on EVs in this topic, the current limitations, and future directions for implementation in a clinical setting as biomarkers or targets of liver disease.

Keywords: nonalcoholic fatty liver disease, hepatocyte, lipotoxicity, inflammation, intercellular communication, extracellular vesicles, biomarkers

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, affecting 25% of the global adult population, especially in industrialized countries (1). In addition, NAFLD is also the most prevalent form of chronic liver disease in childhood, affecting ∼10% of the general pediatric population (2, 3). The classic definition of NAFLD excludes excessive alcohol consumption, which is well known to cause alcoholic liver disease. Growing evidences support the hypothesis that NAFLD is the hepatic manifestation of metabolic syndrome, with insulin resistance as the common pathogenic factor (4). Although some genetic risk factors have been reported (5), the increase in body weight and the presence of several hallmarks of metabolic syndrome

such as adiposity, hyperglycemia, dyslipidemia, and hypertension, may be key determinants in the pathogenesis of NAFLD.

Nonalcoholic fatty liver disease includes a wide signature of liver damage, extending from nonalcoholic fatty liver (NAFL) or steatosis toward nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), lately causing chronic hepatic insufficiency and the need for liver transplantation. Whereas, NAFL is defined by simple liver steatosis, NASH is characterized by the joint presence of steatosis and lobular inflammation with hepatocyte ballooning degeneration, with or without any fibrosis (6, 7). The underlying triggers and mechanisms for the development and progression of NAFLD, an issue under current investigation, are complex and multifactorial. Originally, the "two-hit hypothesis" was formulated in order to explain the progression from simple steatosis to NASH. According to this traditional point of view, the intrahepatic accumulation of lipids secondary to sedentary lifestyle, hypercaloric diets, obesity, and insulin resistance acts as the "first hit" sensitizing the hepatocytes to further injuries or insults. Proinflammatory cytokines, adipokines, bacterial endotoxins, mitochondrial dysfunction, oxidative stress, and/or endoplasmic reticulum (ER) stress represent the "second hit" for the progression to NASH. Subsequently, the "second hit" leads to hepatocyte injury, inflammation, and fibrosis. To date, the most widely accepted hypothesis is the "multiple-hit model" (8), because many other additional elements such as hormones/adipokines secreted from the adipose tissue, nutritional factors, gut microbiota, and genetic and epigenetic factors also contribute to the progression of this disease (9–13). However, liver fat accumulation, caused by obesity and insulin resistance, still seems to represent the first hit. In the adipose tissue, insulin resistance leads to an impaired suppression of lipolysis, causing triglyceride (TG) breakdown and a massive accumulation of free fatty acids (FFAs) and glycerol. Circulating FFAs are taken up by the hepatocytes and esterified into TGs. However, an excessive uptake of saturated FFAs may overwhelm the cellular capacity to store and esterify them into TG, leading to organelle dysfunction, cell injury (lipotoxicity), and apoptotic cell death (lipoapoptosis) of the hepatocytes, processes strongly associated with the progression from NAFLD to NASH.

In most cases, NASH typically develops asymptomatic until the disease progresses to end-stage liver disease at which liver transplantation is the only available therapeutic option. Therefore, early detection of NAFLD may be useful to identify those individuals with potentially silent progressive fatty liver disease. In some cases, the presence of NAFLD has been strongly suspected in individuals showing unexplained elevation of liver enzymes levels or evidence of steatosis by imaging. To date, liver biopsy remains the gold standard method for NAFLD diagnosis that classifies the state of the disease by histologic assessment. However, this highly invasive and harmful procedure to the patient cannot predict disease progression and often leads to late diagnosis.

At present, no pharmacological agents for the treatment of NASH have been currently approved by the US Food and Drug Administration. Therefore, lifestyle and dietary changes leading to weight loss are the first-line strategy to overcome NAFLD. Unfortunately, it has been demonstrated that a high number of patients regain most of the weight after a successful weight loss period, an effect likely due to the unavailability of a full multidisciplinary program focused on long-term weight maintenance for the patient (14, 15). For this reason, there is an urgent need to identify reliable noninvasive biomarkers specific for NAFLD and NASH diagnosis at an earlier time point at which lifestyle interventions and potential newly developed drugs can be used successfully.

To date, it is assumed that the lack of highly effective treatments may be due to the heterogeneity of the population with NAFLD with respect to its primary drivers and coexisting disease modifiers such as cardiovascular diseases (CVDs) or type 2 diabetes mellitus (T2DM). In fact, growing evidences suggest that, during NAFLD, a dichotomous classification of patients with or without NASH may not represent the full spectrum of disease progression due to the aforementioned modifying factors. Recently, the updated term MAFLD, metabolic dysfunction–associated fatty liver disease, has emerged to better define NAFLD pathogenesis and denotes the hepatic manifestation of a multisystem disorder that is heterogeneous in its underlying origin, progression, and outcomes (16, 17). MAFLD represents the overarching umbrella comprising multiple subtypes, but reflecting the dominant driver. As proposed, the criteria for diagnosis are based on evidence of hepatic steatosis in addition to one of the following three criteria, namely, overweight/obesity, presence of T2DM, or evidence of metabolic dysregulation, regardless of the amount of alcohol consumed. Therefore, the identification of the predominant drivers in each patient might allow the implementation of a personalized treatment to ensure the best response with low adverse side effects.

Recent advances in basic and translational research have provided insights into the pathogenic mechanisms driving the progression of NAFLD that involve parenchymal and nonparenchymal liver cells (18). Stressed or dying hepatocytes during lipotoxicity release intracellular molecules named damage-associated molecular patterns or DAMPs, which

Abbreviations: ASGR1, asialoglycoprotein receptor 1; CHOP, C/EBP homologous protein; CVD, cardiovascular disease; CXCL10, C-X-C motif chemokine ligand 10; CYP2E1, cytochrome P450 family 2 subfamily E member 1; DAMPs, Damage-associated molecular patterns; DR5, death receptor 5; EC, endothelial cell; ER, endoplasmic reticulum; EVs, extracellular vesicles; FFAs, free fatty acids; FFC diet, fat-, fructose- and cholesterol-enriched diet; GGPPS, geranylgeranyl diphosphate synthase; HCC, hepatocellular carcinoma; Hep-EVs, hepatocyte-derived EVs; Hep Par 1, hepatocyte paraffin 1; HFHCD, high-fat, high-cholesterol diet; HSCs, hepatic stellate cells; IL, interleukin; IRE1α, inositol requiring enzyme 1α; ITGβ1, integrin β1; ILV, intraluminal vesicle; KCs, Kupffer cells; KLF4, Kruppel-like factor 4; LSECs, liver sinusoidal endothelial cells; miR, mature form of the miRNA; MISEV, Minimal Information for Studies of Extracellular Vesicles; mitoDNA, mitochondrial DNA; MLK3, mixed lineage kinase 3; MR, magnetic resonance; MVBs, multivesicular bodies; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; PA, palmitic acid; PPAR-γ, peroxisome proliferator-activated receptor gamma; ROCK1, rho-associated protein kinase 1; S1P, sphingosine 1-phosphate; T2DM, type-2 diabetes mellitus; TG, triglycerides; TIMP, tissue inhibitor of metalloproteinases; TLR9, Toll-like receptor 9; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VNN1, vanin-1.

activate various cell types such as Kupffer cells (KCs), liver resident macrophages, neutrophils, and hepatic stellate cells (HSCs), boosting inflammation and fibrosis. It is noteworthy to highlight that the location of HSCs and KCs within the space of Disse facilitates their direct contact with other cell types including hepatocytes and liver sinusoidal endothelial cells (LSECs), thus promoting the intercellular transport of soluble mediators and cytokines. According to this, the progression from NASH to more advanced stages is the result of a complex intrahepatic interactome between different cell types via secreted factors, illustrating the complexity of cell–cell signaling in liver physiology and disease. Given this scenario, there is no a single therapeutic target for NAFLD treatment, which explains the lack of an effective therapy for the disease.

EMERGING ROLE OF EXTRACELLULAR VESICLES IN CELL-TO-CELL COMMUNICATION

Through the years, intercellular communication has been thought to be mediated only by direct cell-to-cell interaction or secretion of soluble factors. Nonetheless, it is now well recognized that cells are also capable of releasing, in an evolutionally conserved manner, various types of membrane vesicles as a third type of cellular interactome. These vesicles are generally known as extracellular vesicles or "EVs" (19).

The generic term "EVs" comprises a heterogeneous population of cell-released, nanometer-sized vesicles enclosed by a lipid bilayer membrane. Currently, EVs can be broadly classified into three main categories based on their size and cellular biogenesis: exosomes, microvesicles, and apoptotic bodies (19, 20). Briefly, exosomes are the smallest vesicles (30–150 nm) and are formed as intraluminal vesicles within multivesicular bodies (MVBs), which are released upon fusion with the plasma membrane. Microvesicles (50–1,000 nm) and apoptotic bodies (100–5,000 nm) are larger and formed by outward budding and fission of the plasma membrane, or when plasma membrane blebbing occurs during apoptosis, respectively. **Figure 1** depicts the features commonly used to differentiate EVs subtypes.

Biogenesis of exosomes and microvesicles occurs at distinct sites within the cell. Also, they display similar morphology and overlapping size and share intracellular machinery in their formation. Therefore, this similar composition makes it difficult to identify. This, along with the lack of standardization of both isolation procedures and methods for their characterization, challenges current attempts to devise a more precise nomenclature (20, 21). In this regard, as the number of scientific publications in the field has increased in the last decade (22), the International Society of Extracellular Vesicles (ISEV: www.isev.org) published in 2014 the Minimal Information for Studies of Extracellular Vesicles ("MISEV") guidelines (23) with the aim to unify the nomenclature and the methodologies for EVs studies worldwide. MISEV has been updated in 2018 (24).

Even though EVs are known since late 1980s (25, 26), they have recently remerged in the scientific community on biomedical research. Nowadays, the main interest in the EVs field is focused on their capacity to shuttle between cells, as well as in their specific bioactive cargo molecules such as nucleic acids (i.e., DNAs, RNAs, and noncoding RNAs such as miRNAs), proteins, lipids, sugars, and other metabolites that are transported in response to different stimuli. Of note, it is known that the process of cargo sorting is highly regulated; however, the mechanisms involved remain largely unknown (27). Therefore, each cell type can regulate EV production both quantitatively and qualitatively, depending on its physiological or pathological state. Furthermore, the same cell type may secrete heterogeneous populations of EVs if several mechanisms with distinct activators are involved (19, 20, 28).

Once released into the extracellular milieu, EVs can interact with nearby cells (cell-to-cell communication) or diffuse into bloodstream or other body fluids to act in distant organs (interorgan communication). Ultimately, EVs are able to transmit a unique package of information from donor-to-recipient cells, thereby eliciting functional responses and promoting phenotypic changes that will affect their physiological or pathological status (19, 20).

Extracellular vesicles need to selectively dock at the plasma membrane of specific target cells for triggering their phenotypic effects. In fact, all EVs bear surface molecules that allow them to be recognized by recipient cells. Several studies indicate that surface glycosylation patterns and exposed receptors and ligands (i.e., integrins) may be of relevance for EVs binding to target cells and, therefore, for their subsequent biodistribution (29, 30). Once attached to a target cell, EVs can initiate intracellular signaling pathways through the simple interaction with surface receptors or ligands. For example, EVs bearing surface ligands such as FasL, perforin, or tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) are fully functional in inducing death receptor–mediated apoptosis (31–33). Nonetheless, EVs cargo delivery by vesicle internalization (endocytosis) or fusion with target cells is commonly needed for specific cellular responses. In this regard, EVs can release inside the recipient cell proteins, bioactive lipids, or even active signaling molecules, including enzymes, which activate a plethora of downstream signaling pathways. Moreover, EVs can also load active mRNAs and miRNAs that regulate gene expression through *de novo* translation or posttranslational regulation of target mRNAs, respectively (20, 34).

Extracellular vesicle–mediated intercellular communication is necessary to maintain cellular homeostasis and physiological functions, whereas alterations in this process could be an indicator of pathological states. The fact that EVs cargo can be modified under pathological conditions raises the question whether EVs might have a different biological role in health or disease. Hence, EVs could serve as potential therapeutic targets in the treatment of several pathologies. Moreover, because of disease-associated cargo and ubiquitous presence and stability of EVs in various human biofluids, they may also have clinical relevance as noninvasive biomarkers for disease detection and prognosis (35). On the other hand, EVs, either unmodified or engineered, have also generated considerable attention in

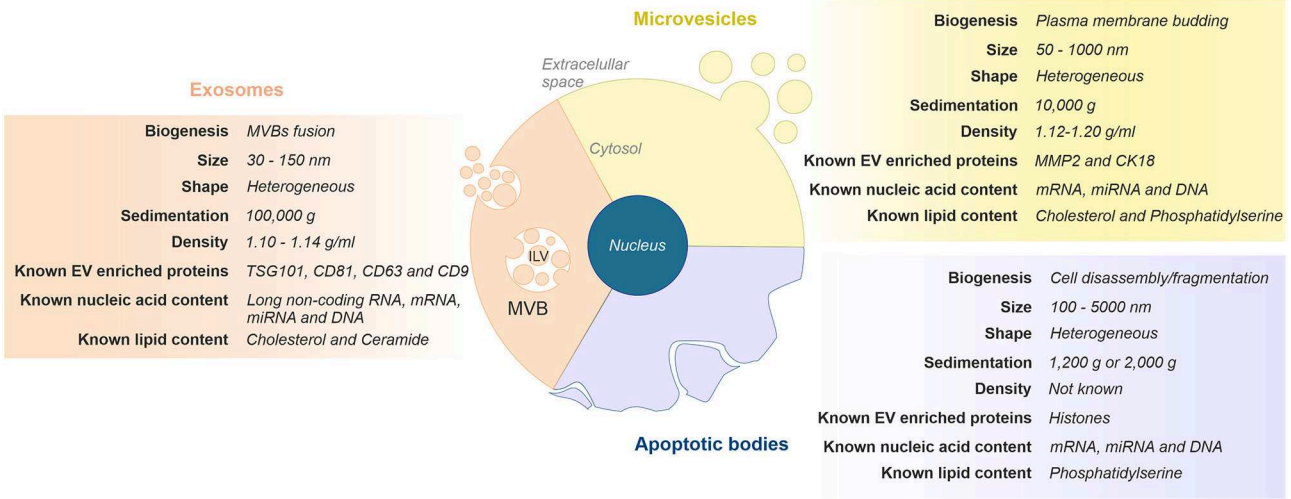

FIGURE 1 | Classification and representative characteristics of extracellular vesicles (EVs) subtypes. Extracellular vesicles can be classified in three main subgroups based on their size and cellular biogenesis: exosomes, microvesicles, and apoptotic bodies. Exosomes are formed via the endosomal pathway and are released upon fusion of MVBs with the plasma membrane. Microvesicles are generated by the outward budding and fission of the plasma membrane. Apoptotic vesicles are released upon cell fragmentation during apoptotic cell death. ILV, intraluminal vesicle; MVB, multivesicular body.

the scientific community because of their potential use for therapeutic purposes (36). Extracellular vesicles are bioavailable, biocompatible, and resistant to RNases and proteases (37). These characteristics make them ideal vehicles for the delivery of drugs, proteins, miRNAs, silencing RNAs (siRNAs), and other molecules. Regarding to liver diseases as a major focus of this review, efforts have been focused in two major areas: on the one hand, the use of EVs as delivery vehicles of drugs to the liver (38) and, on the other, the use of EVs themselves as therapeutic agents to stimulate liver regeneration, modulate inflammation, reduce liver fibrosis, or block hepatocarcinogenesis (39, 40). However, several substantial challenges such as standardization of methodology and selection of the type of EVs for delivery still need to be solved before controlled clinical studies can be carried out (41, 42).

Identification and analysis of cell/tissue–specific molecular patterns is promising for diagnostic, prognostic, and therapeutic purposes. The tissue-specific protein composition of EVs provides opportunities to identify cell type–specific signatures to be used as diagnostic markers. Several protein screening methods available such as two-dimensional gel electrophoresis and mass spectrometry are time consuming, poorly suited for high-throughput screening of many samples, and their sensitivity and reproducibility may be limited. In this sense, Larssen et al. (43) showed that multiplex proximity extension assays (PEAs) are a powerful protein screening tool in EVs research. This technology allows identification, analysis, and validation of potential EVs-associated markers to identify with high specificity and sensitivity the protein profiles of EVs of different origins. Importantly, the ability of this technology to trace cellular origin could be extended to plasma-derived EVs, facilitating efficient and noninvasive diagnostic strategies at early stages of diseases. A limitation of the current study is the fact that the PEA panels are not available to all EV target cells. Thus, further investigation and optimization of PEA to be used in the screening of larger patient cohorts and additional body fluids are needed.

Accumulating evidence supports a role for EVs in a wide range of human diseases, including the spectrum of conditions associated with obesity and metabolic syndrome (44). Moreover, the abundance and the phenotype of blood-circulating EVs have been reported to change in obesity and associated disease states including insulin resistance, T2DM, and NAFLD (45). Several mechanisms implicated in NAFLD progression, such as inflammation, fibrosis, and angiogenesis, all related to metabolic syndrome–associated lipotoxicity, trigger EV production and release by the liver (45). On the one hand, EVs mediate local intercellular communication between the liver cells, thereby driving disease pathogenesis, and on the other, liver-derived EVs could affect distant tissues and organs upon their release to the bloodstream. Thus, liver-derived EVs have promise as biomarkers for diagnostic and prognostic purposes in patients (46). However, the identification of liver-derived EVs in circulation as indicative of metabolic alterations in this organ is still a challenge for basic and clinical researchers.

As mentioned above, NAFLD is not an isolated condition, and generally speaking, this disease occurs as a complication of other metabolic disorders. Therefore, multiple tissues may be affected, and consequently, the contribution of extrahepatic EVs during NAFLD cannot be excluded (i.e., adipocyte- or immune cells–derived EVs). Furthermore, most liver cell types produce EVs including hepatocytes, cholangiocytes, HSCs, and LSECs (35). Nonetheless, as 80% of the liver volume is composed by hepatocytes, their participation to the total pool of liver-derived EVs is likely the most relevant. Therefore, in this review, we will focus on hepatocyte-derived EVs (Hep-EVs) as drivers of NAFLD

pathogenesis or noninvasive biomarkers for NAFLD diagnostic and prognostic.

ROLE OF HEPATOCYTE-DERIVED EVs IN NAFLD

Lipotoxicity is one of the triggers of NAFLD progression because it is a process by which accumulation of toxic lipids species in hepatocytes such as saturated FFAs activates molecular pathways related to cellular stress that can result in cell death (47, 48). In this section, we will analyze different studies with evidences on lipotoxic hepatocyte injury that affects, or even drives, the responses of surrounding liver cells through the release of Hep-EVs. On the other hand, we will highlight the studies currently available that point to the participation of Hep-EVs in NAFLD-associated complications.

Hep-EVs as Key Role in the Progression NAFLD to NASH

As we stated above, there are several key events closely interconnected involved in NAFLD progression to NASH such as inflammation, fibrosis, hepatocyte cell death, and dysregulated angiogenesis. All of these signs are related to metabolic syndrome–associated lipotoxicity which triggers EV production and release by the liver (45). Next, we will review in detail several studies which compile the role of proinflammatory, proangiogenic, and profibrotic Hep-EVs as pathogenic mediators during lipotoxicity in NAFLD (**Figure 2**).

Hep-EVs in Liver Inflammation

Recruitment of monocyte-derived macrophages into the liver contributes to the inflammatory response during NASH (49). However, how hepatocyte lipotoxicity promotes monocyte-derived macrophages chemotaxis, activation, and hepatic inflammation, all of these pathogenic processes being essential in the progression of NAFLD, also remains unclear.

Ibrahim et al. demonstrated that proapoptotic lipotoxic signaling triggered by mixed lineage kinase 3 (MLK3) induces the release of proinflammatory Hep-EVs enriched in potent C-X-C motif chemokine ligand 10 (CXCL10) that, in turn, lead to monocyte-derived macrophages chemotaxis to the liver and may activate KCs during NASH progression (50). Moreover, MLK3-deficient mice fed a fat-, fructose-, and cholesterol-enriched diet (FFC diet) were protected against development of dietary steatohepatitis. This beneficial effect was associated with a reduction in the number of total plasma EVs and EVs containing CXCL10 compared to the wild-type mice. In another study, Kakazu et al. illustrated that palmitate-induced Hep-EVs are enriched in C16:0 ceramide, a bioactive lipid specie generated from palmitate (51). C16:0 ceramide-enriched Hep-EVs were released from damaged hepatocytes in response to lipotoxicity, an effect mediated by the ER stress sensor inositol requiring enzyme 1α (IRE1α). Palmitate-induced EVs were chemoattractive toward macrophages because they also contained sphingosine 1-phosphate (S1P), a ceramide metabolite that activates its receptor in macrophages. They also showed increased C16:0 ceramide in the blood of mouse and humans with NASH. These data provide a mechanistic association between lipotoxic ER stress and disease pathogenesis via EVs and suggest that C16:0 ceramide and S1P content in EVs might be used as biomarkers in NASH patients. In the same line, Hirsova et al. have reported that, upon toxic lipid overload, hepatocytes can initiate a C/EBP homologous protein (CHOP)/death receptor 5 (DR5)/caspase-8/caspase-3 signaling cascade resulting in the activation of Rho-associated protein kinase 1 (ROCK1) and the release of EVs expressing TRAIL on their surface (33). Hence, TRAIL-bearing EVs were able to activate mouse bone marrow–derived macrophages toward an inflammatory phenotype (M1) via nuclear factor κB (NF-κB) signaling. They also showed that the release of Hep-EVs and, therefore, macrophage activation, was decreased by inactivating DR5 signaling pathway or using ROCK1 inhibitors. Likewise, they found that ROCK1 inhibition in mice with NASH led to a reduction of circulating EV levels associated with less liver damage such as inflammation and fibrosis. Moreover, Guo et al. (52) conducted a study that provides insights regarding the mechanism by which lipotoxic Hep-EVs may regulate peripheral blood monocyte adhesion to LSECs and hepatic recruitment and retention during NASH. They found that integrin β1 (ITGβ1), a cell adhesion molecule highly expressed in hepatocytes, plays a role in the progression of NASH. Lipotoxic insult in hepatocytes activates ITGβ1 and facilitates its endocytic trafficking and release of EVs, thereby promoting monocyte adhesion to LSECs, an essential step in hepatic inflammation. They also showed that blocking ITGβ1 in mice fed a FFC diet ameliorates liver inflammation, injury, and fibrosis. Hence, these authors propose that reducing the ability of LSECs to recruit harmful proinflammatory monocytes through ITGβ1 inhibition may serve as an anti-inflammatory therapeutic strategy to combat NASH. On the other hand, Cannito et al. demonstrated that EVs released by fat-laden hepatocytes undergoing lipotoxicity can directly activate the multiprotein platform complex nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome in both hepatocytes and macrophages, resulting in caspase 1 activation and pro–interleukin-1β and pro–interleukin-18 production, ultimately leading to a significant release of IL-1β (53). Since the release of EVs from lipotoxic cells and the subsequent activation of the NLRP3 inflammasome have been suggested to contribute to NAFLD progression, these data point to a novel rational link between lipotoxicity and inflammatory responses.

It is also noteworthy to mention a recent study conducted by Liu et al. showing that lipotoxic hepatocytes release exosomes enriched in miR-192-5p that activate proinflammatory macrophages and hepatic inflammation through the negative regulation of Rictor/Akt/FoxO1 signaling pathway (54). Furthermore, in patients with NAFLD, serum miR-192-5p levels positively correlated with hepatic inflammatory activity score and disease progression. Likewise, serum miR-192-5p levels and the number of M1 macrophages, as well as the expression levels of hepatic proinflammatory mediators, were correlated with disease progression in high-fat, high-cholesterol diet (HFHCD)–induced NAFLD in rats. Thus, they suggested serum

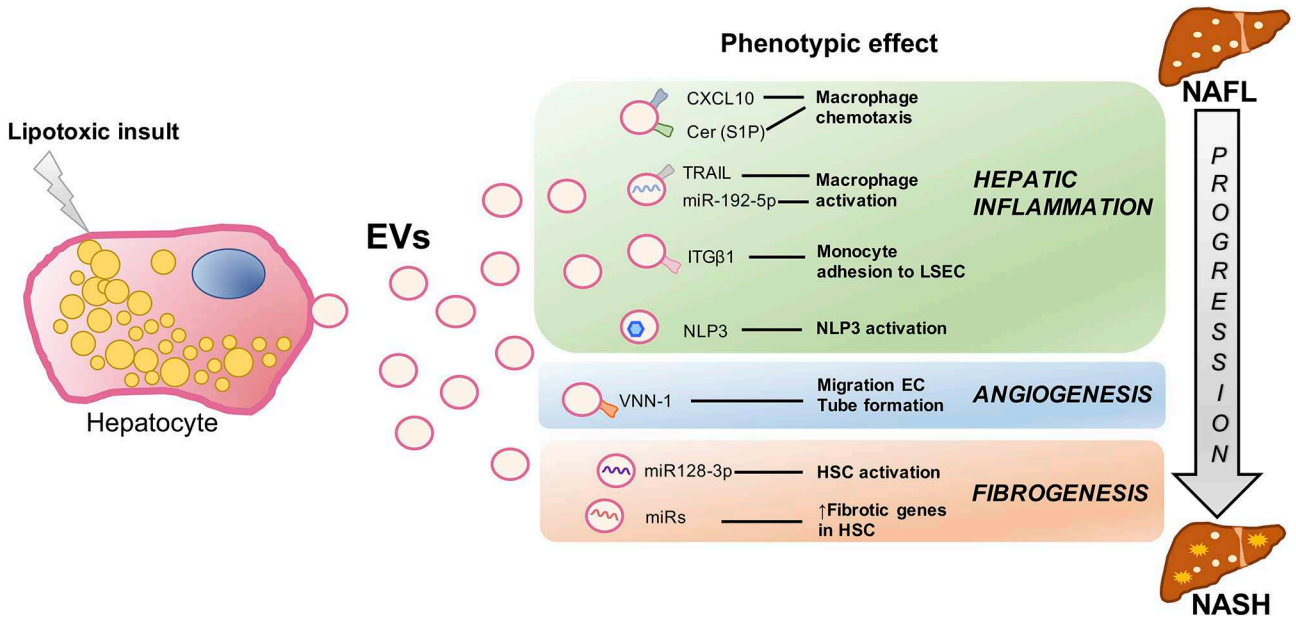

FIGURE 2 | Signaling events mediated by extracellular vesicles during hepatocyte lipotoxicity. Hepatocyte injury induced by lipotoxicity triggers the release of EVs (Hep-EVs) that drive the response of surrounding cells playing an important role during NAFLD progression to NASH such as hepatic inflammation, dysregulated angiogenesis, and fibrosis. Recent *in vitro* and *in vivo* studies have defined multiple Hep-EV cargos responsible of different phenotypic effects in the target cells. CXCL10 and ceramide-enriched EVs mediate monocyte/macrophage chemotaxis to the liver, whereas TRAIL-enriched EVs and miR192-5p contribute to macrophage activation. ITGβ-enriched EVs regulate monocyte adhesion to LSECs, and Hep-EVs can also activate NLRP3 inflammasome. VNN1-1–bearing EVs mediate endothelial cell migration and tube formation and neovascularization, whereas miR-128-3p–laden EVs induce HSC proliferation and activation. EV, extracellular vesicles; CXCL10, C-X-C motif chemokine ligand 10; Cer, ceramide; S1P, sphingosine 1-phosphate; TRAIL, tumor necrosis factor–like apoptosis inducing ligand; ITGβ1, integrin β1, LSEC, liver sinusoidal endothelial cells; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; VNN1, vanin-1; EC, endothelial cells; HSCs, hepatic stellate cells; NAFLD, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

exosomal miR-192-5p as a potential noninvasive biomarker and therapeutic target for NASH.

Hep-EVs Involved in Angiogenesis

Angiogenesis is a pathological feature of NASH and plays a central role in NAFLD progression. However, angiogenesis-inducing molecular and signaling mechanisms, as well as the potential link between lipotoxicity and angiogenesis, remain incompletely understood.

A study reported by Povero et al. (55) provided evidences that hepatocytes overloaded with saturated lipotoxic FFAs secrete proangiogenic signals. They identified Hep-EVs laden with vanin-1 (VNN1), an epithelial ectoenzyme with recognized cell migration and adherence properties, which induced endothelial cell (EC) migration and vascular tube formation, two processes required for angiogenesis. Of relevance, EVs derived from VNN1-deficient HepG2 cells failed to induce significant EC migration and tube formation. Likewise, administration of siRNA targeting VNN1 to mice fed with a methionine- and choline-deficient diet protected against NASH-induced pathological angiogenesis in the liver. Altogether, these findings uncovered a mechanism linking hepatocyte lipotoxicity to angiogenesis and identified a potential therapeutic target for developing novel antiangiogenic strategies for the treatment of NASH, as well as a circulating biomarker of liver damage.

Hep-EVs Involved in Fibrosis

Hepatic stellate cells play a crucial role during liver fibrosis in advanced NAFLD (56). When hepatic steatosis develops, HSCs are activated and express several fibrosis markers such as transforming growth factor β, tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2), and matrix metalloproteinase-2 (57). However, the trigger for HSC activation in NAFLD is still under investigation. The following mentioned studies have suggested that EVs may have important roles in the crosstalk between hepatocytes and HSCs in the progression from simple steatosis to NASH, identifying potential molecular targets for antifibrotic therapeutic interventions.

As shown by a marked up-regulation of profibrogenic genes including collagen-I, α-smooth muscle actin, and TIMP2, as well as proliferation, chemotaxis, and wound healing responses, the study of Povero et al. (55) demonstrated that EVs released from lipid-laden hepatocytes are internalized into HSCs, inducing a phenotypic switch from quiescent to activated HSCs (required for development of liver fibrosis). These changes were associated with the EVs cargo miR-128-3p, which regulates several proteins involved in liver fibrosis and HSC activation, as well as peroxisome proliferator-activated receptor γ (PPAR-γ) that has been proposed as mediator in the maintenance of a quiescent HSCs phenotype in normal liver (58). Interestingly, exposure of HSCs to miR-128-3p–depleted EVs resulted in downregulation

of profibrogenic markers and upregulation of PPAR-γ. Likewise, miR-128-3p–depleted EVs attenuated HSC proliferation and migration. Along these lines, Lee and colleagues have shown enhanced exosomes production and altered exosomal miRNA profile in palmitic acid (PA)–treated hepatocytes that increased the expression levels of fibrotic genes in HSCs. A step further, they confirmed that in exosomes from PA-treated cells, the expression of miRNA-122, one of the most abundant miRNAs in the liver (59, 60), was increased together with miRNA-192 also associated with NASH progression and fibrosis (61, 62). In this study, it was found that direct transfection of miRNA-192 into HSCs increased the expression of fibrosis markers. On the other hand, they found that the expressions of miRNA-122 and miRNA-192 were increased in circulating exosomes from patients with advanced NAFLD compared to those at early stages of the disease (63). Therefore, it was suggested that miRNA profiling in circulating exosomes may serve as a biomarker for the diagnosis of advanced NAFLD or NASH.

In summary, these relevant studies point to the participation of Hep-EVs in the modulation of responses of nonparenchymal cells of the liver including LSECs, HSCs, and KCs as a multiple-hit mechanism resulting in accelerated NASH progression.

Hep-EVs and NAFLD-Associated Complications

The harmful effect of NAFLD is not only limited to damage of the well-known liver functions in metabolism and detoxification processes, among others, but also provides an independent risk for development of atherosclerosis and other related CVDs, which represent the main cause of death in these subjects (64). Although clinical evidences have linked NAFLD and CVD, the underlying molecular mechanisms need to be deciphered. The potential role of Hep-EVs in endothelial inflammation and atherogenesis in the context of NAFLD has been achieved by Jiang et al. (65). They identified miR-1 as mediator of the proinflammatory effect of EVs via downregulation of Kruppel-like factor 4 (KLF4), a transcriptional regulator of vascular homeostasis, and activation of the NF-κB pathway in ECs. Moreover, inhibition of miR-1 with a specific antagomiR-1 in an animal model of atherosclerosis accompanied by fatty liver ($ApoE^{-/-}$ mice fed an high-fat diet) strongly suppressed vascular smooth muscle cells growth, stabilized plaques, and reduced endothelial inflammation leading to a marked amelioration of atherosclerotic plaque formation. This study provides convincing evidence implicating Hep-EVs in the distant communication between the liver and vasculature in NAFLD, and also unravels a molecular mechanism underlying the development of cardiometabolic disease.

In a different line and as previously mentioned, hepatic steatosis through aberrant accumulation of TGs in hepatocytes is the first hit during NAFLD development. Communication among metabolic tissues such as liver and adipose tissue regulates TG distribution in the body, which is critical for maintaining whole-body metabolic homeostasis (66). A recent study suggests that, in the context of lipid overload, the liver orchestrates the cross-talk with adipose tissue via specific EVs-containing miRNAs (67). They propose an interorgan mechanism whereby the liver in response to lipid overload sends an early signal to adipose tissue to modulate metabolic adaptations in order to counteract the excess of lipid deposition and also drives TG redistribution to maintain systemic homeostasis. At the molecular level, this study involves geranylgeranyl diphosphate synthase (GGPPS), an enzyme of the mevalonate pathway, which plays an important role in regulating glucose homeostasis and insulin sensitivity, in the secretion of Hep-EVs containing miRNAs. It was demonstrated that *Ggpps* expression is induced in hepatocytes by acute and chronic HFD consumption allowing geranylgeranylation of the Rab-GTPAse Rab27A, which, in turn, increases EV secretion. Among EVs-derived miRNAs, let-7e-5p enhances adipocyte lipid deposition by increasing lipogenesis and inhibiting lipid oxidation through alet-7e-5p-Pgc1α axis. Furthermore, this phenomenon is inhibited in liver-specific *Ggpps* knockout mice due to reduced Hep-EV secretion. Thus, this seminal study highlights a Hep-EVs–mediated liver–adipose tissue signaling axis that may be necessary for the metabolic adaptations of adipocytes to face lipid overload in order to maintain systemic homoeostasis during NAFLD.

CIRCULATING EVs AS BIOMARKERS OF NAFLD DIAGNOSIS

As stated above, liver biopsy remains the gold standard procedure for diagnosis, staging, and monitoring of NAFLD. However, it is expensive, highly invasive, and inaccurate due to error sampling and carries some morbidity and a rare mortality risk; therefore, it is currently unsuitable for routine use in individuals at risk of NAFLD (68, 69). The paucity of systematic screening for NAFLD has led to massive underdiagnosis in patients progressing to advance NASH or cirrhosis, more severe and irreversible stages of the disease.

Currently, several noninvasive methods are being used in clinical practice to assess NAFLD in order to mitigate the need for liver biopsy. These include imaging techniques such as magnetic resonance (MR)– and ultrasound-based elastography and analysis of serum hepatic enzymes as surrogate markers of liver inflammation and synthetic function. Nonetheless, these techniques lack sensitivity and specificity enough for detection of the early stages and do not always correlate with the severity or extent of hepatocellular injury and/or inflammation (70, 71). Ideal candidate markers should reflect not only the presence of NAFLD, but also its severity, which is vital for early diagnosis and grading progression (45). This diagnostic utility may be further projected to the treatment of NAFLD at early stages in order to decrease the incidence of NASH and cirrhosis.

In this context, circulating EVs may represent an optimal noninvasive blood-based biomarker or, so-called liquid biopsy, for NAFLD diagnosis (35). **Table 1** summarizes potential EVs-based biomarkers for NAFLD. Several protein-based EV biomarkers have been introduced for NAFLD liver damage (72), NASH (33, 50), or HCC (78, 80–82), although to date most studies have focused on characterizing EVs-associated nucleic acids, especially miRNAs and particularly in HCC (85–88). Apart from their use in liver malignancies, EVs-associated miRNAs may also serve as biomarkers in nonmalignant liver diseases such as NASH-induced fibrosis (84). Moreover, because

TABLE 1 | Extracellular vesicle biomarkers in nonalcoholic fatty liver disease (NAFLD).

	NAFLD stage	Vesicle source	Species	Sample	Biomarker	References
Protein-based biomarkers	Liver damage	ND	Human	Serum, plasma	↑ sPTPRG	(72)
	NASH	ND	Mice	Plasma	↑ ASGPR1	(73)
		ND	Mice	Plasma	↑ CXCL10	(50)
		ND	Mice	Serum	↑ CYP2E1	(33, 50)
		Hepatocytes	Mice	Plasma	↑ VNN-1	(55)
		Hepatocytes	Mice	Plasma	↑ ASGR1, CYP2E1	(74)
		Macrophages	Mice	Plasma	↑ Gal3	(74)
		Neutrophils	Mice	Plasma	↑ Ly6G	(74)
		Leukocytes	Human	Serum, plasma	↑ CD14, iNKT	(75, 76)
	Cirrhosis	Hepatocytes	Human	Plasma	↑ CK18	(77)
	HCC	Hepatocytes	Human	Plasma	↑ Hep Par 1	(78, 79)
		ND	Human	Serum	↑ EpCAM, CD133, ASGR1	(80)
		ND	Human	Plasma	↑ANXA2	(81)
		ND	Human	Serum	↑ PIGR	(82)
		ND	Human	Serum	↑ LG3BP	(82)
Nucleic acid–based biomarkers	NASH	Hepatocytes	Rat, human	Serum	↑ miR-192-5p	(54)
		Hepatocytes	Mice	Plasma	↑ miR-122, miR192	(73)
		Hepatocytes	Human	Serum	↑ miR-122, miR192	(63)
		Hepatocytes	Mice, human	Plasma	↑ MitoDNA	(83)
	Fibrosis	ND	Mice	Serum	↓ miR-214 (↓Twist1 ↑CCN2)	(84)
	HCC	ND	Human	Serum	↑ miR-21	(85)
		ND	Human	Serum	↓ miR-718	(86)
		ND	Human	Serum	↑ miR-18a, miR-221, miR-222, miR-224	(87)
		ND	Human	Serum	↓ miR-101, miR-106b, miR-122, miR-195	(87)
		ND	Human	Serum	↑ miR-939, miR-595, and miR-519d	(88)
Lipid-based biomarkers	NASH	ND	Mice, human	Plasma	↑ Ceramide and S1P	(51)

ANXA2, annexin A2; ASGR1, asialoglycoprotein receptor 1; CCN2, connective tissue growth factor; CD133, cluster of differentiation 133; CD14, cluster of differentiation 14; CK18, cytokeratin 18; CXCL10, C-X-C motif chemokine ligand 10; CYP2E1, cytochrome P450 family 2 subfamily E member 1; EpCAM, epithelial cell adhesion molecule; Gal3, galectin 3; HCC, hepatocellular carcinoma; Hep Par 1, hepatocyte paraffin 1; iNKT, invariant natural killer T; LG3BP, galectin-3-binding protein; Ly6G, lymphocyte antigen 6 complex locus G; miR, mature form of the miRNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis ND, not defined; PIGR, polymeric immunoglobulin receptor; S1P, sphingosine 1 phosphate; sPTPRG, soluble protein tyrosine phosphatase receptor gamma; VNN1, vanin-1.

of specific EVs-target cell–tissue interactions, lipid-based EV biomarkers might be important in NAFLD diagnosis as well, even though <3% of circulating lipids are transported in EVs (89). The only data available in this area is shown in the aforementioned study conducted by Kakazu et al. (51) showing increased C16:0 ceramide-enriched EVs in mice and humans with NASH. Nonetheless, circulating EVs are heterogeneous and do not exclusively reflect the specific contribution of the liver. Extrahepatic EVs may, in fact, mask liver-derived EVs, which ultimately, as we have reviewed, have a relevant role in NAFLD pathogenesis. Therefore, the identification of liver-specific markers in EVs might facilitate the detection of low-abundance cargos usually undetected, thereby providing direct information on disease progression, recovery, and treatment responses. In this regard, EV enrichment based on liver-specific markers followed by cargo analysis could represent a good strategy for biomarker discovery in NAFLD.

Circulating Liver-Derived EVs

One of the first studies to overcome this challenge was conducted by Povero et al. in experimental murine models of diet-induced NAFLD and early- and advanced-NASH. These authors observed that levels of circulating EVs increased over time during NASH progression and strongly correlated with several histological features such as cell death, angiogenesis, and fibrosis (73). Consistent with previous findings of other groups (90, 91), in a subsequent analysis Povero et al. revealed that circulating EVs isolated from mice with NAFLD were enriched in miR-122 and miR-192, two miRNAs abundantly expressed in hepatocytes. Based on these results, they proposed that hepatocytes were likely the main source of circulating EVs, concluding that Hep-EVs increased in mice with NAFLD (73). Extracellular vesicle–associated miR-122 and miR-192 were later validated by Lee et al. (63) as a biomarker of NASH progression in sera from NAFLD patients. Nevertheless, Hep-EVs as noted earlier were also enriched in the enzyme VNN-1, which promoted angiogenesis and induced liver damage in NASH, hence representing another potential biomarker (55). Furthermore, in the mentioned study of Liu et al., an increase in Hep-EVs and EV-associated miR-192-5p in serum of HFHCD-fed rat NAFLD model and NASH patients was also observed, therefore proposing miR-192-5p as other biomarker (54). In a previous study, we found that

hepatocyte-derived circulating EVs containing mitochondrial DNA were also increased in plasma from both mice and patients with NASH, and importantly, this work implicated EVs in macrophage activation via Toll-like receptor 9 (TLR9) (83).

A distinct approach to identify liver-derived EVs was achieved by Brodsky et al. (79). They isolated circulating EVs enriched in proteins from liver origin in patients with HCC using flow cytometry and immunolabeling against the protein hepatocyte paraffin 1 (Hep Par 1), an antibody to carbamoyl phosphate synthetase 1 used as tissue marker of HCC. These authors found increased levels of circulating liver-derived EVs in patients with HCC that correlated with the size of the liver tumors. Endothelium-derived EVs were also evaluated, and the same correlation was found (79). In this line, more recently Li et al. (74) identified Hep-EVs using nanoscale flow cytometry detecting hepatocyte selective surface markers on EVs such as asialoglycoprotein receptor 1 (ASGR1) and cytochrome P450 family 2 subfamily E member 1 (CYP2E1). By using this technology, they found increased Hep-EVs in both male and female mice at an early NAFLD stage (12 and 10 weeks of FFC diet feeding) before histologically apparent inflammation and remained elevated over time (24, 36, and 48 weeks). Macrophage- and neutrophil-derived EVs were also analyzed due to the important role of the immune signature in NASH. Macrophage- and neutrophil-derived EVs were significantly elevated at 24 weeks of dietary feeding concomitant with histologic inflammation in the liver. Furthermore, hepatocyte-, macrophage-, and neutrophil-derived EVs strongly correlated with the histologic assessment of NASH and noninvasive MR-based biomarkers of NASH. They also quantified platelet-derived EVs demonstrating sexual dimorphism because they were elevated in male mice at 12, 24, and 48 weeks of dietary feeding, whereas in female mice elevations were found at 24 weeks (74). This work constitutes the first descriptive report of the kinetic changes in hepatocyte-, macrophage-, neutrophil-, and platelet-derived EVs in a mouse model of NASH.

Circulating Immune Cells–Derived EVs

Regarding the immune system in NAFLD progression, it is important to highlight the pioneering study of EVs-based NAFLD diagnosis in humans published by Kornek et al. (75). They suggested for the first time the existence of a correlation between the circulating abundance of specific leukocyte-derived EVs and disease severity, as determined by liver transaminase levels, biopsy grade, and NAFLD activity score. To date, these findings still represent the most compelling study with clinical samples for NAFLD diagnosis and progression based on EVs. Consistently, this study was recently confirmed by Welsh et al. (76), who also reported leukocyte-derived EVs as a marker for liver fibrosis severity in NAFLD. It is noteworthy to mention that Kornek et al. (75) also observed that patients with chronic hepatitis C could be differentiated from patients with NASH using immune cells–derived EVs. This was further supported by another study in which transcriptomic analysis revealed that serum EVs-derived miRNAs were regulated either positively or negatively with the histological features of the disease such as inflammation and fibrosis, therefore differentiating multiple etiologies of liver disease, as well as disease from healthy controls (92). In another study, both circulating Hep-EVs and immune cell–derived EVs were analyzed (77). Extracellular vesicles from hepatocytes or myeloid origin were found increased in patients with cirrhosis compared with healthy individuals. In patients with cirrhosis, plasma Hep-EVs contained high levels of cytokeratin-18 compared with healthy individuals. Moreover, the severity of cirrhosis correlated with the levels of leukoendothelial EVs and Hep-EVs (77).

Taken together, all these studies have established a solid background for EV biomarker discovery in NAFLD diagnosis. However, because of the current notable limitations, there is still a long way to go before EVs-related assays will have translational utilities. Aside from disease and tissue specificity, the lack of generally accepted standardization of the methods for EV isolation and guidelines related to sample collection and handling can interfere with downstream analysis, resulting in high variability that complicates the reproducibility and validation of EVs as biomarkers (35, 45).

CONCLUDING REMARKS

In this review, we have summarized some of the most recent and original studies investigating the key role of EVs released by stressed hepatocytes (Hep-EVs) by targeting nonparenchymal cells such as HSCs, LSECs, and macrophages. This interactome links lipotoxicity with inflammation and angiogenesis, relevant events in the progression of NAFLD to NASH stage. In addition, we compiled several studies on the significant interest of Hep-EVs released into the systemic circulation as potential biomarkers for NAFLD diagnosis and progression. Future studies to examine additional molecular mechanisms involved in EVs biogenesis, release, and dysregulation of target cells, as well as the identification of cargos with potential value as biomarkers for noninvasive diagnosis and monitoring of disease progression, are highly awaited.

AUTHOR CONTRIBUTIONS

IG-M, RA, PR, and AV contributed to the preparation and writing of this review article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Non-alcoholic Fatty Liver Disease and Alcohol-Related Liver Disease: Two Intertwined Entities

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Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide, with a prevalence of 25–30%. Since its first description in 1980, NAFLD has been conceived as a different entity from alcohol-related fatty liver disease (ALD), despite that, both diseases have an overlap in the pathophysiology, share genetic–epigenetic factors, and frequently coexist. Both entities are characterized by a broad spectrum of histological features ranging from isolated steatosis to steatohepatitis and cirrhosis. Distinction between NAFLD and ALD is based on the amount of consumed alcohol, which has been arbitrarily established. In this context, a proposal of positive criteria for NAFLD diagnosis not considering exclusion of alcohol consumption as a prerequisite criterion for diagnosis had emerged, recognizing the possibility of a dual etiology of fatty liver in some individuals. The impact of moderate alcohol use on the severity of NAFLD is ill-defined. Some studies suggest protective effects in moderate doses, but current evidence shows that there is no safe threshold for alcohol consumption for NAFLD. In fact, given the synergistic effect between alcohol consumption, obesity, and metabolic dysfunction, it is likely that alcohol use serves as a significant risk factor for the progression of liver disease in NAFLD and metabolic syndrome. This also affects the incidence of hepatocellular carcinoma. In this review, we summarize the overlapping pathophysiology of NAFLD and ALD, the current data on alcohol consumption in patients with NAFLD, and the effects of metabolic dysfunction and overweight in ALD.

Keywords: non-alcoholic fatty liver disease, steatosis, cirrhosis, NAFLD, NASH, alcohol, alcohol-related liver disease, ALD

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and alcohol-related liver disease (ALD) are the most frequent causes of chronic liver disease worldwide (1, 2). Over the past decade, both entities have been increasing in the U.S. and worldwide, contributing to the rising burden of cirrhosis and hepatocellular carcinoma (HCC) and surpassing the figures of viral hepatitis infection as chief etiologies of these conditions (3). These temporal trend shifts in the contributions of NAFLD

and ALD to the total burden of liver disease are likely related to diverse factors. Among them are the changing epidemiology of viral hepatitis in the last decade, the increasing rates of obesity and type 2 diabetes (T2DM) and the changing patterns of alcohol consumption in the general population. NAFLD and ALD have a number of commonalities and may eventually coexist in the same individual. In this context, it seems timely to review some basic and clinical concepts on these two intertwined conditions.

NAFLD is closely related to obesity and overweight as well as to the presence of metabolic dysfunction, and although the occurrence of steatosis in this setting was recognized in the early 1950s (4), only in 1980 was it pointed out as a possible cause of cirrhosis in a landmark case series study by Ludwig et al. (5). At the present time, NAFLD is defined by steatosis associated with a spectrum of hepatic histopathologic changes including the presence of inflammatory infiltrates and various degrees of fibrosis and cirrhosis (1). These features develop in the absence of known factors that cause fat accumulation such as alcohol consumption (defined as <30 g/day in men and <20 g/day in women), viral liver disease, and hereditary disorders. NAFLD is usually found in patients with comorbidities, such as metabolic syndrome (MetS), obesity, insulin resistance (IR), T2DM, and dyslipidemia (6). It is estimated that between 7 and 30% of patients with NAFLD may develop an inflammatory subtype termed non-alcoholic steatohepatitis (NASH), which is hallmarked by the presence of cell ballooning and lobular inflammation (7). NASH seems to be a more aggressive form of the disease that progresses more commonly to advanced fibrosis and cirrhosis (8). Patients with NAFLD, particularly those with NASH, have an increased mortality due to liver disease, and it is likely that cardiovascular mortality could also be increased (9).

NAFLD has increased significantly worldwide over the last decades, in line with the obesity epidemic and sedentary lifestyles (8, 10–12). Currently, the global prevalence of NAFLD is around 25%, with important differences between the Middle East (32%), South America (31%), United States (24.1%), and Africa (14%) (12–15). Additionally, the prevalence varies in association with metabolic diseases. NAFLD can be detected with ever greater prevalence in ∼90% of obese patients and 65% of overweight patients (13) and in up to 70% of T2DM patients (16).

ALD affects 2–2.5% of the general population and exhibits a greater prevalence in areas with higher alcohol consumption (17). In Western countries, up to 50% of the patients with end-stage liver disease have alcohol as a major etiologic factor (18). According to the World Health Organization in 2018, more than 3 million deaths every year—representing around 5% of global deaths—are attributable to alcohol consumption (19). In the United States in 2006, alcohol-related deaths (excluding accidents) accounted for 22,073 deaths, with 13,000 of those specifically attributed to ALD (20). ALD is caused by heavy chronic alcohol consumption. Heavy or hazardous drinking is defined as consumption of more than 3 standard drinks per day in men, and more than 2 drinks per day in women, or binge drinking (defined as more than 5 standard drinks in men and more than 4 in women over a 2-h period) (21), implying a greater risk of developing health problems associated with alcohol (22–24). Clinical manifestations range from no symptoms to severe acute alcoholic hepatitis (AH) with or without cirrhosis (17).

The relationship between NAFLD and ALD is complex due to overlapping clinical features and lack of positive criteria for NAFLD (25). Of note, the interaction between NAFLD and alcohol consumption has been controversial over the last few years (26). Initially, some studies suggested a protective effect of moderate doses of alcohol (27). However, recent evidence indicates that there is no safe threshold for alcohol consumption in NAFLD patients (28). Moreover, alcohol use is a significant risk factor for the progression of liver disease in these individuals, eventually impacting a mortality in those patients with NAFLD and MetS (29). On the other hand, in ALD, MetS and obesity may increase liver disease progression and the incidence and mortality of HCC (30).

In this review, we aim to summarize current data on the overlapping pathophysiology of NAFLD and ALD as well as the available information on alcohol consumption in patients with NAFLD and the effects of MetS and overweight in ALD. We underscore the need for a change in NAFLD nomenclature in order to account for the dual etiology of liver disease, which is present in a likely significant proportion of patients with concurrent alcohol consumption and metabolic disturbances. Proper consideration of these concepts should impact clinical management.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; ALD, alcohol-related liver disease; FFA, free fatty acids; IR, insulin resistance; ER, endoplasmic reticulum; ROS, reactive oxygen species; EV, extracellular vesicles; DAMPs, damage-associated molecular patterns; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IL-6, interleukin 6; LPS, lipopolysaccharide; MetS, metabolic syndrome; PDGF, platelet-derived growth factor; TLR, toll-like receptors; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor α; TGF-β, transforming growth factor-beta; T2DM, type 2 diabetes mellitus; DNL, *de novo* hepatic lipogenesis; FAO, fatty acid oxidation; ACC, acetyl-CoA carboxylase; CPT, carnitine palmitoyltransferase; IRS, insulin receptor substrate; TRAIL-R2, TRAIL receptor 2; HMGB1, high-mobility-group protein box; SHH, sonic hedgehog; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NLRs, NOD-like receptors; miRs, microRNAs; NR, nuclear receptors; LXR, liver X receptor; PXR, pregnane X receptor; PPARγ, peroxisome proliferator-activated receptor-gamma; FXR, farnesoid X receptor; FGFR4, FGF receptor 4; ASBT, apical sodium-dependent bile acid transporter; BMI, body mass index; ALT, serum alanine aminotransferase; MAFLD, metabolic (dysfunction) associated fatty liver disease; AAFLD, alcohol-associated fatty liver disease.

HOW MUCH ALCOHOL IS BAD IN NAFLD?

Definition of NAFLD, and the distinction from ALD, is based on the amount of alcohol consumed, which has been established arbitrarily, and without definitive evidence. The threshold of alcohol consumption that rules out NAFLD usually is 20 g (2 units per day) in women, 30 g (3 units per day) in men, based on guidelines of scientific associations recommendations (1, 6). Alcohol consumption is reported in up to two-thirds of patients with NAFLD in the United States (31). The effect of alcohol consumption on the prognosis of NASH has been a subject of controversy for many years, with some studies suggesting a protective effect and others suggesting an increased risk of liver disease progression and HCC (32–35) (**Table 1**). Initial

TABLE 1 | Studies assessing the effect of moderate alcohol intake on NAFLD.

References	Description	Findings
Dixon et al. (36)	105 patients who underwent bariatric surgery (cross-sectional cohort study). Alcohol intake was studied by questionnaire and liver disease by biopsy	Moderate alcohol consumption was associated with a decreased incidence of NASH (OR, 0.35; 95% CI, 0.12–1.00)
Suzuki et al. (37)	1,177 male subjects, 5 years of follow-up (cross-sectional and prospective study). Alcohol intake assessment by questionnaire and liver disease by elevation of ALT	Alcohol consumption was negatively associated with elevated ALT (HR 0.4; 95% CI 0.1–0.9)
Dunn et al. (32)	7,211 subjects none alcohol intake and 945 wine drinkers (cross-sectional study). Alcohol consumption assessment by questionnaire and liver disease by raised ALT	Mild wine consumption was associated with 50% reduced risk of elevated ALT (OR 0.51; 95% CI 0.33–0.79) without effect in beer or liquor intake
Gunji et al. (38)	5,599 Japanese men with regular medical survey (cross-sectional study). Alcohol intake defined by Questionnaire and fatty liver detected by US	Mild (40–140 g per week) and moderate (140–280 g per week) alcohol intake reduced the risk of steatosis (OR 0.82; 95% CI 0.68–0.99 and OR 0.75; 0.61–0.93)
Gunji et al. (39)	1,138 Japanese men (≥40 years) (cross-sectional study). Alcohol intake assessment by questionnaires and fatty liver by CT	Alcohol consumption was associated with a reduced risk of steatosis. This reduction was independently of MetS and physical activity
Hiramine et al. (40)	9,886 males on regular health check-ups (cross-sectional cohort). Alcohol intake studied by questionnaire and liver disease by US	Fatty liver increased with obesity and decreased with alcohol intake (light, OR 0.71, 95% CI 0.59–0.86; moderate, OR 0.55, CI 0.45–0.67; heavy, OR 0.44, CI 0.32–0.62)
Moriya et al. (41)	4,957 men and 2,155 women without liver disease (cross-sectional study). Alcohol intake assessment by questionnaire and fatty liver by US and raised ALT	The prevalence of steatosis was lower in drinkers than in non-drinkers' men and women ($p < 0.001$ for both). NAFLD was inversely associated with both amount and frequency of alcohol intake
Hamaguchi et al. (42)	8,571 Japanese men and women (cross-sectional study). Mean BMI, 22.6 kg/m^2 alcohol consumption assessment by questionnaires and fatty liver by US	Light and moderate alcohol intake was inversely associated with fatty liver in men (OR 0.69, 95% CI 0.60–0.79 and OR 0.72, 95% CI 0.63–0.83) and women (OR 0.54, 95% CI 0.34–0.88 and OR 0.43, 95% CI 0.21–0.88)
Dunn et al. (32)	251 modest drinkers and 331 non-drinkers (cross-sectional cohort study). Alcohol intake studied by questionnaires (AUDIT test) and fatty liver by biopsy	Modest drinking reduced the odds of NASH (OR 0.56, 95% CI 0.39–0.84), fibrosis (OR 0.56; 95% CI 0.41–0.77) and ballooning (OR 0.66, 95% CI 0.48–0.92) vs. lifetime non-drinking habits
Hagstrom et al. (43)	120 subjects with demonstrated NAFLD by biopsy (cross-sectional, cohort study). Alcohol intake assessment by questionnaires for lifetime alcohol intake and phosphatidylethanol (PEth) for recent alcohol consumption	Alcohol intake (up to 13 U per week) was associated with reduced risk of fibrosis (OR 0.86 95% CI 0.76–0.97), but high PEth was associated with increased risk of fibrosis (OR 2.77, 95% CI 1.01–7.59)
Kwon et al. (33)	77 subjects with NAFLD demonstrated by biopsy (cross-sectional cohort study). Alcohol intake assessment by retrospective questionnaire and liver disease by biopsy	Lifetime alcohol intake ≥24 g-years was associated with less severe disease (OR 0.26, 95% CI 0.07–0.97)
Moriya et al. (44)	3,773 men and 1,524 women (prospective analysis). Alcohol consumption defined by questionnaire and fatty liver by US	Modest alcohol intake was associated lower incidence of steatosis in men and woman. In men, steatosis was also reduced by alcohol intake in the range ≥280 g per week, after adjustment for confounders (OR 0.68; 95% CI 0.58–0.79)
Mitchell et al. (45)	187 NAFLD patients (cross-sectional, cohort study). Questionnaires for previous and actual alcohol intake and Liver biopsy	Mild alcohol consumption was associated with a decreased risk of advanced fibrosis (OR 0.33, 95% CI 0.14–0.78). Wine intake (not beer drinking) was negatively associated with advanced fibrosis (OR 0.20, 95% CI 0.06–0.69), compared with patients without alcohol intake
Hajifathalian et al. (27)	4,568 subjects follow-up of 70 months (prospective study). Questionnaire for amount and type of alcohol drinking and Hepatic Steatosis Index for determinate the liver disease	Mild alcohol (0.5–1.5 U per day) consumption was associated with decreased overall mortality (HR 0.64, 95% CI 0.42–0.97). However, in NAFLD alcohol consumption ≥1.5 U per day had a harmful effect on mortality (HR 1.45, 95% CI 1.01–2.10)
Sookoian et al. (46)	Meta-analysis. Included 43,175 individuals of 8 studies with high heterogeneity	Modest alcohol consumption was associated with a protective effect in NASH (fixed models: OR 0.69, 95% CI 0.65–0.73; random models: OR 0.68; 95% CI 0.58–0.81)
Ajmera et al. (26)	Critical review of 7 observational studies	Concluded a positive association between moderate alcohol use and decreased NASH and fibrosis. Heavy episodic drinking may accelerate fibrosis progression and moderate alcohol intake may increase the risk of HCC (in patients with advanced fibrosis)
Becker et al. (23)	13,285 men and women (prospective cohort study). Follow-up of 12 years. The alcohol intake assessment by a self-administered questionnaire. Alcohol-induced liver disease by death certificates/hospital registers	Alcohol intake associated with lower risk of liver disease (up to 1–6 U per week). The relative risk was significantly >1 at 7–13 U per week for women and 14–27 for men

(Continued)

TABLE 1 | Continued

References	Description	Findings
Bellentani et al. (47)	6,917 subjects of the general population (community-based study). The alcohol intake assessment by questionnaire and liver disease by blood test and clinical	Increased risk of chronic liver disease and cirrhosis with alcohol intake above 30 g/day
Bellentani et al. (48)	257 participants (Dionysos Study, Cross-sectional, Cohort). Validated questionnaire for alcohol intake and NAFLD by US	Fatty liver risk 2.8-fold higher in drinkers (95% CI, 1.4–7.1) and 4.6-fold higher in obese persons (95% CI, 2.5–11.0). In subjects obese and alcohol intake was 5.8-fold higher (95% CI, 3.2–12.3)
Bedogni et al. (49)	144 subjects without and 336 with fatty liver (cohort study). Follow-up of 8.5 years. Questionnaire for amount of alcohol intake	The alcohol intake increases the incident steatosis by 17%, steatosis remission decreased by 10%, and mortality increased by 10%
Eckstedt et al. (34)	71 patients with NAFLD by biopsy (cohort study). Follow-up of 13.8 years. Alcohol intake assessment by validated questionnaire and oral interview. The outcome was fibrosis progression by biopsy	Episodic and continuous heavy drinking was more common among those with fibrosis progression. Binge drinking predicted fibrosis progression
Aberg et al. (30)	6,732 subjects without liver disease, follow-up of 11.4 years (cohort study). Alcohol intake studied by questionnaire and outcome was liver disease progression, HCC, liver-related death	Alcohol intake (below the risk threshold) remained as a significant independent predictor of liver disease progression and HCC
Chang et al. (50)	58,927 Korean adults with NAFLD and low fibrosis (cohort study). Followed for a median of 4.9 years. Fibrosis was assessed using non-invasive indices including NAFLD fibrosis score (NFS) and Fibrosis-4 Index (FIB-4)	Non-heavy alcohol consumption, especially moderate alcohol consumption, was significantly and independently associated with worsening of non-invasive markers of fibrosis
Younossi et al. (29)	4,264 individuals with hepatic steatosis (retrospective cohort study). Mean age, 45.9 years; 51% male; 76% white; 46% with MetS; 6.2% with excessive alcohol use. Steatosis determined by US and alcohol intake by questionnaire	The presence of MetS [adjusted hazard ratio (aHR), 1.43; 95% CI, 1.12–1.83] and excessive alcohol consumption (aHR, 1.79; 95% CI, 1.21–2.66) were independently associated with an increased risk of death in individuals with hepatic steatosis; any lower average amount of alcohol consumption was not associated with mortality (all $P > 0.60$)
Ajmera et al. (51)	285 participants were modest alcohol users and 117 were abstinent (Longitudinal study). Follow-up period of 47 months. Liver was studied by biopsies and alcohol intake by questionnaire	Modest alcohol use was associated with less improvement in steatosis (adjusted odds ratio, 0.32; 95% CI, 0.11–0.92; $p = 0.04$) and level of aspartate transaminase, as well as lower odds of NASH resolution, compared with no use of alcohol
Verrill et al. (52)	100 patients with biopsy-proven alcohol-induced liver cirrhosis (retrospective study)	Abstinence from alcohol at 1 month after diagnosis of cirrhosis was the more important factor determining survival with a 7-year survival of 72% for the abstinent patients vs. 44% for the patients continuing to drink. Early drinking status is the most important factor determining long-term survival in alcohol-related cirrhosis
Sookoian et al. (53)	A Mendelian randomization study using a validated genetic variant (rs1229984 A;G) in the alcohol dehydrogenase (ADH1B) gene as a proxy of long-term alcohol exposure	The analysis of association with the disease severity showed that carriers of the A-allele had lower degree of histological steatosis (1.76 ± 0.83 vs. 2.19 ± 0.78, $P = 0.03$) and lower scores of lobular inflammation (0.54 ± 0.65 vs. 0.95 ± 0.92, $P = 0.02$) and NAFLD-Activity Score (2.9 ± 1.4 vs. 3.7 ± 1.4, $P = 0.015$) compared with non-carriers. The analysis suggests no beneficial effect of moderate alcohol consumption on NAFLD disease severity
Yi et al. (54)	504,646 Korean subjects in health maintenance visits (Cohort study). Follow-up of 10.5 years. Questionnaires for alcohol consumption, ICD-X codes for liver disease	HCC risk increases with age and alcohol consumption (for any 20 g per day)
Askgaard et al. (55)	55,917 subjects (between 50 and 64-year-old), Danish study (1993–2011). Alcohol consumption and pattern from questionnaire. Follow-up 14.9 years	Recent daily drinking associated with an increased risk of ALD cirrhosis in men (HR, 3.65; 95% CI, 2.39–5.55), compared to drinking 2–4 days per week

evidence for the protective effect of moderate alcohol intake in NASH dates back to 2001. Dixon et al. suggested that moderate alcohol consumption reduces the risk of NAFLD in the severely obese, probably by reducing IR (OR, 0.35; 95% CI, 0.12–1.00). Steatosis was diagnosed by laparoscopic biopsies during bariatric surgery and NASH was present only in 25% (26/105) of the cases (36). In 2007, Suzuki et al. performed a cross-sectional study in men without chronic liver diseases to determine the association between alcohol consumption (none, light, moderate, and excessive) and elevated serum aminotransferase levels. They concluded that excessive alcohol consumption was associated with increased aminotransferase levels, while light and moderate alcohol intake may protect against the development of elevated aminotransferases (37). However, aminotransferases are poor screening tools for NAFLD. The third National Health and Nutrition Examination Survey (NHANES) compared patients

with no alcohol intake (n = 7,211) vs. patients who drank wine exclusively (up to 10 g/day) (n = 945). In this study, the low-dose wine consumption (but not beer or liquor) was associated with a decreased risk of elevated aminotransferase levels (OR 0.62; 95% CI 0.41–0.92) (56). In 2009, Gunji et al. developed a cross-sectional study in the Japanese population, including a large series of asymptomatic male subjects. Alcohol intake was defined through a questionnaire, and steatosis status was assessed by aminotransferases or ultrasonography (US) (38). They reported an inverse association between alcohol consumption and steatosis, with a protective effect of light and moderate alcohol intake. Later, the same group obtained similar results defining steatosis through a CT scan (independent of MetS or physical activity) (39). Another cross-sectional study in males on regular health check-ups was conducted by Hiramine et al. They utilized a questionnaire to determine the alcohol intake and classified the subjects according to alcohol consumption as none, light, moderate, and heavy drinkers (0, <20, 20–59, and ≥60 g/day, respectively). Steatosis was defined by US. They also concluded that alcohol consumption plays a protective role against fatty liver in men. It is interesting that the analysis of the drinking patterns revealed that the prevalence of fatty liver was inversely associated with the frequency of alcohol consumption (≥21 days/month) (OR 0.62, CI 0.53–0.71), but not with the volume of alcohol consumed (40). Moriya et al. reported a significant inverse correlation between drinking frequency and the prevalence of fatty liver ($p < 0.001$) in the Japanese population. These authors described that drinking <20 g on 1–3 days/week was associated with a lower prevalence of fatty liver assessed by US (adjusted odds ratio, 0.47; 95% confidence interval, 0.23–0.96). This study included men and women, obtaining the same results for both (men: OR 0.59; 95% CI 0.52–0.68; women: OR 0.60; 95% CI 0.45–0.80) (41). These results were consistent with the study by Hamaguchi et al., who defined the prevalence of steatosis by CT scan (42).

At least 4 cross-sectional studies suggested a protective role of alcohol consumption after defining steatosis by liver biopsy (32, 43, 45, 57). In the NIH NASH Clinical Research Network, modest alcohol consumption was associated with less steatohepatitis, hepatocellular ballooning, and fibrosis (32). In another study, alcohol intake up to 13 U/week was associated with lower fibrosis stage in NAFLD (OR 0.86 per U/week, 95% CI 0.76–0.97). Nevertheless, an elevated phosphatidylethanol (a biomarker for recent alcohol consumption) was associated with higher stages of fibrosis (43). Finally, Mitchell et al. had similar results, but particularly with wine consumption (not with beer) and non-binge pattern (45). The association of modest alcohol intake with survival in NAFLD has also been evaluated. This analysis was made using the NHANES data (1988–2010). NAFLD was diagnosed by hepatic steatosis index (HSI) in 4,568 subjects. Modest alcohol consumption was associated with a significant decrease in all-cause mortality (after a median follow-up of 70 months, and adjustment for race, physical activity, education level, T2DM, and fiber and polyunsaturated fatty acid intake) [hazard ratio (HR) 0.64, 95% CI 0.42–0.97], whereas drinking ≥1.5 drinks per day was to be associated with an increased in mortality (HR 1.45, 95% CI 1.01–2.10) (27). Currently, only one meta-analysis has been published (46), which included 43,175 individuals. It concluded that modest alcohol consumption was associated with a protective effect in NASH (fixed models: OR 0.69, 95% CI 0.65–0.73; random models: OR 0.68; 95% CI 0.58–0.81) based on 8 heterogeneous studies. The critical review by Ajmera et al. (26) of 7 observational studies concluded a positive association between moderate alcohol use and decreased NASH and fibrosis; however, heavy episodic drinking may accelerate fibrosis progression and moderate alcohol intake may increase the risk of HCC (in patients with advanced fibrosis). However, the studies had significant methodological limitations, including incomplete adjustment for confounding factors.

Despite that some studies suggest a beneficial effect of moderate alcohol consumption on the occurrence and progression of NAFLD, more recent evidence suggests that there is no safe limit for alcohol consumption and that the alcohol intake is associated with a higher risk of liver disease progression, including HCC (23, 30, 34, 36, 47–49, 54, 55, 58–63). The association between alcohol intake and liver damage has been reported widely since 1957 (64). Many studies showed that alcohol consumption is associated with increased prevalence and progression of NASH. In the Dionysos Study, 144 subjects without steatosis and 336 with steatosis were followed up for 8.5 years. The most relevant risk factor for steatosis incidence and remission, as well as a predictor of mortality in these patients with fatty liver, was alcohol intake (20 g/day). The incidence of fatty liver increased by 17%, steatosis remission decreased by 10%, and mortality increased by 10% in the fatty liver cohort (49). Follow-up liver biopsy in 71 patients with NAFLD showed that fibrosis progression was associated with episodic drinking (at least once per month) and higher weekly alcohol consumption. Also, the heavy episodic drinking ($p < 0.001$) and IR ($p < 0.01$) were independently associated with significant fibrosis progression. The study concluded that moderate alcohol consumption was associated with fibrosis progression in NAFLD, and the authors advised to refrain from heavy episodic drinking in patients with NAFLD (34). Recent studies showed that there is no safe limit for alcohol consumption and suggested that even light alcohol consumption is not safe in NAFLD. In 2018, a systematic analysis from the Global Burden of Diseases, Injuries, and Risk Factors Study, which included 28 million individuals and 649,000 cases with outcomes, also suggested a detrimental effect of moderate alcohol intake (65). A Finnish cohort study of 6,732 individuals without baseline liver disease, after a follow-up of 11.4 years, demonstrated that non-risky alcohol intake (<3 units per day in men and 2 units per day in women) was associated with a significant increase in the risk of liver disease progression (30). A large-scale cohort was performed in 58,927 young and middle-aged Korean individuals with NAFLD (with low baseline fibrosis scores), who were followed for a median of 4.9 years. The progression from low to intermediate or high probability of advanced fibrosis was assessed using non-invasive index including NAFLD fibrosis score (NFS) and Fibrosis-4 Index (FIB-4). They demonstrated that light (1.0–9.9 g/d) or moderate (10.0–29.9 g/d for men and 10.0–19.9 g/d for women) alcohol consumption compared with none (0 g/d) was significantly and independently associated with worsening of fibrosis. The

effect was higher with moderate alcohol consumption (50). The authors also suggested that a safe limit of alcohol use may not exist. In a study that analyzed NHANES III including 4,264 adults with hepatic steatosis diagnosed by US examination, the overall mortality was significantly higher among subjects with excessive alcohol intake (32.2%) vs. subjects with non-excessive alcohol consumption (22.2%) after 5 and 20 years of follow-up ($p = 0.003$). The association of excessive alcohol use with mortality was significant in individuals who have MetS (aHR, 2.46; 95% CI, 1.40–4.32) but not without it ($p = 0.74$) (29). The impact of alcohol consumption is associated not only with incidence of steatosis, fibrosis progression, and mortality but also with less improvement in steatosis in patients with NASH and increased liver malignancies. In a longitudinal analysis of liver biopsies from patients with NAFLD, the low and modest alcohol use was associated with less improvement in steatosis, higher levels of AST, and less NASH resolution, compared with no alcohol intake (51). Another study suggested that total abstinence even prevents disease progression and is the more important factor determining survival in patients with established cirrhosis (52). Additionally, data from a Mendelian randomization study using a validated genetic variant (rs1229984 A:G) in the alcohol dehydrogenase (ADH1B) gene as a proxy of long-term alcohol exposure was used, thereby minimizing measurement bias and confounding factors. They found that carriers of the A-allele consumed significantly lower amounts of alcohol compared with non-carriers. Additionally, A-allele carriers had a lower degree of histological steatosis, lobular inflammation, and NAFLD-Activity Score (NAS) compared with non-carriers. They suggested that there is no beneficial effect of moderate alcohol consumption on NAFLD disease severity (53).

The association between alcohol intake and HCC development has been evaluated. Mild or moderate alcohol consumption may be a cofactor for the development of HCC in NAFLD. In a study that included 504,646 Korean patients (age 40–80 years) on routine health checkups, HCC incidence was associated with hepatitis B and C infection, and each 20 g/day of alcohol intake increased the risk of HCC by 6, 8, 16, and 30%, respectively, in individuals aged <50, 50–59, 60–69, and 70–80 years (54). An analysis of the participants in the Health 2000 and FINNRISK (1992–2012) databases was performed, which linked national registers for hospital admissions, malignancies, and death regarding liver, cardiovascular, and malignant disease, as well as all-cause mortality. They concluded that alcohol consumption is associated with a dose-dependent risk of advanced liver disease and neoplasia but a dose-dependent decrease in cardiovascular outcomes (21% risk reduction with ≤1 unit per day intake limited to non-smokers) (58). Alcohol intake is also associated with extrahepatic cancers, particularly breast, oral, pharyngeal, and colorectal cancer (66–69).

In summary, although some studies suggest a beneficial effect of light and moderate alcohol consumption on the occurrence and progression of NAFLD, most of them are cross-sectional studies, limiting their interpretation. These studies defined the association observed on the grounds of present alcohol intake history; nevertheless, NAFLD and ALD are processes that require long-term exposure and the damage could be driven by previous alcohol history. This is limited by the recall bias. Some studies have significant methodological limitations (including incomplete adjustment for confounding factors, as metabolic history) and several potential biases (especially in retrospective analyses), limiting their validity. On the other hand, the evidence supporting no benefit or even a detrimental effect of alcohol intake is based on solid longitudinal studies. The association between alcohol intake with fibrosis progression and cancer in these studies seems appealing and less biased than observed in cross-sectional analyses.

HOW OBESITY AND METABOLIC SYNDROME AFFECT ALD?

In recent years, the association between alcohol consumption and NAFLD progression has been clearly established. Emerging evidence has demonstrated that obesity and MetS increase the progression of ALD and HCC incidence and mortality. Indeed, a synergism between alcohol and obesity has been suggested. A cross-sectional study (48) including 257 participants of the Dionysos Study used a validated food questionnaire and ultrasound assessment of NAFLD. The prevalence of steatosis was increased in heavy drinkers (46.4% [95% CI, 34–59%]) and obese (75.8% [CI, 63–85%]) compared with controls (16.4% [CI, 8–25%]). Those heavy drinkers who are obese had an even higher prevalence of NAFLD, 94.5% (CI, 85–99%), which suggest an additive effect. Obesity doubles the risk of steatosis in heavy drinkers (48). This synergistic effect was also observed in 2 of the long-term Midspan prospective cohort studies (9,559 men) in Scotland. The body mass index (BMI) and alcohol consumption were strongly associated with liver disease mortality in analyses adjusted by other confounders ($p = 0.001$ and $p < 0.0001$, respectively). After a median follow-up of 29 years, consumers of 15 U/week or more exhibited higher rates of liver disease irrespective of BMI. In mild users (1–14 U/week), an excess of liver disease was only observed in subjects with obesity, with a synergistic effect between alcohol and BMI (synergy index, 2.89; [95% CI, 1.29–6.47]) (60). In the analysis of NHANES III, previously commented in this review, the presence of MetS [adjusted hazard ratio (aHR), 1.43; [95% CI, 1.12–1.83]] and excessive alcohol consumption (HR, 1.79; [95% CI, 1.21–2.66]) were independently associated with an increased mortality in subjects with steatosis. Additionally, alcohol intake and the presence of MetS had a synergistic effect (29). It is necessary to consider the alcohol intake as an aggravating element of overweight and obesity. A mild alcohol intake can contribute with 100–300 kcal/day, directly to weight gain and obesity, irrespective of the type of alcohol consumed (69, 70).

The association between BMI and HCC (incidence and mortality) has been demonstrated through two prospective population-based studies in Taiwan. The first of them was a prospective study that included 2,260 Taiwanese men positive for HBV infection, followed up for 14 years. HCC was diagnosed by imaging or histopathology (Cancer Registry). In this study, alcohol intake (any amount) had synergistic effects with the risk of incident HCC in analyses adjusted for age (HR, 3.41; 95% CI, 1.25–9.27; $p < 0.025$) and multiple variables (HR, 3.40; 95% CI, 1.24–9.34; $p < 0.025$). Also, the risk of HCC increased in

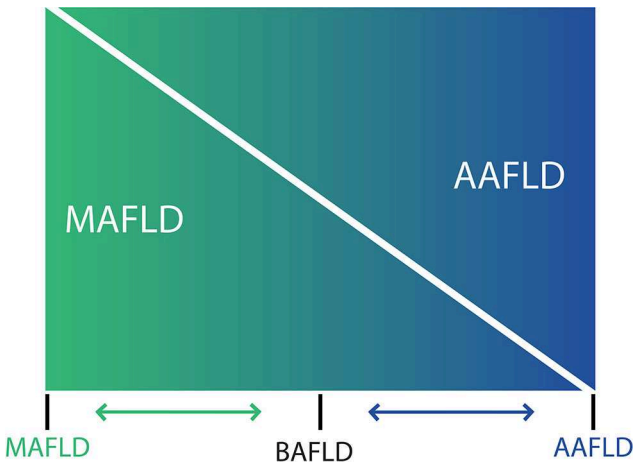

FIGURE 1 | Spectrum of fatty liver diseases. In non-alcoholic fatty liver disease (NAFLD) and alcohol-related liver disease have been generally conceived as different entities, and despite that both conditions share an overlapping pathophysiology and frequently coexist in clinical practice, thresholds to diagnose NAFLD makes difficult to account for a dual etiology in a given patient. For that reason, a nomenclature change has been proposed [see (71) in the main text] considering that at the ends of the spectrum of fatty liver disease, there are patients with true ALD (now named alcohol-associated fatty liver disease, AAFLD) and some with true NAFLD with alcohol consumption near-zero (now named metabolic associated fatty liver disease, MAFLD) but that the vast majority of patients are between these two extremes. Thus, in clinical practice there will be patients with ALD that have metabolic cofactors (AAFLD with MetS) and patients with NAFLD that consume alcohol, which contributes to the disease process (MAFLD with alcohol component). In the middle, a large group of patients have both conditions (NAFLD and ALD) with some showing an equal contribution of alcohol and metabolic factors (proposedly named as both alcohol and metabolic associated fatty liver disease, BAFLD).

overweight (HR, 2.4; 95% CI, 1.3–4.4), obese (HR, 2.0; 95% CI, 1.1–3.7), and extremely obese (HR, 2.9; 95% CI, 1.0–8.0) alcohol users (p for trend = 0.046) (62). Later, Loomba et al. conducted a prospective, population-based study of 23,712 Taiwanese, followed for 11.6 years for the incidence of HCC, and the study concluded with similar results. Alcohol consumption and obesity (BMI $\geq$30) showed a synergistic association with the risk of incident HCC in both unadjusted analyses (HR = 7.19, 95% CI: 3.69, 14.00; $p < 0.01$) and multivariable-adjusted analyses [age, sex, smoking, serum alanine aminotransferase (ALT), serum hepatitis B surface antigen, anti-hepatitis C virus antibody, and T2DM] (HR = 3.82, 95% CI: 1.94, 7.52; $P < 0.01$). Finally, the study concluded that obesity and alcohol have a synergic effect increasing the risk of incident HCC (63).

NEW NAMES FOR NAFLD/ALD: A CHANGING NOMENCLATURE FOR FATTY LIVER DISORDERS

The use of the acronym NAFLD as an umbrella term is now recognized as a problematic issue in the field of hepatology (31). This is largely due to the significant heterogeneity of patients grouped under that denomination as well as by the absence of positive criteria for NAFLD, which makes it difficult to classify subjects with metabolic alterations drinking beyond the threshold set for NAFLD. The latter also impedes the recognition of dual etiology for liver disease in individuals with both moderate or excessive alcohol consumption and metabolic disturbances. For this reason, and in order to better characterize the disease, NAFLD nomenclature has recently been revised and a new consensus-driven acronym proposed (71). Thus, the term MAFLD, which stands for metabolic (dysfunction)-associated fatty liver disease, was suggested as a more appropriate overarching term. This revised nomenclature should allow for more precise study designs leading to decreased variability of study groups and to a better understanding of the natural history of the disease. Positive criteria to diagnose MAFLD have been also proposed by the same expert group (72), which considers the evidence of fat accumulation in the liver and presence of evidence of metabolic dysregulation. The latter is defined by the presence of at least two metabolic risk abnormalities (dyslipidemia, hypertension, abdominal obesity, prediabetes, IR, or elevated high sensitivity C-reactive protein). Since ALD also comprises a spectrum of liver lesions, some adaptations may be needed in order to acknowledge the dual etiology of patients with fatty liver disease (i.e., concomitant MAFLD and ALD). Thus, the spectrum of fatty liver disease (**Figure 1**) should include patients with true ALD (alcohol-associated fatty liver disease, AAFLD), patients with predominant ALD but with metabolic cofactors (ALD with MetS), those with true NAFLD with alcohol consumption near zero (MAFLD) (71), and patients with NAFLD but with alcohol consumption contributing to the disease process (i.e., MAFLD with alcohol component). A final group will be composed of those patients with both MAFLD and ALD, equally contributing (or not possible to determine which one predominates) to the disease process (both alcohol and metabolic associated fatty liver disease, BAFLD). Similarly, the term BASH has been used to describe both alcohol and metabolic associated steatohepatitis (73). Each group probably has different clinical manifestations, course, liver prognosis, and mortality. This approach recently suggested by Eslam et al. (31) proposes that patients with fatty liver and predominance of metabolic dysfunction could be stratified according to alcohol intake and patients with alcohol predominant fatty liver according to the presence of coexisting metabolic comorbidities when included in clinical studies. These distinctions may help to a more robust understanding of the natural history of these different patient populations. In **Figure 2**, we suggest an algorithm to be applied in patients with fatty liver, which intend to account for dual etiology and predominance in the setting of metabolic dysfunction and alcohol consumption.

PATHOGENESIS OF NAFLD AND ALD: OVERLAPPING ASPECTS AND SALIENT DIFFERENCES

Although NAFLD and ALD are two distinct biological entities, they have a number of commonalities in their pathogenetic mechanisms leading to activation of both hepatic inflammatory

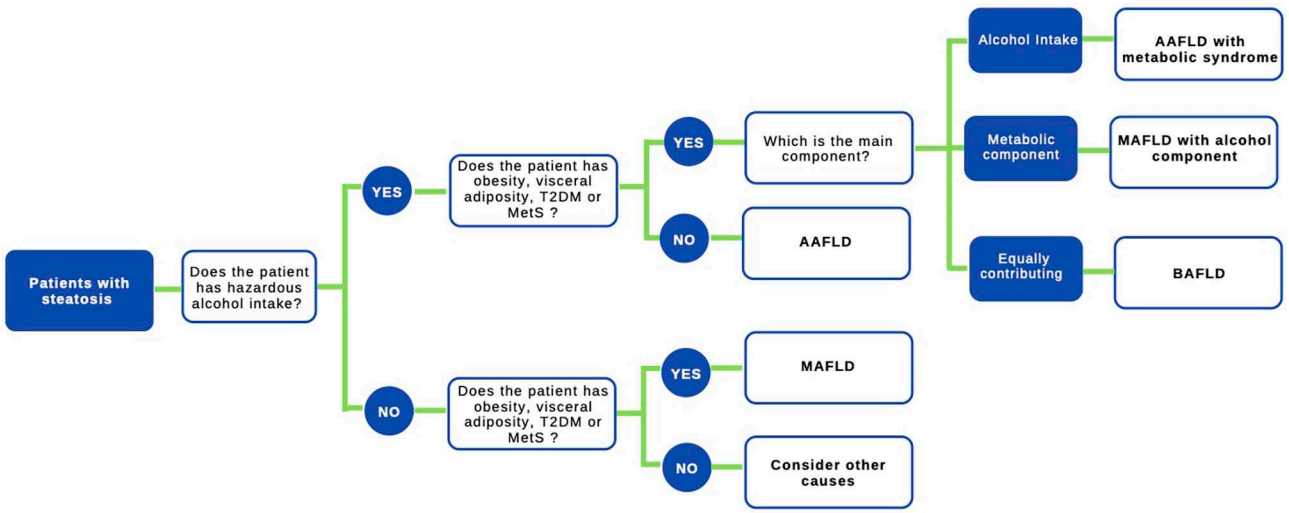

FIGURE 2 | Proposed algorithm to approach patients with liver steatosis. A staggered algorithm to approach patients with liver steatosis is shown. This algorithm considers alcohol intake and metabolic cofactors (obesity, T2DM, and MetS) and classifies patients in AAFLD alcohol-associated fatty liver disease (true ALD) if hazardous alcohol intake is present, AAFLD with metabolic component (predominant ALD but with metabolic cofactors), MAFLD metabolic associated fatty liver disease (NAFLD with alcohol consumption near zero), MAFLD with alcohol component (NAFLD but with alcohol consumption contributing to the disease process), and finally BAFLD both alcohol and metabolic associated fatty liver disease (patients with both NAFLD and ALD equally contributing or no possible to determine which predominates). Criteria to diagnose MAFLD are suggested in (72) in the main text.

and fibrogenetic pathways that fuel disease progression (74, 75) (**Figure 3**). Disturbed lipid handling by the hepatocyte resulting in intracellular accumulation of potentially toxic bioactive lipid species is an essential phenomenon in both NAFLD and ALD followed by the occurrence of cellular stress [i.e., endoplasmic reticulum (ER) stress and mitochondrial dysfunction] and death, which in turn triggers the innate immune response and activation of hepatic stellate cells (HSCs), resulting in inflammation and excessive collagen production and deposition (76). This general sequence is highly heterogeneous in both entities and is modulated by different genetic and epigenetic factors, some of which are also common for NAFLD and ALD. This accounts for the broad phenotypic spectrum seen in both diseases. In the following paragraphs, we summarize the main mechanisms of liver injury at play in NAFLD and ALD and underscore their similarities and differences (77–80).

Hepatic Fat Accumulation and Lipotoxicity

Increased lipid droplets (i.e., steatosis) inside the hepatocytes is the earliest histological finding in NAFLD and ALD (78). These lipid droplets are enriched in fully saturated triglycerides and result in the typical histological pattern of macrovesicular steatosis common to both entities (7). Excessive accumulation of triglycerides and other lipid species relates to a dysregulated hepatic lipid flux consisting in an increased hepatocellular lipid uptake, synthesis, and degradation [i.e., fatty acid oxidation (FAO)], induced by a positive caloric balance and IR in NAFLD and ethanol consumption in ALD (81). The main regulators of this process are SREBP1c (82) and PPARα and PPARδ, which are critical regulators of FAO (83). In NAFLD, excessive calorie intake increases the size and number of adipocytes and renders them insulin resistant, leading to uncontrolled lipolysis and decreased fatty acid uptake, thus promoting the release of free fatty acids (FFA) into the circulation. FFA are later uptaken by hepatocytes promoting lipid droplet formation. Of note, some data suggest that fatty acid transporters are upregulated in the setting of NAFLD (84). Also, *de novo* hepatic lipogenesis (DNL) seems to be upregulated in most subjects with NAFLD, which relates to inactivation of peroxisome proliferator-activated receptor-alpha (PGC1-α) and upregulation of SREBP1c (82, 85–87). Interestingly, DNL may be accompanied by a compensatory enhancement of FAO, but studies in this regard are conflicting. Human data from patients with NAFLD show that FAO may be enhanced, unchanged or decreased (84). In ALD, ethanol consumption induces a multilevel disturbance of hepatic lipid metabolism (88). One of the best-studied effects, in murine models, is the increase in SREBP1c expression (a key transcription factor in hepatic lipogenesis) (85), leading to increased expression of hepatic lipogenic genes and increased DNL. Other important enzymes controlling lipid fluxes such as acetyl-CoA carboxylase, ACC (limiting enzyme in DNL), and carnitine palmitoyltransferase, CPT (limiting enzyme for mitochondrial β-oxidation, that regulates lipid degradation), have a pivotal role in both diseases in human studies (88, 89). Ethanol increases the activity of ACC and suppresses the rate of palmitic acid oxidation, producing modifications in fatty acid metabolism and steatosis (90). Additionally, an increased ACC expression has been described in experimental murine models of NAFLD (91). Accumulation of saturated fatty acids as well as other harmful lipids such as ceramides, diacylglycerols, and lysophosphatidylcholine, among others, promote the occurrence of lipotoxicity, a phenomenon defined

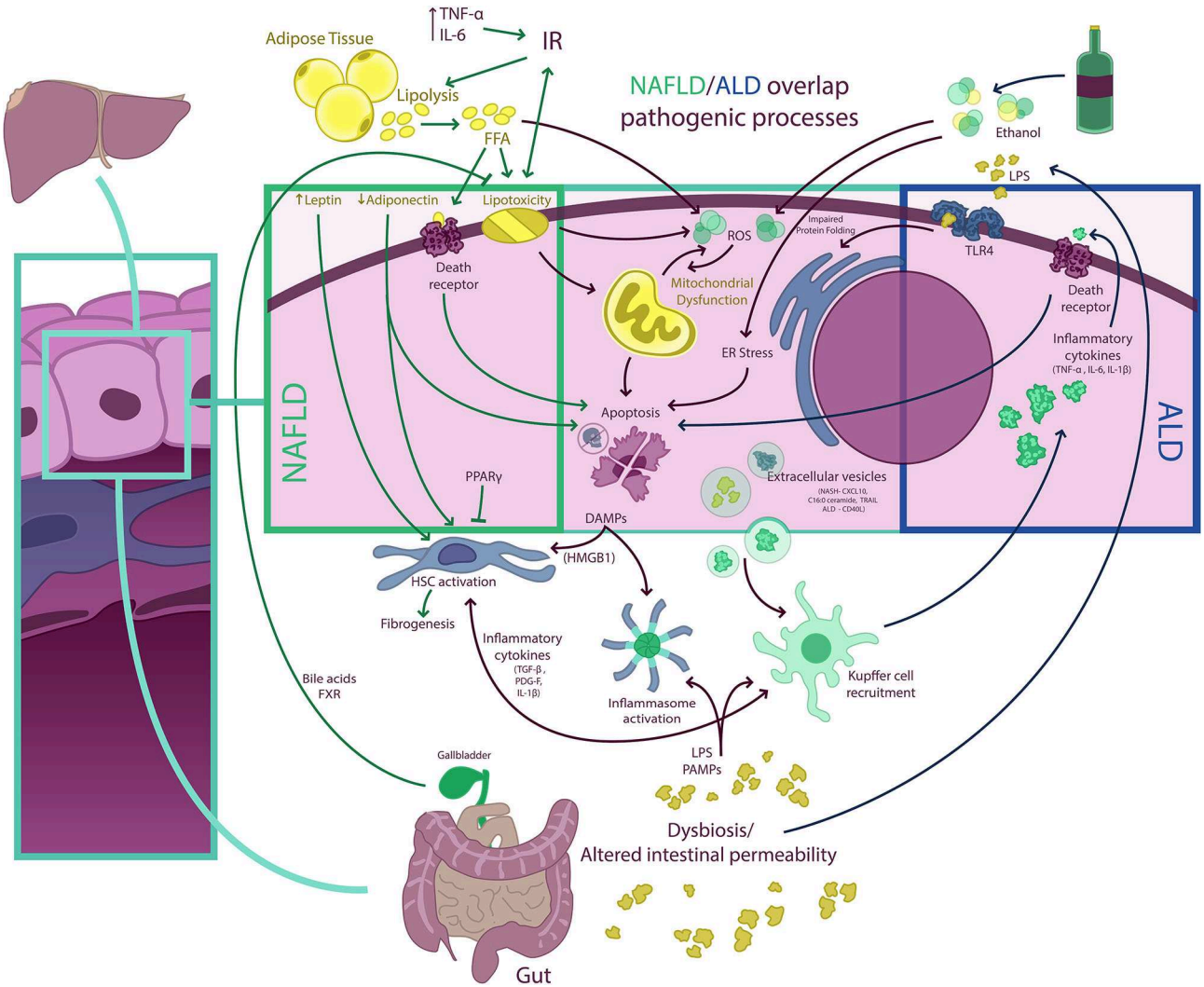

FIGURE 3 | NAFLD/ALD overlapping pathogenic processes. Free fatty acids (FFA) and ethanol have a myriad of effects on hepatocytes determining, among other phenomena, the occurrence of mitochondrial dysfunction, endoplasmic reticulum (ER) stress (resulting in impaired protein folding), and excessive production of ROS, which result in hepatocellular injury, activation of other cell death pathways, and inflammasome activation. Damaged hepatocytes release damage-associated molecular pattern (DAMP) molecules [e.g., high-mobility group box 1 (HMGB1)] that signal to Kupffer and hepatic stellate cells (HSC), fueling inflammation and fibrogenesis. Extracellular vesicle (EV) release from hepatocytes also contributes to both Kupffer cell and HSC activation. In non-alcoholic fatty liver disease (NAFLD), insulin resistance (IR) is a central phenomenon promoting adipose tissue lipolysis and an increased FFA flux to the liver. This FFA overflow surpasses the storage capacity of hepatocytes and determines the occurrence of lipotoxicity and activation of cell death pathways. Also, adipose tissue dysfunction is associated with a proinflammatory state with elevated levels of circulating cytokines [e.g., tumor necrosis factor-α (TNF-α) and interleukin (IL)-6] and with an imbalance in circulating levels of adipose tissue-derived adipokines (i.e., a decrease in adiponectin and an increase in leptin). This may increase IR and contribute to HSC activation. Of note, some nuclear receptors such as peroxisome proliferator-activated receptors (PPARs) and farnesoid X receptor (FXR), a bile acid receptor, may have anti-inflammatory and anti-steatotic effects, which are being exploited therapeutically. Both NAFLD and ALD (alcohol-related liver disease) are associated with intestinal dysbiosis and altered gut permeability, which results in the pass of bacterial products [e.g., pathogen-associated molecular patterns (PAMPs), lipopolysaccharides (LPS), and others] into portal circulation. In the liver, LPS and PAMPS may activate different Toll-like receptors (TLRs) as well as promote inflammasome assembly determining amplification of inflammatory responses. In ALD, proinflammatory cytokines released from macrophages may also activate cell death receptors and induce apoptosis.

by the appearance of leading to cellular dysfunction and death (92). Lipotoxicity plays an important pathophysiological role in NAFLD/NASH (93) and likely drives disease progression through different mechanisms such as direct cytotoxicity, increased IR and hyperinsulinemia, cell signaling modification [through hepatic nuclear factor-α or toll-like receptors (TLR)], ER stress, upregulation of autophagic processes, and trigger of different cell death pathways (e.g., apoptosis, necrosis, pyroptosis; see below). Cell injury and death, in murine and human models, determine release of damage-associated molecular pattern (DAMP) molecules, leading to macrophage recruitment and a secondary inflammatory response (94). In ALD, although accumulation of FFA occurs similarly to NAFLD, the lipotoxic phenomenon has not been well characterized and information on

the nature of intrahepatic lipid species and their cellular effects is scarce (95).

Insulin Resistance

IR plays a pivotal role in the pathogenesis of NAFLD. IR in adipose tissue is associated with an increase lipolysis in adipocytes, leading to excess FFA release into the circulation and to a higher uptake by the hepatocytes, which is a driver of steatosis. In turn, increased hepatic fat content promotes IR in hepatocytes by decreasing insulin-stimulated tyrosine phosphorylation of both insulin receptor substrate (IRS)-1 and IRS-2, which leads to increased gluconeogenesis and hepatic glucose production, which leads to hyperinsulinemia. The latter stimulates the transcription factor SREBP-1c, activating most genes involved in DNL, further increasing steatosis (96). Worsening IR is considered a potential driver of disease progression in NAFLD (97), and targeting IR is one of therapeutic strategies explored for NAFLD/NASH treatment according to evidence from *in vitro* and mouse models (98). With regard to the relationship between alcohol consumption and IR, data are limited. Both clinical and experimental information suggests that insulin signaling is impaired in ALD. Chronic alcohol consumption disrupts whole-body lipid metabolism, and several studies, in clinical and preclinical mouse models, suggest that alcohol may promote IR and increase the risk of T2DM (99). Indeed, it is likely that the presence of IR, which is prevalent in patients with ALD, may increase the risk of advanced liver disease through multiple mechanisms. However, current data is limited, and more studies are needed to confirm if targeting insulin signaling pathways pharmacologically may be beneficial in the setting of ALD.

Cell Death Signaling

Another important process involved in the pathogenesis of NAFLD/ALD is the activation of cell death pathways (100–102). This pathway can be activated by both intrinsic and extrinsic signaling via surface receptors belonging to the tumor necrosis factor-alpha (TNF-α) receptor family (103–105). Specifically, TRAIL (TNF-related apoptosis-inducing ligand) receptor 2 (TRAIL-R2) has been associated with hepatocyte lipoapoptosis, probably as an effect of toxic lipids (free cholesterol, ceramides, and FFA), that promote reorganization of plasma membrane domains and ligand-independent activation of TRAIL-R2 signaling leading to cell death (106–109). In addition to apoptosis, other lytic forms of cell death may be at play in NAFLD/NASH, such as necroptosis, pyroptosis, and ferroptosis, which are related to cell-membrane permeabilization (101). The TRAIL pathway has been also implicated in ALD pathogenesis. After ethanol consumption, TRAIL expression leads to hepatic steatosis and TRAIL-mediated steatosis that can be inhibited by the neutralizing TRAIL antibody (110). Also, ER stress, induced by alcohol consumption and lipotoxicity, and reactive oxygen species may activate Bcl2 initiators of apoptosis members (108) and inhibit guardian members, inducing cell deaths, through caspase activation in mouse models (111). Finally, activation of apoptosis signal-regulating kinase 1 (ASK-1), which leads to phosphorylation of p38 and JNK and activation of several stress response pathways, has been involved in apoptosis occurrence in both NASH and alcoholic hepatitis. This is of particular interest since inhibitors of this pathway are available (i.e., selonsertib). However, studies conducted to date have yielded negative results (112).

Other cell death pathways that have been studied in liver diseases is necroptosis, which is a form of programmed necrosis. Necroptosis is defined as a non-apoptotic cell-death-receptor-mediated death observed in some cell types and is dependent of a kinase cascade that involves a family of proteins known as receptor interaction protein kinases (RIPKs), in particular RIPK3 activation of mixed-lineage-kinase-domain-like (MLKL) (113). This pathway has been explored in mouse models of NAFLD and ALD with different results (113–116). Of note, RIPK3-dependent and RIPK1-independent activity activation has been described in ALD, while in NASH models studies are contradictory (116–118). Currently, new studies assessing the role of necroptosis in fatty liver are underway (119).

Autophagy, or cellular self-digestion, is a cellular pathway crucial for development, differentiation, homeostasis, and survival of cells. This process is used to eliminate potentially harmful proteins and organelles and to remove intracellular microbial pathogens. In ALD and NAFLD, a dysregulation of this process with a decreased autophagic function that can lead to liver cell death, steatohepatitis, and HCC exist. In the setting of NASH, it has been demonstrated that palmitic acid suppresses autophagy, while oleic acid may promote it. Also, mice with genetic deletion of *Atg7* (a critical autophagy mediator) have shown increased hepatic fat content accumulation (120). The autophagic degradation of intracellular lipid droplets may play a role in buffering FFA toxicity and maintaining hepatic lipid homeostasis (120–123). In ALD, a normal functioning of autophagy is associated with attenuated alcohol-induced injury and less lipotoxicity (122).

Immune Response

Innate immune cells have a fundamental role in the pathogenesis of both NAFLD and ALD, sharing important characteristics but with some substantial differences (124, 125). Release of DAMPs from hepatocytes activate innate immune cells, particularly resident macrophages (i.e., Kupffer cells) (123) in *in vitro* studies using cultured HepG2 cells and primary mouse hepatocytes. Additionally, some specific DAMPs such as high-mobility-group protein box 1 (HMGB1) have shown to activate TLR4 in NASH and ASH, playing a pivotal role during the early progression of NAFLD (126, 127). Other DAMPs, for example, sonic hedgehog (SHH) ligands, have been also associated with progression of NAFLD and fibrosis in human studies (128).

Neutrophils are another key element in ASH and NASH. Neutrophil elastase (NE), a protease released by neutrophils, produces cellular IR, and the deletion of NE produces less tissue inflammation and is associated, in mouse models, with lower adipose tissue neutrophil and macrophage content (129). In ALD, neutrophils induce progression through the release of ROS, proteases, and proinflammatory mediators (130). Additionally, neutrophils have been associated with portal hypertension as these immune cells promote the formation of microvascular

thrombosis, through neutrophil extracellular traps (NETs). Occurrence of microvascular thrombosis and fibrin may drive portal hypertension through space effects in liver sinusoids (131).

Finally, the monocyte chemoattractant protein-1 (132) is another important component in ASH and NASH. The monocyte chemotactic protein-1 (MCP-1) is an inflammatory chemokine released by hepatocytes, Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells in response to alcohol, producing chemoattraction of macrophages and maintaining mononuclear infiltration in mouse models (133–136).

Inflammasome

Inflammasomes are multiprotein complexes that are mainly expressed in hepatocytes and myeloid cells (i.e., Kupffer cells) (137) that sense pro-inflammatory signals through NOD-like receptors (NLRs) and activate caspase-1, the effector protein (138–141). Caspase-1 cleaves pro-interleukins (IL-1β, IL-18, and IL-23), which results in sterile inflammation and lytic hepatocyte cell death (i.e., pyroptosis). Activation of the NLRP3 inflammasome has been found in murine models of both NAFLD and ALD (142–144). Also in rodent models of NAFLD/NASH, mRNAs encoding the NLRP3 inflammasome complex are elevated and overexpression of NLRP3 is associated with greater degrees of liver injury (145–147). Despite the similarities, the cell types involved and the trigger signals for NLRP3 activation appear to be somewhat different between ALD and NAFLD (148). In ALD, inflammasome and IL-1 production is increased at very early stages of the disease, which seems not to occur early in NASH (147). Also, in ALD, inflammasome activation is predominantly seen in Kupffer cells, while in NASH NLRP3 is mainly activated by hepatocytes (149–151). Finally, inflammasome component deficiency, in mouse models, protects against inflammation, steatosis, and liver injury in both ALD and NASH (152–154).

Extracellular Vesicles and MicroRNAs

In response to injury, damaged cells release extracellular vesicles (EVs) which are membrane-surrounded structures (66) released by almost all types of cells. EVs can contain a wide variety of cargoes [e.g., proteins, lipids, and nucleic acids [coding and non-coding RNA] and mitochondrial DNA] that mediate intercellular communication. In the setting of liver damage, hepatocytes increase EV release, which may act on different target cells leading to pivotal pathobiological processes, such as activation of macrophages, endothelial cells, and HSCs, thus promoting proinflammatory, angiogenic, and fibrotic responses (155–158). Observations made in mouse models suggest that these EV-mediated processes are relevant events in the pathogenesis of both NAFLD and ALD (159–161). Moreover, EVs are promising candidates to serve as disease biomarkers. Also, their therapeutic use as a liver-specific delivery method of different compounds is being studied (155). For an in-depth discussion of current knowledge about the role of EVs in NAFLD and ALD, the reader is referred to a recent review (155).

Changes in microRNA (miR) expression are involved in pathogenesis ALD and NAFLD. In NASH, it has been shown that an upregulation of miR-34a and a downregulation of let7d (miR precursor) decrease FAO, promoting fat synthesis in murine models (83, 162, 163), while miR-122 has been linked with steatosis and fibrosis (164). On the other hand, miR-132 may trigger fibrogenesis secondary to ethanol intake, and miR-155 is associated with ethanol-induced inflammation, probably mediated by TNF-α (165). In mouse models of NASH, miR-155 is also induced without a defined role (166, 167).

Microbiota

Another interesting factor in the pathogenesis of ALD and NAFLD is the effect of the intestinal microbiota. The number of microorganisms inhabiting the gastrointestinal tract has been estimated to exceed 10^{14}, with extremely diverse features (168, 169). Dysbiosis has been described in both ALD and NAFLD; however, the exact role in the NAFLD and ALD disease processes remains unclear (170). The intestinal microbiota composition is associated with the stage of fibrosis and also on the ethanol consumption pattern, being different between chronic, binge, and "social" drinkers (132, 171, 172). Chronic alcohol consumption disrupts tight-junction proteins and increases intestinal permeability, resulting in increased translocation of endotoxins (lipopolysaccharides) and bacterial DNA into the portal circulation, which increases even more by the overgrowth of gram-negative bacteria. This process activates Kupffer cells through activation of TLRs (TLR4 and TLR9) (173, 174), which may also contribute to steatosis and hepatic fibrosis via stimulation of TLR9-dependent profibrotic pathways in mouse models (152, 175). The peptidoglycan and flagellin, other bacteria-derived toxins, also have an impact on TLR signaling producing proinflammatory cytokines (176). Changes in microbiota have been described in NAFLD (mainly decreased *Bacteroidetes* and increased *Prevotella* and *Porphyromonas* species) (177). This dysbiosis may be an important factor in causing NASH, in mouse models and human, through different mechanisms like deregulating energy homeostasis, modulation of choline and bile acid metabolism, and generation of bacteria-derived toxins, such as lipopolysaccharide (LPS), and increased hepatic TNF-a expression (through TLR4 and TLR9-dependent profibrotic pathways) in hepatic Kupffer cells (178–181). Therefore, microbiota plays an important role in ALD and NAFLD, but with some differences. The TLR4 can activate two distinct pathways: one pathway is MyD88-dependent (producing activation of NF-κB and proinflammatory cytokines), and the other pathway is MyD88-independent (inducing type I IFNs and NF-κB) (151). MyD88-dependent signaling seems to have a relevant role in NAFLD, but not in ALD (*in vitro* and *in vivo* in murine models) (152, 182, 183). Furthermore, a role may have the adipocytokines that can inhibit MyD88-dependent pathways in macrophages (184).

Bile Acids and Nuclear Receptors

Nuclear receptors (NR) are ligand-activated transcription factors that have a key role in regulating lipid homeostasis and inflammation in the NAFLD/NASH process. The NRs act as receptors for fatty acids, cholesterol, oxysterols, and xenobiotics and regulate the cell metabolism, cell differentiation, and cellular homeostasis. The principal NR studied in NAFLD/NASH are

liver X receptor (LXR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor-gamma (PPARγ), Farnesoid X receptor (FXR), and HNF4α, hepatocyte nuclear factor 4α (185–189). PPARα has been studied in NAFLD and ALD. PPARα induces FAO in the mitochondria, thus decreasing steatosis. PPARγ ligands can inhibit inflammatory responses by decreasing IL-6, TNF-α, and IL-1β secretion and iNOS production in macrophages and Kupffer cells (187, 188, 190). Polymorphism in the PPARγ gene is associated with the susceptibility to NAFLD. LXR and RXRα also play a role in ALD due to their actions in lipid homeostasis and inflammation, with a particular role of RXRα in alcohol detoxification. HNF4α is constitutively active through the binding of integral fatty acids. HNF4α has an important role in the maintenance of hepatocyte differentiation and the regulation of bile acid and lipid homeostasis genes, mainly in mouse models (191–193).

Bile acids (BA) are not only detergents that stimulate hepatic bile flow and biliary excretion and aid in the digestion and absorption of fats from the intestinal lumen but also relevant signaling molecules that act on hepatic and extrahepatic tissues to regulate lipid and carbohydrate metabolic pathways (77). FXR is highly expressed in the liver, small intestinal mucosa, and kidneys, with effects on glucose and lipid metabolism; acts as a sensor for BA; and regulates the BA synthesis, protecting hepatocytes from the toxic effect of BA and reducing the triglyceride levels (194). FXR has anti-inflammatory and anti-steatotic effects, promoting FAO through upregulation of PPARα and repressing lipogenesis (by the modulation of SREBP-1c expression). Activation of FXR in the ileal enterocytes after active intestinal BA uptake also has important metabolic implications via FXR-stimulated local production of FGF15 (FGF19 in humans) (195). In hepatocytes, FGF15/19 is a major regulator of BA synthesis, through FGF receptor 4 (FGFR4) (196), and also decreases hepatic lipogenesis and indirectly stimulates mitochondrial FAO, in mouse models (196, 197). FXR also has a beneficial role in glucose metabolism, and it is important in vascular remodeling (198–200). Experimental models with FXR-null mice fed a high-cholesterol/high-fat diet develop massive steatosis (201) and exhibit decreased insulin sensitivity. Conversely, treatment with the selective, non-steroidal FXR agonist GW4064 improved IR and glucose homeostasis in obese *ob/ob* and diabetic *db/db* mice (202). Many other specific BA-activated receptors, including members of the nuclear receptor superfamily (FXR, NR1H4), a vitamin D receptor (NR1I1), PXR (NR1I2), members of the G protein–coupled receptor superfamily (TGR5 and sphingosine 1 receptor 2), and transporters such as ileal apical sodium-dependent bile acid transporter (ASBT), have a role in insulin sensitivity and NAFLD pathogenesis and are a target for novel therapies.

CONCLUSIONS

NAFLD and ALD share a number of features and often coexist. Alcohol consumption is often a confounding factor in patients with NAFLD due to inaccurate reporting of the magnitude of alcohol intake and the ill-defined impact of alcohol consumption, even within the arbitrary thresholds considered to diagnose NAFLD, on liver disease progression in these patients. Although initially some studies suggested protective effects in moderate doses, current evidence shows that there is no safe threshold for alcohol consumption in the setting of NAFLD. On the other hand, the presence of MetS and obesity increases the progression of ALD as well the incidence of HCC and mortality. Considering the high prevalence of obesity and MetS and the changing patterns of alcohol consumption worldwide, which may impact the incidence of advanced liver disease, it is necessary to better define both diseases, acknowledge the presence of a dual etiology of liver disease in a group of patients, and develop a multidisciplinary approach focused on preventive measures.

AUTHOR CONTRIBUTIONS

FI, AK, and OM contributed to the review concept and drafting of the manuscript. MA and JA contributed to the review concept, critical revision of the manuscript for important intellectual content, supervision, and final version approval. All authors contributed to the article and approved the submitted version.

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REVIEW
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miRNA Dysregulation in the Development of Non-Alcoholic Fatty Liver Disease and the Related Disorders Type 2 Diabetes Mellitus and Cardiovascular Disease

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According to the World Health Organization, the continuing surge in obesity pandemic creates a substantial increase in incidences of metabolic disorders, such as non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus, and cardiovascular disease. MicroRNAs (miRNAs) belong to an evolutionarily conserved class of short (20–22 nucleotides in length) and single-stranded non-coding RNAs. In mammals, miRNAs function as critical post-transcriptional negative regulators involved not only in many biological processes but also in the development of many diseases such as NAFLD and comorbidities. More recently, it has been described that cells can secrete miRNAs in extracellular vesicles, transported by body fluids, and uptaken by other tissues regulating gene expression. Therefore, this could be a mechanism of signaling involved not only in physiological pathways but also in the development of diseases. The association of some miRNA expression profiles with certain disorders has made them very interesting molecules for diagnosis, prognosis, and disease management. The finding of specific miRNA signatures to diagnose NAFLD and related diseases could anticipate the risk of development of related complications and, actually, it is the driving force of present health strategies worldwide. In this review, we have included latest advances in knowledge about the miRNAs involved in the development of NAFLD and related diseases and examined how this knowledge could be used to identify new non-invasive biomarkers and new pharmacological interventions.

Keywords: non-alcoholic fatty liver disease, microRNAs, type 2 diabetes mellitus, cardiovascular disease, obesity

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INTRODUCTION

Nowadays, due to the high prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide, it is considered as the most common hepatic disorder in western countries, with a prevalence of 15–30% (1–4). This condition represents the hepatic manifestation of metabolic syndrome since it is strongly associated with obesity, insulin resistance (IR), hypertension, and dyslipidemia (1). More precisely, NAFLD is defined as the presence of steatosis in >5% of hepatocytes,

determined by liver histology, without any evidence of other factors related to the development of fatty liver, such as drug and alcohol abuse, viral hepatitis, or autoimmunity (5, 6).

Obesity and type 2 diabetes mellitus (T2DM) are common and well-established risk factors for NAFLD since increased body mass index (BMI) and visceral adiposity are critically involved in NAFLD development. Although it is true that NAFLD patients do not necessarily develop T2DM, the association between both diseases has been thoroughly described. This significant association has been attributed to IR, the main cause for lipid overflow to the liver (7, 8). Moreover, NAFLD is associated with increased mortality in relation to cardiovascular disease (CVD), neoplastic processes, and hepatic failure (9).

The earliest stage is characterized by benign steatosis (fatty liver) (9), which can progress to non-alcoholic steatohepatitis (NASH), a potentially severe condition from which 10–25% of patients finally develop cirrhosis. This stage is defined by lobular and portal inflammation, hepatocyte ballooning, and variable degrees of fibrosis, cirrhosis, and, ultimately, hepatocellular carcinoma (HCC) (7). Due to the high prevalence mentioned above, NASH is predicted to surpass hepatitis C as the leading cause of liver transplantation in the near future (2–4). Several factors influence the progression from steatosis to NASH, including lipotoxicity, oxidative stress, and activation of the immune system, although many others may be involved and remain unclear (10).

Bearing in mind all of these, identification of patients who might be at an increased risk of adverse outcomes is critical; therefore, the increase in knowledge of serum biomarkers could be of great interest in order to allow an early diagnosis. The treatment of NASH patients should be a priority, especially for those who develop concomitant fibrosis since they are prone to have adverse outcomes. In that sense, treatment goals depend on the severity of the disease due to the likelihood of progression and the comorbidities that each patient might have. Lifestyle-focused treatment strategies are essential regardless of the disease stage, keeping in mind that other complementary therapies could be beneficial according to the patient circumstances (10).

Even though certain genetic risk factors are linked to steatohepatitis and fibrosis degree, they are fairly infrequent. As a result, they are not currently suitable to identify appropriate individuals for therapy since they do not account for a large-enough proportion of the variability in the phenotype of the disease (11–13).

In the recent years, there has been great interest in the function and the usefulness of microRNAs (miRNAs) as clinical tools. The miRNAs are small (20–22 nucleotides), non-coding, highly conserved endogenous RNAs that regulate gene expression at the post-transcriptional level. It has been demonstrated that the expression of the majority of mammal genes is regulated by miRNAs; therefore, these molecules have crucial roles in numerous physiological processes such as cell growth, embryonic development, and apoptosis. It has been demonstrated that an altered expression of some miRNAs is enough to promote the development and the progression of pathophysiological processes including NAFLD and T2DM (14, 15). This review summarizes the data published regarding the role of miRNAs in the development of NAFLD and associated metabolic diseases such as T2DM and CVD, exploring their role in diagnosis and as potential targets for treatment. Therefore, further characterization of the mechanisms that regulate the expression and the function of these small non-coding RNA molecules is essential to develop new therapeutic strategies. Furthermore, not only do these miRNAs regulate gene expression in the tissues in which they are expressed but also they are secreted to the bloodstream in extracellular vesicles to regulate gene expression in different tissues. As it has been described that the dysregulation of some miRNA expression is involved in the development of NAFLD and the associated complications, much effort is essential to increase the knowledge regarding the underlying mechanisms, which would allow an increase in the therapeutic potential for this group of diseases.

BIOLOGY OF miRNAs

As mentioned before, miRNAs belong to an evolutionarily conserved class of short (20–22 nucleotides in length) and single-stranded non-coding RNAs. In mammals, miRNAs take part in gene expression regulation by its repression at the post-transcriptional level. Consequently, they are involved in a huge number of biological processes including cell growth, tissue differentiation, cell proliferation, embryonic development, and apoptosis (16, 17). Such regulation can be carried out owing to their ability to target the 3′ untranslated region of a gene mRNA, resulting in translational repression, mRNA degradation, or mRNA cleavage, based on the complementarity between the miRNA and its target (18). It is known that ∼1–4% of the protein-encoding loci in the human genome also encode one or more miRNAs and a single miRNA can regulate as many as 200 cognate mRNAs.

miRNAs are synthesized by RNA polymerase II in the nucleus and, immediately, they are cleaved into pre-miRNAs by the endonuclease Drosha. After processing, the Ran-GTPase Exportin-5 exports pre-miRNAs into the cytoplasm, allowing Dicer to process them into 20–22-nucleotide-long mature miRNAs. Then, miRNAs are loaded into Argonaute-2 of the RNA-induced silencing complex in order to direct the post-transcriptional repression of target mRNAs (16).

Aberrant miRNA expression can foster the onset and the progression of a range of pathophysiological processes (17). Indeed the literature has recently highlighted the role of circulating miRNAs as potential diagnostic and prognostic biomarkers (18). Several studies have evaluated their levels in different chronic diseases including chronic liver disease (19–26). For instance, patients with hepatitis B virus-related hepatocellular carcinoma and/or chronic type B hepatitis showed increased levels of serum miR-21, miR-122, and miR-223, pointing toward their potential use as diagnostic biomarkers for liver injury and potential target for treatment (27). Moreover, between all the miRNAs described in the literature, only miR-133a/b, miR-208a/b, and miR-499 were well-established as possible biomarkers. Recently, within liver damage diseases, other authors have highlighted the importance of miR-122,

miR-29a, and miR-34a as relevant biomarkers for the diagnosis of this spectrum of diseases (28–30).

The unexpected stability of miRNAs in the circulation points toward a signaling pathway in which miRNAs are selectively secreted by one cell and taken up by a distant target cell to regulate gene expression. This intriguing idea of circulating miRNAs regulating distant cell-to-cell communication has been intensively investigated. In this sense, a recent and elegant paper demonstrates that adipose-derived circulating miRNAs regulate gene expression in other tissues such as the liver (31). However, the mechanisms involved in these processes are poorly understood. Huge effort is needed in order to know the specific targets of miRNAs in cells and the molecular mechanisms playing a role in the development and the progression of NAFLD and related outcomes.

miRNAs INVOLVED IN NAFLD

Thanks to the efficient distribution and accumulation of exogenously administered small RNAs in the liver, it is likely that liver diseases will have miRNA-based therapies in the near future (32).

Many studies have described that aberrant miRNA expression is a main feature of liver diseases, including NAFLD, viral hepatitis, and HCC (**Figure 1** and **Table 1**). Moreover, it has been demonstrated that changes in miRNA expression are well-correlated with NAFLD progression (26, 33, 34). As mentioned before, circulating miRNAs are quite stable in body fluids, and therefore it would be reasonable to think that changes in the circulating miRNA pattern could represent a real-time signal of how NAFLD evolves, reflecting variations at the histological and the molecular levels (34). Hence, establishing the exact miRNA signature in NAFLD is fundamental to unravel the development mechanism and to enable the early diagnosis and severity evaluation of this disease.

A study explored the dynamics of miRNA expression during NAFLD progression in mice fed a high-fat diet (HFD) for a long term. miR-125a-5p and miR-182 were found to be altered in the early stages of NAFLD, and miR-340-5p, miR-484, miR-574-3p, and miR-720 were related to liver damage and tumor development. miRNA expression profiles were also different depending on the NAFLD stage, suggesting that its progression involves diverse miRNAs (35).

miR-122

miR-122 is the main miRNA in the liver and is involved in many biological processes. A mouse model of liver damage, by feeding it with a methyl-deficient diet, showed a decreased expression of miR-122 (36). The same was found in NASH patients but not in normal subjects or simple steatosis patients (29). In addition, mice with *Mir122a* deletion induced steatosis that led to NASH, fibrosis, and HCC, suggesting an essential role of this miRNA in NAFLD initiation and progression (36). In that report, the authors showed that miR-122 is essential in processes such as fatty acid, triglyceride, and cholesterol metabolism and also in terminal differentiation of hepatocytes by regulating its targets, including *FASN, ACC, SCD1*, and *SREBPs*, among others (36–40). miR-122 knockout mice showed decreased lower serum cholesterol and triglyceride levels but increased cholesterol and triglyceride content within the liver. The alterations in very-low-density lipoprotein assembly and secretion are responsible for changes in cholesterol and triglyceride levels in the serum and the liver in a miR-122-dependent manner (36). However, another study found that inhibiting miR-122 with antisense oligonucleotides in HFD and normal-diet mice decreased the expression of lipogenic genes and increased hepatic fatty acid oxidation, ameliorating hepatocyte steatosis (40). This controversy has been observed in several studies and could be due to the use of distinct models and inhibition approaches. It is of great necessity to further study deeper on how miR-122-dependent and miR-122-independent pathways can regulate hepatic lipid metabolism *in vivo*.

Moreover, miR-122 could also be important in NAFLD diagnosis since it has been reported that miR-122, along with miR-17 and miR-20a/b, plasma levels were increased in T2DM patients with NAFLD compared to those diabetic patients without NAFLD complication (39).

In addition, miR-122 plays a role in the fibrogenic and the carcinogenic signaling pathways of NAFLD. Reduced miR-122 expression resulted in the activation of MEKK-3, vimentin, and hypoxia-inducible factor-1α, factors involved in epithelial to mesenchymal transition, a fundamental process related to chronic inflammation, fibrosis, and metastasis (41). Furthermore, it has also been described that the loss of miR-122 fosters liver fibrosis and promotes the activation of oncogenic miRNAs (37). All these results demonstrate that miR-122 could be considered as an important biomarker in NAFLD diagnosis and staging.

miR-335

A different work reported that, in mice, miR-335 could be a biomarker for steatosis since higher levels of this miRNA were well-correlated with hepatic steatosis in genetically obese mice (*ob/ob* and *db/db*) (42). Although the quantity and the quality of reports regarding the role of miRNAs in NAFLD are increasing every week, much effort is needed in order to clarify the multifaceted roles of these molecules.

miR-34a

Another miRNA that seems to be important in NAFLD development is miR-34a. For instance, one study performed in 34 patients showed that this miRNA was only detected in the serum of NAFLD/NASH patients, but not in healthy subjects, which has been also supported by Liu et al. (43, 44). Indeed this aberrant increase negatively impacts the signaling of the fibroblast growth factors 19 and 21, and it is positively correlated with BMI in obese patients (45, 46). Moreover, miR-34a expression is triggered by lipids, and as sirtuin 1 has been identified as one of its targets, this miRNA seems to have a role in aggravating the manifestations of NAFLD and NASH, mainly through hepatocyte apoptosis induction by increasing p53 acetylation (47, 48). This evidence

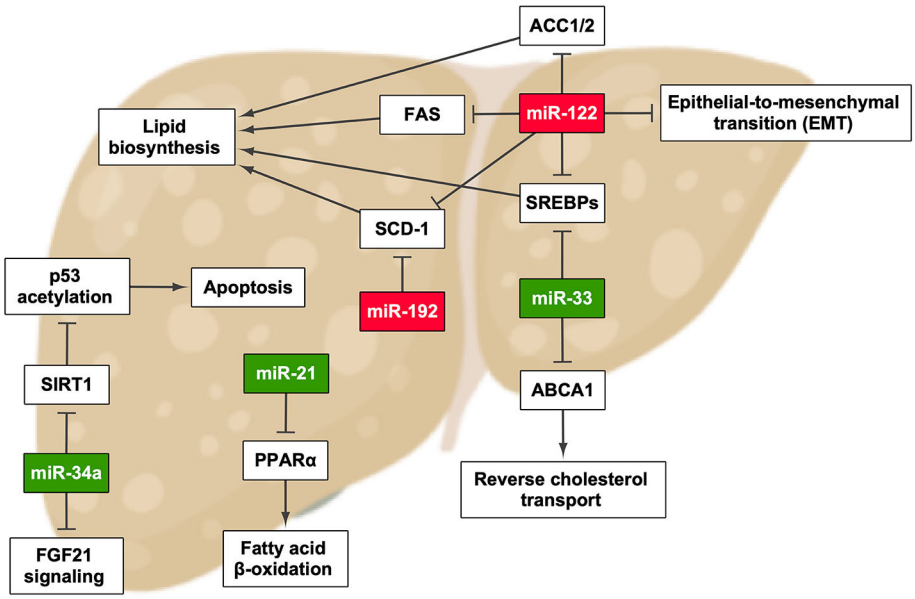

FIGURE 1 | miRNAs that have been described to promote the development and/or progression of non-alcoholic fatty liver disease and their effect in pathways involved in this process. Green highlights indicate the upregulation of miRNA, whereas red highlights indicate its downregulation. ACC1/2, acetyl-CoA carboxylase 1/2; FAS, fatty acid synthase. Created with BioRender.com.

suggests that miR-34a silencing might be supposed as a new therapeutic strategy for NAFLD treatment.

miR-192

Among other miRNAs involved in NAFLD, miR-192 stands out due to its implication in lipid synthesis through targeting stearoyl-CoA desaturase 1; therefore, its upregulation would be an approach to treat the disease (49, 50). It has been also described that, like miR-122, miR-192 expression is lower in the liver of NASH patients in contrast with NAFLD patients (29, 44, 51).

miR-21

Becker et al. assessed the serum profiles of two cohorts consisting of 137 NAFLD/NASH patients and 61 healthy controls, and the results showed an increase in circulating miR-21 levels in patients suffering from NASH compared to healthy controls and NAFLD patients (52). The authors mentioned that it might be due to increased fibrosis in NASH patients; thus, miR-21 plays a pathogenic role by targeting peroxisome proliferator-activated receptor alpha, an important factor for the progression of the disease (53, 54).

miR-33

The NAFLD patients also show upregulated miR-33 expression in the liver (55), and it has been found to be a regulator of lipid metabolism and transport and insulin signaling pathways, its main targets being sterol regulatory element-binding proteins (SREBPs) and ATP binding cassette subfamily A member 1 (56–58). Moreover, it has been demonstrated that miR-33 inhibition attenuates atherosclerosis progression (57, 58).

miRNAs INVOLVED IN TYPE 2 DIABETES MELLITUS

As has been mentioned above, in the same way that the prevalence of NAFLD in obese patients can exceed 90% (7, 10, 59), patients that suffer from T2DM are at a high risk of developing NAFLD. Classically, high plasma glucose levels are present, owing to metabolic dysregulations that lead to impaired fasting glucose, a pre-diabetic state that might progress to T2DM, characterized by IR and reduced insulin secretion from pancreatic β cells (60). Blood glucose levels exert a real-time regulatory effect on many pancreatic β cells genes, allowing accurate insulin expression and secretion (61). The physiological release, the transcription and mRNA stability, translation, and processing of insulin are all regulated by glucose concentrations in β cells. Moreover, the relative quantity of miRNA transcripts is responsive to variable glucose concentrations (62). The exposure to long-term high-glucose conditions significantly modifies the expression of a large number of miRNAs in a cultured pancreatic β cell line (MIN6). The expression of miR-124a, miR-107, and miR-30d increased, whereas miR-296, miR-484, and miR-690 expression descended under prolonged high glucose conditions. However, many other miRNAs have been involved in several subcellular events essential for glucose-stimulated insulin secretion (GSIS) (63). In this review, we have included the most relevant miRNAs involved in T2DM (**Figure 2** and **Table 1**).

miR-375

One of the first miRNAs specific of β cells was miR-375, a highly expressed miRNA in pancreatic islets in humans and mice involved in insulin secretion and glucose homeostasis. This

TABLE 1 | Summary of miRNAs aberrantly expressed in non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM), and cardiovascular disease (CVD).

miRNA	Expression changes	Organism	Sample	References
NAFLD				
miR-21	↑	Human	Serum	(52–54)
miR-33	↑	HepG2 and Huh7 cells, Mice, rat, and human	Liver	(55–57)
miR-34a	↑	Mice, rat, and human	Serum	(43–48)
miR-122	↓	Primary cultured murine Hepatocytes, mice, and human	Liver	(29, 36–40)
miR-125a-5p	↓	Mice	Liver	(35)
miR-155	↓	Mice	Liver	(87)
miR-182	↑	Mice	Liver	(35)
miR-192	↓	Rat and human	Liver	(29, 36, 37, 39–41, 44, 49–51)
miR-223	↑	Human	Serum	(27)
miR-335	↑	Mice	Liver	(42)
miR-340-5p	↓	Mice	Liver	(35)
miR-484	↑	Mice	Liver	(35)
miR-574-3p	↑	Mice	Liver	(35)
miR-720	↓	Mice	Liver	(35)
T2DM				
let-7	↑	C2C12 cells and mice	Liver and skeletal muscle	(74, 75)
miR-1	↓	Mice and human	Skeletal muscle	(72, 73)
miR-7a	↑	MIN6 cells, mice, and human	Pancreas	(66, 67)
miR-17	↑	Human	Serum	(39)
miR-20a/b	↑	Human	Serum	(39)
miR-29	↑	3T3-L1 and Huh7 cells, mice, and rat	Liver, adipose tissue, and skeletal muscle	(69–71)
miR-30d	↑	MIN6 cells	Pancreas	(63)
miR-107	↑	MIN6 cells	Pancreas	(63)
miR-124a	↑	MIN6 cells	Pancreas	(63)
miR-133a	↓	Mice and human	Skeletal muscle	(72, 73)
miR-145	↓	Human	Circulating monocytes	(89–92)
miR-200	↑	MIN6 cells and mice	Pancreas	(68)
miR-296	↓	MIN6 cells	Pancreas	(63)
miR-375	?	MIN6 cells and mice	Pancreas	(64, 65)
miR-484	↓	MIN6 cells	Pancreas	(63)
miR-690	↓	MIN6 cells	Pancreas	(63)
CVD				
let-7	↓	Human	Atherosclerotic plaques and serum	(93, 96)
miR-1	↓	Human	Atherosclerotic plaques	(96)
miR-15a-5p	↑	Human	Serum	(95)
miR-16-5p	↑	Human	Serum	(95)
miR-17	↓	Human	Serum	(92)
miR-21	↑	Human	Atherosclerotic plaques	(96)
miR-22	↓	Human	Atherosclerotic plaques	(96)
miR-34a	↑	Human	Serum	(51)
miR-92a	↓	Human	Serum	(92)
	↑	Human	Atherosclerotic plaques	(96)
miR-93a-5p	↑	Human	Serum	(95)
miR-99a	↑	Human	Atherosclerotic plaques	(96)
miR-122	?	Human	Serum	(76, 82, 83)
miR-126	↓	Human	Serum	(92)
miR-132	↓	Human	Serum	(86)
miR-133a	↑	Human	Serum	(92)
miR-143	↑	Human	Serum	(86)
miR-145	↓	Mice and human	Serum, murine aorta, and human carotid	(89–92)
miR-146a-5p	↓	Human	Serum	(95)
miR-155	↑	Mice	Aorta	(87)
	↓	Human	Serum	(88, 92, 93)
miR-208	↑	Human	Serum	(92)
miR-212	?	Human	Serum	(94)
miR-372	?	Human	Serum	(94)
miR-454	?	Human	Serum	(94)
miR-744	?	Human	Serum	(94)

study demonstrated that the knockdown of miR-375 increases GSIS in murine pancreatic β cell line (MIN6) and primary β cells (64). Next, the same laboratory result described that the deletion of miR-375 induced a marked decrease in β cell number, provoking a severe diabetic state. Conversely, miR-375 is increased in the pancreatic islets of *ob/ob* mice, which is consistent with the characteristic compensatory pancreatic hyperplasia of the prediabetic stage (65). With these results, the authors concluded that miR-375 is key for physiological glucose metabolism, β cell proliferation, and turnover (64, 65).

miR-7a and miR-200

Another β-cell-specific miRNA is miR-7a, which has been shown as a negative regulator of β cell proliferation by regulating the protein mammalian target of rapamycin (mTOR) signaling pathway. Wang et al. showed that the decrease of miR-7a activity resulted in increased mTOR signaling and β cell replication in murine pancreatic islets, implying that miR-7a could represent a potential therapeutic target for T2DM treatment (66). miR-7a not only regulates β-cell proliferation but also its functionality since it was found to inhibit GSIS. Moreover, the increased expression of miR-7a in β cells in mice induced diabetes. In contrast, these authors described that both obese and diabetic

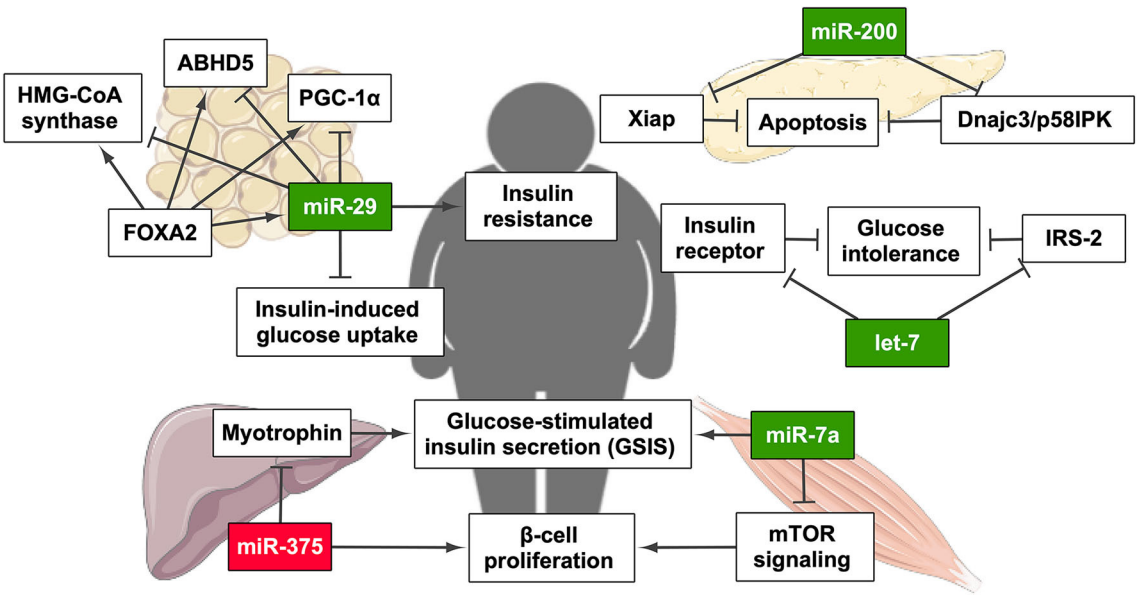

FIGURE 2 | miRNAs related to the establishment and/or progression of type 2 diabetes mellitus (T2DM) and their regulatory effect on T2DM-related pathways. Green highlights indicate the upregulation of miRNA, whereas red highlights indicate its downregulation. PGC-1α, PPARγ coactivator 1α. This figure has been edited from Servier Medical Art. Servier is licensed under a Creative Commons Attribution 3.0 Unported License. Created with BioRender.com.

mouse models and islets from obese and moderately diabetic patients suffered from a decrease in β cell miR-7a expression. All these data suggest that the levels of miR-7a vary depending on the stage of T2DM development, being decreased when β cell proliferation is needed as it occurs in the first stages of insulin resistance compensation (67). In the same way as miR-7a, another remarkable family of miRNAs is miR-200. These miRNAs are highly expressed in the pancreatic islets of the diabetic *db/db* mouse model. Overexpression of miR-200 in mouse β cells induced severe apoptosis that prompted to T2DM under stressed conditions by inhibiting Dnajc3/p58IPK and the caspase inhibitor Xiap. Indeed β cell apoptosis and T2DM pathogenesis are controlled by miR-200 family members (68). Many other miRNAs have been shown to regulate insulin expression and secretion.

miR-29

In addition to its role in β cell function and development, it has been widely described that miRNAs are also involved in IR in target tissues. For instance, miR-29 expression is enhanced by hyperinsulinemia and/or hyperglycemia in adipocyte-derived 3T3-L1 cells (69). Moreover, in the rat model of T2DM Goto–Kakizaki, the expression of this miRNA was increased in the liver, adipose tissue, and skeletal muscle. In the same report, the authors demonstrated that the overexpression of miR-29 in 3T3-L1 cells diminishes insulin-induced glucose uptake (70). Finally, it has been demonstrated that miR-29 expression is partially regulated by Forkhead Box A2 (FOXA2), and miR-29 also modifies FOXA2-mediated regulation genes including *PPARGC1A*, *HMGCS2*, and *ABHD5*, key players in lipid metabolism (71).

miR-1 and miR-133

Some other miRNAs involved in insulin resistance are miR-1 and miR-133a. These miRNAs are specifically expressed in the muscle, and their expression is regulated by insulin through SREBP1c and myocyte enhancer factor 2C (MEF2C) (72). Moreover, insulin-induced SREBP1c activation in human skeletal muscle triggered the subsequent downregulation of miR-1 and miR-133 through the inhibition of MEF2C. Consequently, insulin is unable to regulate miR-1 and miR-133a in the skeletal muscle in T2DM, perhaps due to the altered activation of SREBP1c (72, 73).

Let-7

Furthermore, the let-7 family of miRNAs is also involved in global glucose homeostasis since they regulate the expression of *INSR* and *IRS2* genes, among others (74, 75). Frost and Olson showed that the suppression of the let-7 family ameliorated glucose intolerance in a mouse model of diet-induced obesity by enhancing insulin signaling in the muscle and the liver (74). Another group demonstrated that let-7g overexpression produces glucose intolerance; however, insulin resistance changes are not detected. By repressing likewise the activity of the let-7 family, thanks to the overexpression of their let-7 negative regulators, LIN28 isoforms, glucose tolerance, and insulin sensitivity were improved (75). Therefore, this miRNA family supposes a new therapeutic target for T2DM treatment.

Despite the increasing amount of reports describing the role of specific miRNAs in T2DM, not much is known about *in vivo* situations (76). In consequence, it is of great interest to validate these situations in animal models in pathophysiological conditions.

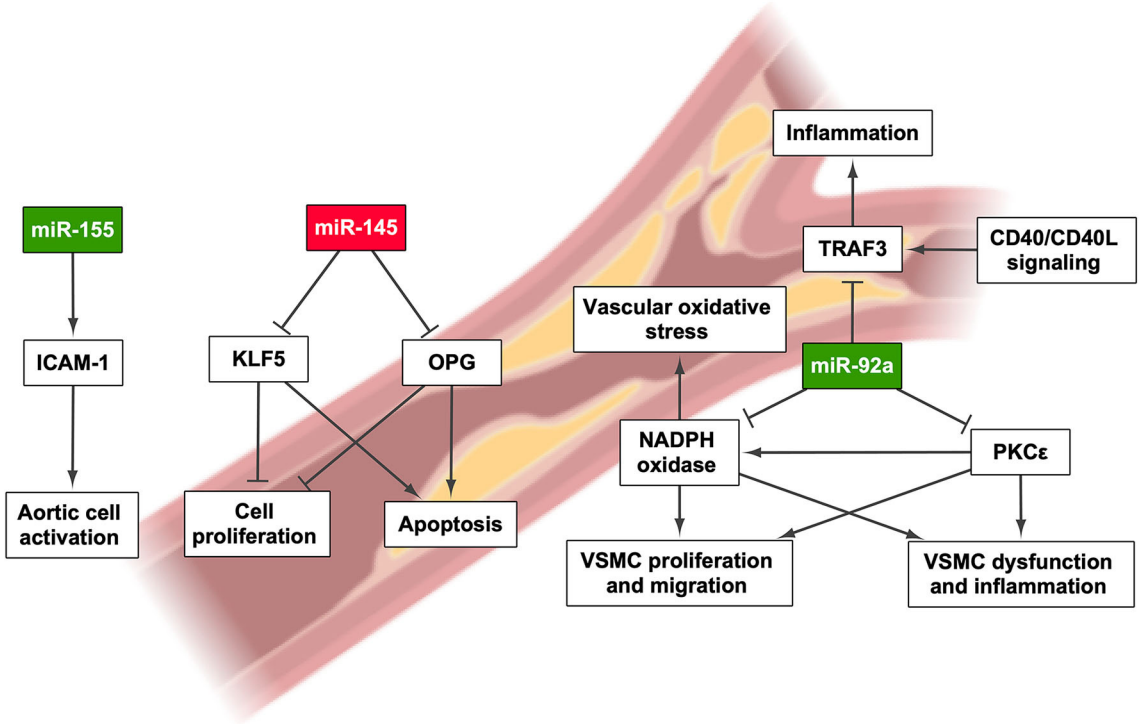

FIGURE 3 | miRNAs involved in the onset and/or progression of cardiovascular disease and their effect on pathways that regulate their development. Green highlights indicate the upregulation of miRNA, whereas red highlights indicate its downregulation. Created with BioRender.com.

miRNAs INVOLVED IN CARDIOVASCULAR DISEASE

Despite the fact that the subjacent pathogenic mechanism linking NAFLD and CVD is still to be determined, several studies have found that NAFLD increases the chances of suffering CVD (77–79). Therefore, NAFLD may cause chronic systemic inflammation, leading to the development of subclinical atherosclerosis and, eventually, CVD (79). The main links between NAFLD and CVD seem to be obesity, fat accumulation in the liver, and secretion of hepatic proteins, including hepatokines, proprotein convertase subtilisin/kexin type 9, and coagulation factors (80). In fact, miRNA secretion from the liver might play an important role in CVD development, especially in relation to CAD, as has been mentioned throughout this review (81) (**Figure 3** and **Table 1**).

miR-122

Aside from its role in the development of liver diseases, miR-122 also stands out due to its involvement in CVD since it is able to regulate lipid metabolism genes. Nevertheless, the experimental results obtained in clinical studies related to vascular complications are sparse. miR-122 levels are elevated in patients with hyperlipidemia and associated with CAD (82) and acute coronary syndrome (76). Moreover, how statins might reduce the levels of miR-122 in circulation has been described (83). However, other studies have reported negative associations between miR-122 and cardiovascular outcomes (84, 85).

miR-34a, miR-132, and miR-143

As has been previously described, miR-34a is a potential target for treating NAFLD, but it has been also analyzed in CAD patients, either with or without NAFLD. miR-34a expression is upregulated in patients with CAD, and this is aggravated when the subjects also suffer from NAFLD (51). On the other hand, Mehta et al. described that patients with NAFLD and CAD had a decrease in serum miR-132 levels and an increase in miR-143 expression; thus, they could be used as CAD markers (86).

miR-155 and miR-145

Another miRNA that seems to be involved in cardiovascular disease is miR-155. One study showed the increased levels of this miRNA in aortic samples of *ApoE*$^{-/-}$ mice, which develop atherosclerosis spontaneously. miR-155 and *ApoE* simultaneous knockout results in mice showing a decrease in atherosclerosis development but with increased obesity and aggravated NAFLD, suggesting that an increase of this miRNA is only detrimental in vascular tissue. In contrast, miR-155 upregulation promotes aortic endothelial cell activation by the induction of the expression of intercellular adhesion molecule 1 (87). According to this finding, a study carried out in 400 individuals showed

elevated levels of miR-155 in the serum of patients with coronary heart disease and a positive correlation between the expression of this miRNA and the severity of the disease (88). miR-145 has also been pointed out as a miRNA whose expression is altered in CAD. In a study including 195 individuals, the miR-145 circulating levels were decreased in CAD patients, showing a progressive decrease as the disease aggravates (89). Interestingly, this miRNA has a role in attenuating T2DM and atherosclerosis. Bearing in mind that miR-145 is abundantly expressed in the vessel walls, its role in atherosclerosis may be protective since its target genes are osteoprotegerin and Kruppel-like factor 5, thus regulating the inflammatory response (90). In this regard, Lovren et al. described a decrease in the expression of miR-145 in the aorta of ApoE$^{-/-}$ mice and the carotids of patients with atherosclerosis. It has previously been described that miR-145 promotes plaque stability by increasing the number of vascular smooth muscle cells, collagen content, and fibrous cap area as well as decreasing the number of macrophages and the necrotic area (91).

Indeed miR-145 and miR-155 have been mentioned many times with other miRNAs as candidates for diagnosis of CAD because of their expression patterns in the blood of CAD patients. The miR-145 and miR-155 levels were lower in the plasma of 67 patients, along with miR-126, miR-17, and miR-92a, whereas miR-133a and miR-208a were increased (92). This was later supported by a study that showed that the levels of miR-145, miR-155, and let-7 family member let-7c are lower when comparing CAD patients and healthy subjects (93). Although miR-145 and miR-155 expression seems to be generally altered in CVD, the involvement of the latter in this process remains unclear because of the inconsistencies found regarding its expression levels.

Other miRNAs

Plenty of other miRNAs have been described as potential biomarkers for CAD and other CVDs. For instance, an analysis of serum samples from non-atherosclerotic and atherosclerotic patients revealed that miR-454, miR-744, miR-372, and miR-212 are differentially expressed between these two groups, although only miR-212 showed a discrimination power when combined with other atherosclerosis risk factors (94). In contrast, a study performed in 50 CAD patients showed the elevation of miR-93a-5p, miR-15a-5p, and miR-16-5p levels and the decrease of miR-146a-5p level in plasma (95). Parahuleva et al. predicted the impact of miR-92a on atherosclerosis and pointed out many target genes, such as NADPH oxidase and its impact on vascular oxidative stress, TNF receptor associated factor 3, which modulates CD40 signaling in atherogenesis, as well as protein kinase C ε and its implication in inflammation and smooth muscle cell dysfunction (96–99). Indeed the atherosclerotic plaque itself shows altered miRNA expression: higher levels of miR-21, miR-92a, and miR-99a and a lower expression of miR-1, miR-22, and let-7f were found in the atherosclerotic plaques of 12 patients, opening a new path for the search of putative biomarkers in cardiovascular alterations associated with NAFLD (96).

miRNA AS BIOMARKERS IN THE DIAGNOSIS OF PATIENTS WITH NAFLD/NASH

Owing to the critical role of miRNAs as important epigenetic factors and their regulation of lipid and cholesterol biosynthesis in hepatocytes, these molecules are recently considered to be powerful biomarkers to diagnose hepatic diseases. miRNA involvement likewise ought to be strongly evaluated as follow-up and therapeutic tools in order to urgently find non-invasive strategies capable of replacing the only reliable method to differentiate between conditions, liver biopsy (100). Consequently, a key requirement to prove miRNA sensitivity and specificity would be large clinical cohorts suffering from NAFLD or related diseases. Despite the current drawbacks that miRNA-based technology presents, another useful feature that miRNAs possess is being targets for mimics and antisense oligonucleotides in case of undergoing an alteration in a specific disease (101). In addition, miRNAs are molecules that circulate into the bloodstream, where their expression levels are stable and easily quantitated, adding another reason to consider them as suitable clinical biomarkers. Specifically, the more proper option would be performing combined multiple miRNA analyses, as miRNA serum expression is also subject to the presence of non-liver-related disorders (102). In this sense, different authors have proposed miRNA panels for a high diagnostic accuracy of NAFLD. Whereas, Tan et al. proposed miR-122-5p, miR-1290, miR-27b-3p, and miR-192-5p for the diagnosis of NAFLD, Pirola et al. found that miR-192-5p or miR-122, miR-192, miR-19a, miR125, and miR-375 could also be an alternative panel (29, 103). However, other authors have suggested the combination of RNA panels with classical biomarkers for the prediction of NASH, specifically miR-122, miR-192, and miR-21 together with ALT and cytokeratin-18-Asp396 (52).

Furthermore, miRNAs could also be useful tools to assess NAFLD progression. Indeed miR-192 and miR-375 are associated with NAFLD activity score and classical biomarkers such as cytokeratin-18 (103). miR-122 in serum may likewise be a better predictor of NAFLD severity than classical biomarkers, such as ALT and cytokeratin-18 (29).

For all these reasons, miRNAs are becoming one of the few opportunities to advance and help, together with other classical biomarkers, in the diagnosis and prognosis of NAFLD.

CONCLUSIONS

In the last decade, an increasing number of papers have highlighted the great diversity of functions that miRNAs have in the pathophysiology of metabolic diseases such as NAFLD, T2DM, and CVD. Indeed apart from the high amount of human genes regulated by miRNAs (104), miRNAs themselves are aberrantly expressed in many human diseases (105–108). In spite of the number of miRNAs identified in mouse and humans and the fact that some of them have been involved in hepatic pathogenesis, data regarding their specific roles are still scarce, and more efforts are needed to unravel the precise

mechanisms. This task is quite challenging since a single miRNA can regulate hundreds of potential mRNA targets and different miRNAs can act on a particular mRNA target in a synergic or antagonic manner.

In this sense, manipulating miRNA expression whose targets are well-defined could represent a stage-specific therapy for NAFLD patients. For instance, both miR-122 and miR-21 are involved in the regulation of genes related to lipid metabolism, thus positioning them as candidates for the treatment of the earliest NAFLD stages, characterized by liver steatosis. Other miRNAs such as miR-34a, which regulates targets that participate in oxidative stress and inflammation, might be better suited for the treatment of individuals that have already progressed toward NASH. Moreover, since the development of NAFLD is tightly associated with the onset of T2DM and CVDs and all of them are promoted by subjacent systemic inflammation and metabolic alterations, it would be reasonable to expect that these diseases share alterations in the expression of some miRNAs. Therefore, the CVD-related miR-92a and miR-155 or the T2DM-associated let-7 and miR-200 could suppose novel and yet undiscovered targets for NAFLD treatment, with the potential to alleviate not only hepatic alterations but also other metabolic syndrome manifestations.

Finally, despite the recent advances that have been made about circulating miRNAs as potential biomarkers for NAFLD and the related diseases T2DM and CVD, in our opinion, the real challenge is to find more accurate correlations between the circulating levels of these miRNAs and the disease stage. In this way, these molecules could serve as powerful non-invasive diagnostic and prognostic tools that nowadays are a must for clinicians to avoid liver biopsy. Unfortunately, by now, much effort is still needed for this important challenge.

AUTHOR CONTRIBUTIONS

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

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Psychological Biomarkers and Fibrosis: An Innovative Approach to Non-alcoholic Fatty Liver Disease

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Background: It is unknown how perceived social support and the progression of liver damage influence the psychosocial profile of patients with non-alcoholic fatty liver disease (NAFLD). In the present study, we therefore investigated which biomarkers influence the quality of life, mental health, and coping strategies of NAFLD patients.

Methods: Quality of life (SF-12 and CLDQ-NAFLD), mental health (HADS and BDI-II), and coping strategies (COPE-28) were evaluated by high or low perceived social support (MSPSS) and the presence of non-alcoholic steatohepatitis (NASH) and significant fibrosis in 492 biopsy-proven NAFLD patients. The results were compared with quality of life normality tables for the general Spanish population. We also determined whether liver histology and biopsychosocial variables predicted participants' quality of life.

Results: Interactive effects were found in vitality ($p = 0.05$), activity ($p = 0.005$), anxiety ($p = 0.04$), and denial ($p = 0.04$), with NASH patients showing a higher-risk biopsychosocial profile when they perceived less social support. Furthermore, patients with low perceived social support showed lower quality of life, worse mental health, and more maladaptive coping than those with high perceived social support, regardless of NASH presence. Patients with significant fibrosis showed lower quality of life compared to those without or the general Spanish population. Patients with significant fibrosis also reported worse mental health and more maladaptive coping. Lastly, significant fibrosis, female sex, greater anxiety and depressive symptoms, and worse physical and mental health-related quality of life were found to be independent determinants of worse disease-specific quality of life in these patients.

Conclusions: Low perceived social support, significant fibrosis, and female sex were independently associated with a higher-risk psychosocial profile in NAFLD. These findings support the role of psychological biomarkers based on quality of life, mental health, and coping strategies in the management of these patients and suggest the potential benefits of a psychological intervention.

Keywords: NAFLD, fibrosis, quality of life, mental health, coping, perceived social support

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) causes a stronger negative impact on patients' quality of life (QoL) than do viral, alcoholic, autoimmune, or cholestatic liver diseases (1, 2), especially impairing physical functioning or the ability to perform daily activities (3–6). Mental health is also affected by an increase in anxiety and depressive symptoms (7). Similarly, although coping strategies have not been studied in NAFLD, maladaptive coping, such as denial of the disease, anger or getting upset after the diagnosis, disengagement, or giving up (8), is often found among chronic liver patients. The influence of perceived social support on these variables has not been approached. However, in chronic liver diseases, such as hepatitis B or C, satisfactory support implies improved patient progress and recovery (9, 10) and a decrease in the frequency and intensity of depressive symptoms (11).

The fibrosis stage is the main predictor of mortality associated with NAFLD (12), although the results are contradictory. Some studies have found worse QoL in patients with non-alcoholic steatohepatitis (NASH) and advanced fibrosis than those with NAFLD without advanced fibrosis, with cirrhotic patients complaining of the most decline in their QoL of all severity levels (2, 4, 13). However, Huber et al. (14) did not find any significant effect of fibrosis stage on QoL. In addition, the relationship between fibrosis and mental health in NAFLD patients is not clear either. Several studies have found an association between the presence of fibrosis and anxiety and depressive symptoms (7, 15, 16), while Kim et al. (17) found no relationship. Furthermore, female sex has been associated with a worse physical and mental QoL than does male sex (2, 18).

In view of the shortage of psychological studies in NAFLD, we decided to analyze the differences in QoL, mental health, and the coping strategies of patients with the absence or presence of NASH by perceived social support (high or low). We also studied the influence of liver disease severity levels on these variables using data from the general Spanish population to compare QoL. Finally, we determined whether certain histological and biopsychosocial variables predicted participants' QoL. We hypothesized that patients would have worse QoL, more anxiety and depressive symptoms, and more maladaptive coping when they have low perceived social support, NASH, or significant fibrosis. Furthermore, we hypothesized that the presence of determinants of liver damage (moderate or severe steatosis, lobular inflammation, hepatocellular ballooning, and significant fibrosis) and a higher-risk biopsychosocial profile (female sex, older age, presence of obesity, worse physical and mental health-related QoL, greater anxiety and depressive symptoms, maladaptive coping strategies, and low perceived social support) would be associated with a greater negative impact on the disease-specific QoL of NAFLD patients.

MATERIALS AND METHODS

Participants

This research was approved by the Ethics Committee of the Virgen del Rocío University Hospital of Seville. All patients gave their informed consent for participation, and the research was conducted in accordance with the 1975 Declaration of Helsinki guidelines of good practice. As shown in **Figure 1**, we selected a group of 492 patients with biopsy-proven NAFLD (290 men and 202 women) with a mean age of 54.90 ± 11.74 years. The sociodemographic characteristics of the groups are shown in **Supplementary Tables 1**, **2**. Data from the general Spanish population were also considered for QoL (SF-12) (19).

Measures

The 12-Item Short-Form Health Survey (SF-12v.2)

This scale comprised 12 items with either three- or five-point Likert-type scales (20, 21). It evaluates the following eight dimensions of health-related QoL: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health. It also calculates two summary measures using Quality Metric Health Outcomes^{TM} Scoring Software 5.0: physical component summary (PCS) and mental component summary (MCS). All scores ranged from 0 (worst state of health) to 100 (best state of health). Higher scores indicate better health-related QoL. In our sample, the Cronbach's alpha for the dimensions varied from 0.72 to 0.95. The Cronbach's alpha values for the PCS and MCS were 0.92 and 0.88, respectively (20).

Chronic Liver Disease Questionnaire—Non-alcoholic Fatty Liver Disease

The Chronic Liver Disease Questionnaire—Non-alcoholic Fatty Liver Disease (CLDQ-NAFLD) comprised 36 items rated on either a one- or a seven-point Likert-type scale (22). It evaluates specific QoL for NAFLD and NASH patients. It provides information referring to the total score on the scale and six domains: abdominal symptoms, activity, emotional, fatigue, systemic symptoms, and worry. Higher scores indicate better disease-specific QoL. In our sample, the Cronbach's alpha was 0.94 for the total instrument and from 0.68 to 0.89 for the domains.

Hospital Anxiety and Depression Scale

The Hospital Anxiety and Depression Scale (HADS) is made up of 14 items, seven on the anxiety subscale and seven on the depression subscale, with either zero- or three-point Likert-type scales (23). It evaluates anxiety and depressive symptoms. The test provides two total scores, one for anxiety and the other for depression. Scores range from 0 to 21 for each subscale. Higher scores indicate more anxiety and depressive symptoms. We used the Spanish version of this instrument (24). In our sample, the Cronbach's alpha was 0.81 for the anxiety subscale and 0.87 for the depression subscale.

Beck Depression Inventory—II

The Beck Depression Inventory—II (BDI-II) has 21 items answered on a four-point (0–3) scale, except for items 16 and 18, which have seven categories (25). It evaluates the severity of depression during the past 2 weeks. A total score of 0–63 is found. Higher scores show more severe depression. We used the Spanish

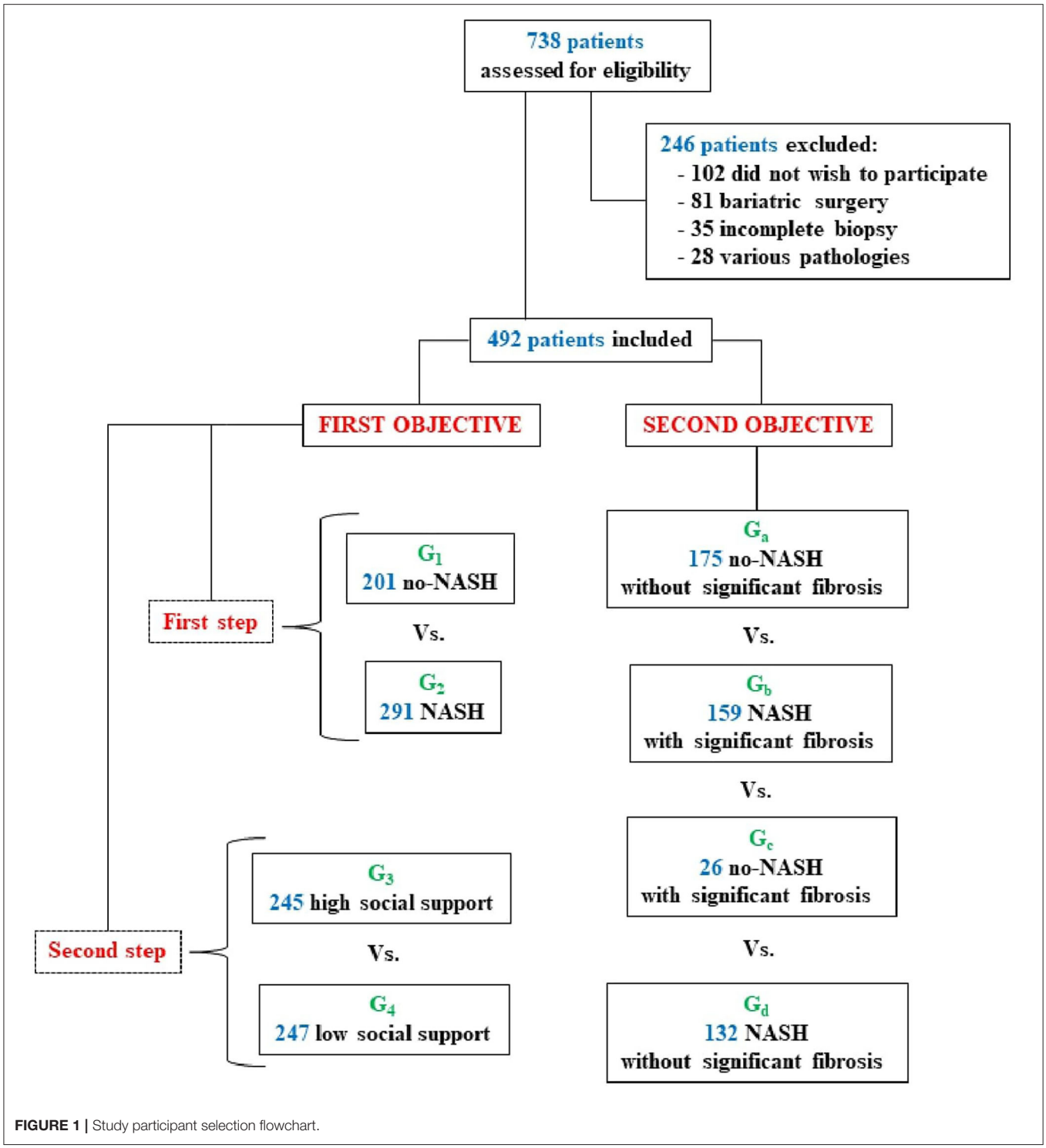


FIGURE 1 | Study participant selection flowchart.

version of this instrument (26). In our sample, the Cronbach's alpha was 0.91.

The Brief COPE

The Brief COPE (COPE-28) comprised 28 items with either zero- or three-point Likert-type scales (27). It evaluates 14 coping strategies: active coping, planning, instrumental support, emotional support, self-distraction, venting, disengagement, positive reframing, denial, acceptance, religion, substance use, humor, and self-blame. We used the Spanish version of this instrument (28). On all the subscales, higher scores indicate more use of the coping strategy. In our sample, the Cronbach's

alpha was 0.80–0.99 on the different subscales, except for positive reframing which was 0.45.

Multidimensional Scale of Perceived Social Support

The Multidimensional Scale of Perceived Social Support (MSPSS) consists of 12 items with either a one- or a seven-point Likert-type scale (29). It evaluates perceived support from three different sources: family, friends, and partner or other significant persons. It also provides information on the total scale. We used the Spanish version of this instrument (30). Higher scores show more perceived social support. In our sample, the Cronbach's alpha was 0.94 for the total instrument and was 0.95–0.99 for the various dimensions of social support.

Procedure

The 492 study patients were selected from 12 hospitals in six autonomous regions of Spain (Andalusia, Madrid, Castile and Leon, Catalonia, Cantabria, and Valencia). The same procedure was followed for all patients in the study. The researcher responsible at each participating hospital was contacted, and this contact provided a list of candidate NAFLD patients for the study. Then, these patients were phoned to make an appointment for the evaluation. All patients were evaluated by the same psychologist using the same psychological measures, always applied in the same order: a psychosocial interview and the SF-12, CLDQ-NAFLD, HADS, BDI-II, COPE-28, and MSPSS instruments. The inclusion criteria were as follows: (a) over 18 years of age; (b) informed consent; (c) no difficulties in understanding the evaluation instruments; (d) no severe or disabling psychopathological condition; and (e) diagnosed with biopsy-proven NAFLD. As all of the patients had undergone liver biopsy, they could be classified into groups by the hepatologist's criteria (G_1 = absence of NASH and G_2 = presence of NASH) and liver severity levels based on NASH and significant fibrosis (F2, F3, and F4) (G_a = no-NASH without significant fibrosis, G_b = NASH with significant fibrosis, G_c = no-NASH with significant fibrosis, and G_d = NASH without significant fibrosis) (**Figure 1**). Patients were classified as NASH or not after clinical assessment by the hepatologist, who provided a histological diagnosis by liver biopsy based on hepatocellular ballooning and lobular inflammation levels. The two perceived social support groups (G_3 = high and G_4 = low) were formed in two stages: firstly, each patient's total score on the MSPSS scale, which varied from 1 to 7, where higher scores show greater social support, was taken. These scores were then arranged in ascending order and the percentages accumulated were used to divide the sample into two groups at the 50.2 percentile (G_3 = high and G_4 = low) (**Figure 1**). Finally, to determine how liver histology and biopsychosocial variables predicted disease-specific QoL, the following histological variables provided by liver biopsy were analyzed: hepatic steatosis severity (a mild group of steatosis patients with 6–33% and a moderate-to-severe group with steatosis equal to or more than 34%); lobular inflammation (absence or presence depending on the number of foci per HPF, from none to more than one); hepatocellular ballooning (absence or presence based on the existence of ballooned cells); and significant fibrosis (absence, F0–F1, or presence, F2–F4, depending on the fibrosis stage). The biopsychosocial variables analyzed were sex (male or female), age, obesity, physical and mental health-related QoL (PCS and MCS; SF-12), anxiety and depressive symptoms (total anxiety and total depression; HADS, BDI-II), coping strategies (scores for all 14 coping strategies; COPE-28), and perceived social support (total scale score; MSPSS).

Statistical Analysis

Pearson's chi-square test was applied to the sociodemographic variables to compare categorical variables (sex, marital status, education, and employment) and the *t*-test for independent samples or a one-way ANOVA (Welch's *U)* with Games–Howell *post hoc* pairwise analysis to compare the age variable.

A 2 × 2 factorial ANOVA (Snedecor's *F*) was performed to evaluate the influence on QoL (SF-12 and CLDQ-NAFLD), mental health (HADS and BDI-II), and coping strategies (COPE-28) exerted by NASH (absence or presence) and perceived social support (high or low). And to compare these variables (QoL, mental health, and coping strategies) between the NAFLD severity groups, a one-way ANOVA (Snedecor's *F* or Welch's *U*) as an omnibus test was computed depending on whether or not they met the assumption of homoscedasticity. For *post hoc* multiple comparisons, Tukey's honestly significant difference or the Games–Howell test was applied. The *t*-test for independent samples was also applied for comparison with the general Spanish population. Cohen's *d* (for continuous variables) and *w* (for categorical variables) were computed as effect size indexes.

Binary logistic regression analysis was used to determine the contribution of the histological and biopsychosocial variables to disease-specific QoL. The independent variables in the regression model were hepatic steatosis severity, lobular inflammation, hepatocellular ballooning, significant fibrosis, sex, age, obesity, PCS (SF-12), MCS (SF-12), total anxiety (HADS), total depression (HADS and BDI-II), the 14 coping strategies measured with the COPE-28, and total perceived social support (MSPSS). For categorical variables, reference groups were formed for patients with moderate to severe steatosis, presence of lobular inflammation, hepatocellular ballooning or significant fibrosis, female sex, and the presence of obesity. The total score on the CLDQ-NAFLD questionnaire (total CLDQ-NAFLD) was analyzed as the dependent variable. This score was arranged in ascending order and the cumulative percentages were used to divide the sample into two groups (better and worse QoL) at the 50th percentile. The results for binary logistic regression were reported as odds ratios (OR) at 95% confidence intervals. A two-sided p-value < 0.05 was considered statistically significant. All the data were analyzed with the SPSS Statistics v.25 program.

RESULTS

Sociodemographic Variables

There were no important between-group differences (null or small effect sizes) in NASH, social support, or severity (**Supplementary Tables 1**, **2**). The only difference in severity was that age was higher in G_b (NASH with significant fibrosis) than in G_d (NASH without significant fibrosis; $p < 0.001$, $d = 0.557$). Age

TABLE 1 | Quality of life (SF-12) of non-alcoholic fatty liver disease (NAFLD) patients based on non-alcoholic steatohepatitis (NASH; absence and presence) and social support level (high and low) variables.

SF-12	NASH: M^a (SD)		Social support level: M^a (SD)		Main effects		Interaction effects
	Absence (G_1) $n = 201$	Presence (G_2) $n = 291$	High (G_3): $n = 245$	Low (G_4): $n = 247$	NASH: $F_{(1,488)}$ p (d^b)	Social support level: $F_{(1,488)}$ p (d^b)	$F_{(1,488)}$ (p)
Physical functioning	76.85 (33.03)	65.71 (33.09)	81.73 (33.34)	60.83 (33.79)	13.53 <0.001 (0.337 S)	47.55 <0.001 (0.623 M)	3.73 (0.06)
Role-physical	81.53 (28.50)	73.21 (28.49)	86.67 (28.80)	68.08 (29.07)	10.17 0.002 (0.292 S)	50.76 <0.001 (0.642 M)	2.97 (0.09)
Bodily pain	79.18 (27.22)	70.86 (27.29)	81.43 (27.55)	68.61 (27.82)	11.09 0.001 (0.304 S)	26.33 <0.001 (0.463 S)	1.36 (0.24)
General health	52.06 (24.24)	46.72 (24.22)	56.77 (24.57)	42 (24.83)	5.74 0.02 (0.220 S)	43.97 <0.001 (0.598 M)	1.73 (0.19)
Vitality	62.71 (25.80)	52.24 (25.76)	66.35 (25.98)	48.59 (26.40)	19.62 <0.001 (0.406 S)	56.45 <0.001 (0.678 M)	3.98 (0.05)
Social functioning	88.85 (21.97)	85.57 (22.00)	94.43 (22.23)	79.99 (22.47)	2.63 0.11 (0.149 N)	51.01 <0.001 (0.646 M)	1.80 (0.18)
Role-emotional	83.02 (24.53)	78.74 (24.39)	89.31 (24.73)	72.45 (24.99)	3.65 0.06 (0.175 N)	56.45 <0.001 (0.678 M)	1.36 (0.24)
Mental health	73.00 (21.27)	68.01 (21.15)	77.60 (21.44)	63.41 (21.69)	6.57 0.01 (0.235 S)	53.07 <0.001 (0.658 M)	2.35 (0.13)
PCS	48.89 (10.21)	45.67 (10.06)	49.94 (10.17)	44.63 (10.37)	11.98 0.001 (0.318 S)	32.57 <0.001 (0.517 M)	2.51 (0.11)
MCS	52.26 (9.36)	50.57 (9.38)	54.61 (9.55)	48.21 (9.59)	3.86 0.05 (0.180 N)	55.44 <0.001 (0.669 M)	1.64 (0.20)

A 2 × 2 factorial ANOVA (Snedecor's F) was applied.

SF-12, 12-Item Short-Form Health Survey; PCS, physical component summary; MCS, mental component summary.

[a] *Higher scores show better quality of life.*

[b] *Effect sizes: N, null; S, small; M, medium.*

was also higher in G_c (no-NASH with significant fibrosis) than in G_a (no-NASH without significant fibrosis; $p = 0.003$, $d = -0.725$) or G_d (NASH without significant fibrosis; $p = 0.001$, $d = 0.778$) (**Supplementary Table 2**).

Influence of NASH and Social Support Variables on QoL, Mental Health, and Coping Strategies

The results are shown in **Table 1** (SF-12), **Table 2** (CLDQ-NAFLD), **Table 3** (HADS and BDI-II), and **Table 4** (COPE-28). Four statistically significant interactive effects were found: vitality ($p = 0.05$; **Table 1**), activity ($p = 0.005$; **Table 2**), anxiety ($p = 0.04$; **Table 3**), and denial ($p = 0.04$; **Table 4**). As observed in **Table 5** and **Figure 2**, the simple effects showed that NASH patients had less vitality ($p < 0.001$, $d = 0.873$), less activity ($p < 0.001$, $d = 0.805$), more anxiety ($p < 0.001$, $d = -0.786$), and more denial ($p < 0.001$, $d = -0.638$) when they perceived less social support. However, in patients without NASH, there were no differences depending on perceived social support, except in the vitality variable ($p < 0.001$, $d = 0.505$), which was lower in patients with low social support. Moreover, when social support was high, there were no differences between patients with and without NASH, but when social support was low, patients with NASH had lower scores in vitality ($p < 0.001$, $d = 0.590$) and activity ($p < 0.001$, $d = 0.600$).

The main effects by relevant effect sizes (medium and large) were that, regardless of whether NASH was present or not, patients with low social support had worse QoL (SF-12 and CLDQ-NAFLD) than those with high social support on most of the variables, except bodily pain, abdominal symptoms, and worry, in which there were no differences between the two groups (**Tables 1**, **2**). In mental health (HADS and BDI-II), patients with low social support had higher scores in anxiety

TABLE 2 | Quality of life (CLDQ-NAFLD) of non-alcoholic fatty liver disease (NAFLD) patients based on non-alcoholic steatohepatitis (NASH; absence and presence) and social support level (high and low) variables.

CLDQ-NAFLD	NASH: M^a (SD)		Social support level: M^a (SD)		Main effects		Interaction effects
	Absence (G_1): $n = 201$	Presence (G_2): $n = 291$	High (G_3): $n = 245$	Low (G_4): $n = 247$	NASH: $F_{(1,488)}$ p (d^b)	Social support level: $F_{(1,488)}$ p (d^b)	$F_{(1,488)}$ (p)
Abdominal symptoms	5.64 (1.42)	5.51 (1.53)	5.88 (1.41)	5.28 (1.57)	0.91 0.34 (0.088 N)	19.36 <0.001 (0.402 S)	0.201 (0.65)
Activity	5.86 (1.28)	5.45 (1.19)	5.99 (1.25)	5.33 (1.26)	13.32 <0.001 (0.332 S)	33.92 <0.001 (0.526 M)	7.97 (0.005)
Emotional	5.90 (0.99)	5.62 (1.02)	6.17 (0.94)	5.35 (0.94)	9.14 0.003 (0.279 S)	82.23 <0.001 (0.872 L)	3.34 (0.07)
Fatigue	5.55 (1.13)	5.13 (1.19)	5.86 (1.25)	4.82 (1.26)	14.60 <0.001 (0.362 S)	89.58 <0.001 (0.829 L)	2.61 (0.11)
Systemic symptoms	5.98 (0.85)	5.64 (0.85)	6.10 (0.94)	5.52 (0.94)	17.60 <0.001 (0.400 S)	47.72 <0.001 (0.617 M)	1.04 (0.31)
Worry	6.30 (0.99)	6.10 (1.02)	6.39 (0.94)	6.00 (0.94)	5.22 0.02 (0.199 N)	19.42 <0.001 (0.415 S)	0.012 (0.91)
Total	5.87 (0.85)	5.58 (0.85)	6.06 (0.78)	5.38 (0.78)	14.54 <0.001 (0.341 S)	75.10 <0.001 (0.872 L)	3.04 (0.08)

A 2 × 2 factorial ANOVA (Snedecor's F) was applied.
[a]*Higher scores show better quality of life.*
[b]*Effect sizes: N, null; S, small; M, medium; L, large.*

TABLE 3 | Mental health (HADS and BDI-II) of non-alcoholic fatty liver disease (NAFLD) patients based on non-alcoholic steatohepatitis (NASH; absence and presence) and social support level (high and low) variables.

	NASH: M^a (SD)		Social support level: M^a (SD)		Main effects		Interaction effects
	Absence (G_1): $n = 201$	Presence (G_2): $n = 291$	High (G_3): $n = 245$	Low (G_4): $n = 247$	NASH: $F_{(1,488)}$ p (d^b)	Social support level: $F_{(1,488)}$ p (d^b)	$F_{(1,488)}$ (p)
HADS							
Total anxiety	3.20 (3.40)	3.78 (3.41)	2.46 (3.44)	4.52 (3.46)	3.29 0.07 (−0.170 N)	42.52 <0.001 (−0.597 M)	4.079 (0.04)
Total depression	2.26 (3.26)	3.09 (3.24)	1.22 (3.29)	4.13 (3.30)	7.66 0.006 (−0.255 S)	94.63 <0.001 (−0.883 L)	2.916 (0.09)
BDI-II							
Total depression	6.17 (6.95)	7.58 (6.99)	3.38 (7.04)	10.38 (7.07)	4.89 0.03 (−0.202 S)	12.69 <0.001 (−0.992 L)	2.272 (0.13)

A 2 × 2 factorial ANOVA (Snedecor's F) was applied.
[a]*Higher scores show worse mental health.*
[b]*Effect sizes: N, null; S, small; M, medium; L, large.*

($p < 0.001$, $d = -0.597$) and depressive symptoms measured with both HADS ($p < 0.001$, $d = -0.883$) and BDI-II ($p < 0.001$, $d = -0.992$) (**Table 3**). And in coping strategies (COPE-28), by relevant effect sizes (medium), patients with low social support scored higher in disengagement ($p < 0.001$, $d = -0.702$) and lower in active coping ($p < 0.001$, $d = 0.756$), planning

TABLE 4 | Coping strategies (COPE-28) of non-alcoholic fatty liver disease (NAFLD) patients based on non-alcoholic steatohepatitis (NASH; absence and presence) and social support level (high and low) variables.

COPE-28	NASH: M^a (SD)		Social support level: M^a (SD)		Main effects		Interaction effects
	Absence (G_1): $n = 201$	Presence (G_2): $n = 291$	High (G_3): $n = 245$	Low (G_4): $n = 247$	NASH: $F_{(1,488)}$ p (d^b)	Social support level: $F_{(1,488)}$ p (d^b)	$F_{(1,488)}$ (p)
Active coping	1.96 (0.71)	1.87 (0.68)	2.21 (0.78)	1.62 (0.78)	1.95 0.16 (0.129 N)	74.35 <0.001 (0.756 M)	0.10 (0.92)
Planning	1.29 (0.99)	1.34 (1.02)	1.63 (0.94)	1.01 (1.10)	0.27 0.60 (−0.050 N)	45.29 <0.001 (0.606 M)	1.35 (0.25)
Instrumental support	1.15 (0.99)	1.14 (1.02)	1.40 (0.94)	0.88 (0.94)	0.02 0.88 (0.010 N)	33.89 <0.001 (0.553 M)	0.33 (0.56)
Emotional support	1.03 (0.99)	1.10 (1.02)	1.33 (0.94)	0.79 (0.94)	0.56 0.45 (−0.070 N)	35.66 <0.001 (0.574 M)	0.10 (0.75)
Self-distraction	0.67 (0.99)	0.82 (1.02)	0.68 (0.94)	0.80 (0.94)	2.53 0.11 (−0.149 N)	1.83 0.18 (−0.128 N)	1.23 (0.27)
Venting	0.98 (0.99)	1.02 (1.02)	1.17 (1.09)	0.83 (1.10)	0.15 0.70 (−0.040 N)	13.55 <0.001 (0.310 S)	0.75 (0.39)
Disengagement	0.30 (0.57)	0.31 (0.51)	0.14 (0.47)	0.47 (0.47)	0.11 0.74 (−0.018 N)	50.74 <0.001 (−0.702 M)	0.19 (0.66)
Positive reframing	1.35 (0.99)	1.16 (1.02)	1.58 (0.94)	0.93 (0.94)	4.33 0.04 (0.189 N)	52.02 <0.001 (0.691 M)	0.47 (0.49)
Denial	0.18 (0.42)	0.24 (0.34)	0.12 (0.47)	0.30 (0.47)	2.63 0.10 (−0.157 N)	21.32 <0.001 (−0.383 S)	4.14 (0.04)
Acceptance	2.04 (0.71)	1.99 (0.68)	2.29 (0.78)	1.75 (0.78)	0.64 0.42 (0.072 N)	61.92 <0.001 (0.692 M)	1.44 (0.23)
Religion	0.84 (1.13)	0.99 (1.19)	0.90 (1.25)	0.94 (1.26)	2.00 0.16 (−0.129 N)	0.117 0.73 (−0.032 N)	0.01 (0.94)
Humor	1.11 (0.99)	1.00 (1.02)	1.29 (1.09)	0.82 (0.94)	1.45 0.23 (0.109 N)	24.21 <0.001 (0.462 S)	0.02 (0.89)
Self-blame	0.55 (0.71)	0.50 (0.68)	0.39 (0.78)	0.66 (0.78)	0.43 0.51 (0.072 N)	18.35 <0.001 (−0.346 S)	1.94 (0.16)

A 2 × 2 factorial ANOVA (Snedecor's F) was applied.

[a] *Higher scores show more use of the coping strategy.*

[b] *Effect sizes: N, null; S, small; M, medium.*

($p < 0.001$, $d = 0.606$), instrumental support ($p < 0.001$, $d = 0.553$), emotional support ($p < 0.001$, $d = 0.574$), positive reframing ($p < 0.001$, $d = 0.691$), and acceptance ($p < 0.001$, $d = 0.692$) (**Table 4**).

Influence of Liver Disease Severity on QoL, Mental Health, and Coping Strategies

In QoL (SF-12 and CLDQ-NAFLD), there were statistically significant differences between the severity levels in physical functioning ($p < 0.001$), role-physical ($p < 0.001$), bodily pain ($p < 0.001$), general health ($p < 0.001$), vitality ($p < 0.001$), social functioning ($p = 0.01$), role-emotional ($p = 0.01$), mental health ($p = 0.001$), PCS ($p < 0.001$), MCS ($p = 0.03$), abdominal symptoms ($p < 0.001$), activity ($p < 0.001$), emotional ($p < 0.001$), fatigue ($p < 0.001$), systemic symptoms ($p < 0.001$), worry ($p < 0.001$), and total CLDQ-NAFLD ($p < 0.001$). Specifically, by relevant effect sizes (medium and large), as shown in **Tables 6**, **7** and **Figures 3**, **4**, G_a (no-NASH without significant

TABLE 5 | Simple effects in vitality (SF-12), activity (CLDQ-NAFLD), anxiety (HADS), and denial (COPE-28).

		Vitality (SF-12)		Activity (CLDQ-NAFLD)		Anxiety (HADS)		Denial (COPE-28)	
		p	Cohen's *d*[a]	*p*	Cohen's *d*[a]	*p*	Cohen's *d*[a]	*p*	Cohen's *d*[a]
Social support level	High–low	Absence NASH (G_1): $n = 201$							
		<0.001	0.505 M	0.05	0.283 S	0.004	−0.410 S	0.09	−0.250 S
		Presence NASH (G_2): $n = 291$							
		<0.001	0.873 L	<0.001	0.805 L	<0.001	−0.786 M	<0.001	−0.638 M
NASH	Absence–presence	High social support (G_3): $n = 245$							
		0.08	0.223 S	0.56	0.075 N	0.88	0.020 N	0.77	0.045 N
		Low social support (G_4): $n = 247$							
		<0.001	0.590 M	<0.001	0.600 M	0.007	−0.352 S	0.01	−0.395 S

SF-12, 12-Item Short-Form Health Survey; CLDQ-NAFLD, Chronic Liver Disease Questionnaire—Non-alcoholic Fatty Liver Disease; HADS, Hospital Anxiety and Depression Scale; COPE-28, Brief COPE.

[a]*Cohen's d: N, null effect size; S, small effect size; M, medium effect size; L, large effect size.*

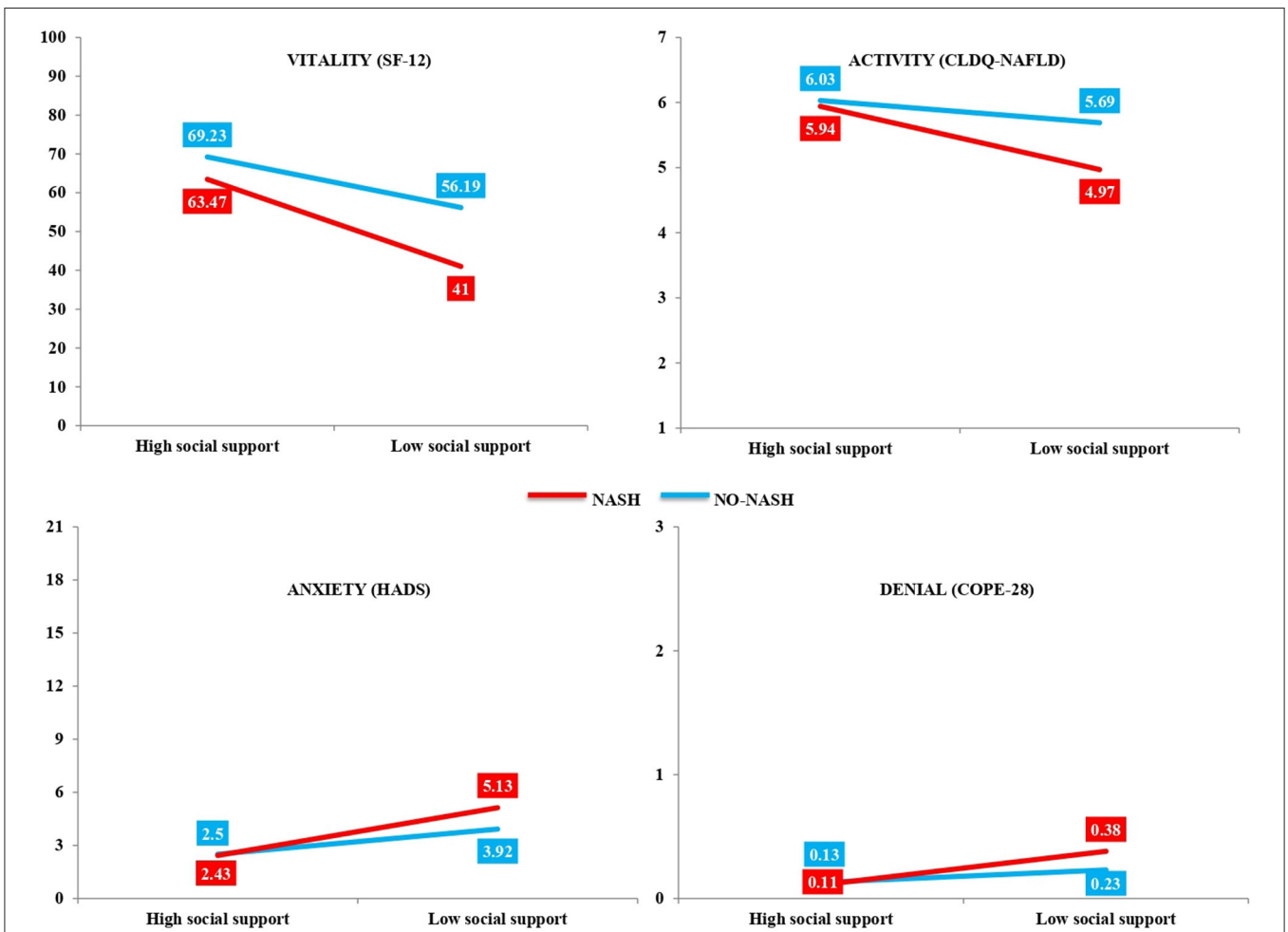


FIGURE 2 | Interactive effects of non-alcoholic steatohepatitis (NASH; absence or presence) and level of social support (high or low) factors. Analysis of the influence of NASH and social support on the quality of life, mental health, and coping strategies of non-alcoholic fatty liver disease (NAFLD) patients showing interactive effects in vitality ($p = 0.05$), activity ($p = 0.005$), anxiety ($p = 0.04$), and denial ($p = 0.04$).

fibrosis) scored higher in physical functioning ($p < 0.001$, $d = 0.660$), role-physical ($p < 0.001$, $d = 0.603$), bodily pain ($p < 0.001$, $d = 0.566$), vitality ($p < 0.001$, $d = 0.638$), PCS ($p < 0.001$, $d = 0.647$), activity ($p < 0.001$, $d = 0.679$), emotional ($p < 0.001$, $d = 0.517$), fatigue ($p < 0.001$, $d = 0.736$), systemic symptoms ($p < 0.001$, $d = 0.750$), worry ($p < 0.001$, $d = 0.573$),

TABLE 6 | Comparison of quality of life (SF-12) in non-alcoholic fatty liver disease (NAFLD) severity groups: non-alcoholic steatohepatitis (NASH; with and without significant fibrosis) and no-NASH (with and without significant fibrosis).

SF-12	G_a–G_b: p (d^a)	G_a–G_c: p (d^a)	G_a–G_d: p (d^a)	G_b–G_c: p (d^a)	G_b–G_d: p (d^a)	G_c–G_d: p (d^a)
Physical functioning	<0.001 (0.660 M)	0.04 (0.610 M)	0.69 (0.127 N)	0.98 (−0.082 N)	<0.001 (−0.547 M)	0.14 (−0.489 S)
Role-physical	<0.001 (0.603 M)	0.26 (0.422 S)	0.99 (0.034 N)	0.79 (−0.189 N)	<0.001 (−0.579 M)	0.33 (−0.395 S)
Bodily pain	<0.001 (0.566 M)	0.57 (0.297 S)	0.79 (0.108 N)	0.68 (−0.238 S)	0.001 (−0.447 S)	0.83 (−0.190 N)
General health	<0.001 (0.480 S)	0.32 (0.373 S)	0.99 (0.036 N)	0.94 (−0.124 N)	0.001 (−0.439 S)	0.43 (−0.332 S)
Vitality	<0.001 (0.638 M)	0.26 (0.362 S)	0.14 (0.257 S)	0.65 (−0.220 S)	0.007 (−0.375 S)	0.92 (−0.125 N)
Social functioning	0.01 (0.335 S)	0.43 (0.143 N)	0.98 (0.012 N)	1.00 (0.013 N)	0.05 (−0.296 S)	0.54 (−0.314 S)
Role-emotional	0.01 (0.336 S)	0.97 (0.093 N)	1.00 (0.010 N)	0.58 (−0.255 S)	0.02 (−0.346 S)	0.98 (−0.088 N)
Mental health	0.001 (0.410 S)	0.98 (0.085 N)	0.96 (0.058 N)	0.38 (−0.220 S)	0.01 (−0.360 S)	1.00 (−0.026 N)
PCS	<0.001 (0.647 M)	0.06 (0.574 M)	0.81 (0.102 N)	0.97 (−0.094 N)	<0.001 (−0.542 M)	0.17 (−0.465 S)
MCS	0.03 (0.299 S)	1.00 (0.029 N)	0.95 (0.066 N)	0.55 (−0.267 S)	0.16 (−0.239 S)	1.00 (0.035 N)

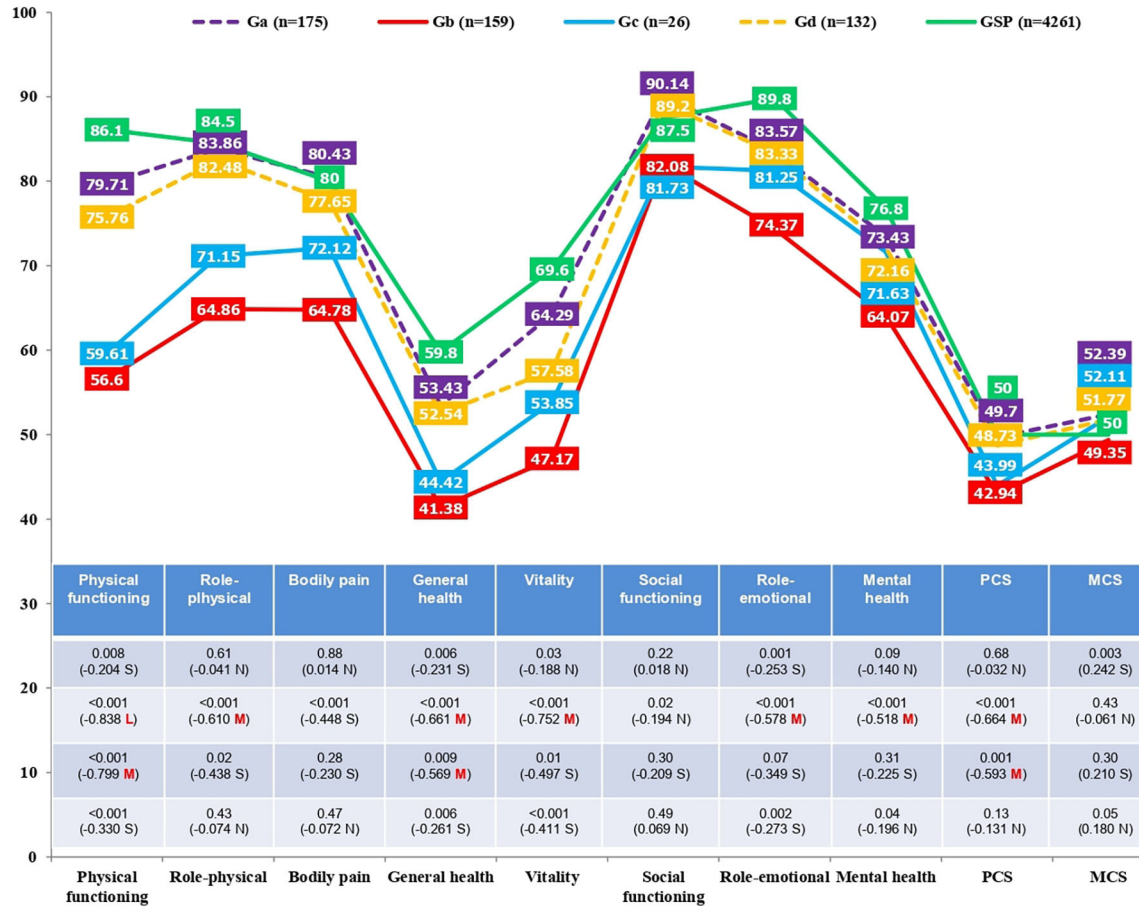
Tukey's honestly significant difference or Games–Howell was applied depending on whether or not they met the assumption of homoscedasticity.
G_a, no-NASH without significant fibrosis; G_b, NASH with significant fibrosis; G_c, no-NASH with significant fibrosis; G_d, NASH without significant fibrosis; PCS, physical component summary; MCS, mental component summary; SF-12, 12-Item Short-Form Health Survey.
[a]*Effect sizes: N, null; S, small; M, medium.*

TABLE 7 | Comparison of quality of life (CLDQ-NAFLD) between non-alcoholic fatty liver disease (NAFLD) severity groups: non-alcoholic steatohepatitis (NASH; with and without significant fibrosis) and no-NASH (with and without significant fibrosis).

CLDQ-NAFLD	G_a–G_b: p (d^a)	G_a–G_c: p (d^a)	G_a–G_d: p (d^a)	G_b–G_c: p (d^a)	G_b–G_d: p (d^a)	G_c–G_d: p (d^a)
Abdominal symptoms	0.006 (0.358 S)	0.73 (0.229 S)	0.37 (−0.186 N)	0.93 (−0.134 N)	<0.001 (−0.559 M)	0.30 (−0.412 S)
Activity	<0.001 (0.679 M)	0.06 (0.575 M)	0.91 (0.083 N)	0.91 (−0.138 N)	<0.001 (−0.633 M)	0.12 (−0.523 M)
Emotional	<0.001 (0.517 M)	0.94 (0.111 N)	1.00 (0.010 N)	0.15 (−0.428 S)	<0.001 (−0.521 M)	0.96 (−0.104 N)
Fatigue	<0.001 (0.736 M)	0.03 (0.671 M)	0.85 (0.095 N)	0.99 (−0.056 N)	<0.001 (−0.651 M)	0.06 (−0.586 M)
Systemic symptoms	<0.001 (0.750 M)	0.15 (0.514 M)	0.67 (0.131 N)	0.86 (−0.166 N)	<0.001 (−0.638 M)	0.33 (−0.409 S)
Worry	<0.001 (0.573 M)	0.14 (0.537 M)	0.94 (−0.066 N)	1.00 (−0.008 N)	<0.001 (−0.633 M)	0.10 (−0.594 M)
Total	<0.001 (0.769 M)	0.11 (0.546 M)	1.00 (0.000 N)	0.85 (−0.178 N)	<0.001 (−0.804 L)	0.12 (−0.570 M)

Games–Howell post hoc pairwise analysis was applied.
G_a, no-NASH without significant fibrosis; G_b, NASH with significant fibrosis; G_c, no-NASH with significant fibrosis; G_d, NASH without significant fibrosis; CLDQ-NAFLD, Chronic Liver Disease Questionnaire—Non-alcoholic Fatty Liver Disease.
[a]*Effect sizes: N, null; S, small; M, medium; L, large.*

and total CLDQ-NAFLD ($p < 0.001$, $d = 0.769$) than G_b (NASH with significant fibrosis). G_a (no-NASH without significant fibrosis) also scored higher in physical functioning ($p = 0.04$, $d = 0.610$), PCS ($p = 0.06$, $d = 0.574$), activity ($p = 0.06$, $d = 0.575$), fatigue ($p = 0.03$, $d = 0.671$), systemic symptoms ($p = 0.15$, $d = 0.514$), worry ($p = 0.14$, $d = 0.537$), and total CLDQ-NAFLD ($p = 0.11$, $d = 0.546$) than G_c (no-NASH with significant fibrosis). G_b (NASH with significant fibrosis) scored



Physical functioning	Role-physical	Bodily pain	General health	Vitality	Social functioning	Role-emotional	Mental health	PCS	MCS	Intergroups comparisons
0.008 (-0.204 S)	0.61 (-0.041 N)	0.88 (0.014 N)	0.006 (-0.231 S)	0.03 (-0.188 N)	0.22 (0.018 N)	0.001 (-0.253 S)	0.09 (-0.140 N)	0.68 (-0.032 N)	0.003 (0.242 S)	G_a-GSP p (d^*)
<0.001 (-0.838 L)	<0.001 (-0.610 M)	<0.001 (-0.448 S)	<0.001 (-0.661 M)	<0.001 (-0.752 M)	0.02 (-0.194 N)	<0.001 (-0.578 M)	<0.001 (-0.518 M)	<0.001 (-0.664 M)	0.43 (-0.061 N)	G_b-GSP p (d^*)
<0.001 (-0.799 M)	0.02 (-0.438 S)	0.28 (-0.230 S)	0.009 (-0.569 M)	0.01 (-0.497 S)	0.30 (-0.209 S)	0.07 (-0.349 S)	0.31 (-0.225 S)	0.001 (-0.593 M)	0.30 (0.210 S)	G_c-GSP p (d^*)
<0.001 (-0.330 S)	0.43 (-0.074 N)	0.47 (-0.072 N)	0.006 (-0.261 S)	<0.001 (-0.411 S)	0.49 (0.069 N)	0.002 (-0.273 S)	0.04 (-0.196 N)	0.13 (-0.131 N)	0.05 (0.180 N)	G_d-GSP p (d^*)

FIGURE 3 | Comparison of quality of life (SF-12) in the non-alcoholic fatty liver disease (NAFLD) severity groups and the general Spanish population (GSP). *Cohen's *d*: *N*, null effect size; *S*, small effect size; *M*, medium effect size; *L*, large effect size. G_a, no-NASH without significant fibrosis; G_b, NASH with significant fibrosis; G_c, no-NASH with significant fibrosis; G_d, NASH without significant fibrosis; *GSP*, general Spanish population; *PCS*, physical component summary; *MCS*, mental component summary; *SF-12*, 12-Item Short-Form Health Survey.

lower in physical functioning ($p < 0.001$, $d = -0.547$), role-physical ($p < 0.001$, $d = -0.579$), PCS ($p < 0.001$, $d = -0.542$), abdominal symptoms ($p < 0.001$, $d = -0.559$), activity ($p < 0.001$, $d = -0.633$), emotional ($p < 0.001$, $d = -0.521$), fatigue ($p < 0.001$, $d = -0.651$), systemic symptoms ($p < 0.001$, $d = -0.638$), worry ($p < 0.001$, $d = -0.633$), and total CLDQ-NAFLD ($p < 0.001$, $d = -0.804$) than G_d (NASH without significant fibrosis). G_c (no-NASH with significant fibrosis) also scored lower in activity ($p = 0.12$, $d = -0.523$), fatigue ($p = 0.06$, $d = -0.586$), worry ($p = 0.10$, $d = -0.594$), and total CLDQ-NAFLD ($p = 0.12$, $d = -0.570$) than G_d (NASH without significant fibrosis). Similarly, the G_b (NASH with significant fibrosis) and G_c (no-NASH with significant fibrosis) groups differed considerably from the general Spanish population (GSP) in some dimensions of QoL measured with the SF-12 (**Figure 3**). More precisely, G_b (NASH with significant fibrosis) scored lower in physical functioning ($p < 0.001$, $d = -0.838$), role-physical ($p < 0.001$, $d = -0.610$), general health ($p < 0.001$, $d = -0.661$), vitality ($p < 0.001$, $d = -0.752$), role-emotional ($p < 0.001$, $d = -0.578$), mental health ($p < 0.001$, $d = -0.518$), and PCS ($p < 0.001$, $d = -0.664$) than the GSP. G_c (no-NASH with significant fibrosis) also scored lower in physical functioning ($p < 0.001$, $d = -0.799$), general health ($p < 0.009$, $d = -0.569$), and PCS ($p < 0.001$, $d = -0.593$) than the GSP.

As shown in **Table 8**, differences in mental health (HADS and BDI-II) were found in total anxiety ($p = 0.01$) and total depression in the HADS ($p < 0.001$) and BDI-II ($p < 0.001$). Specifically, by relevant effect sizes (medium), G_b (NASH with significant fibrosis) showed higher scores in total depression than groups G_a (no-NASH without significant fibrosis) (HADS: $p < 0.001$, $d = -0.531$; BDI-II: $p < 0.001$, $d = -0.501$) or G_d (NASH without significant fibrosis) (HADS: $p < 0.001$, $d = 0.573$; BDI-II: $p < 0.001$, $d = 0.628$).

In coping strategies (COPE-28), shown in **Table 9**, differences were found in active coping ($p < 0.001$), planning ($p = 0.03$), disengagement ($p < 0.001$), positive reframing ($p = 0.001$), denial ($p = 0.004$), acceptance ($p < 0.001$), and humor ($p = 0.02$). By relevant effect sizes (medium), group G_d (NASH without

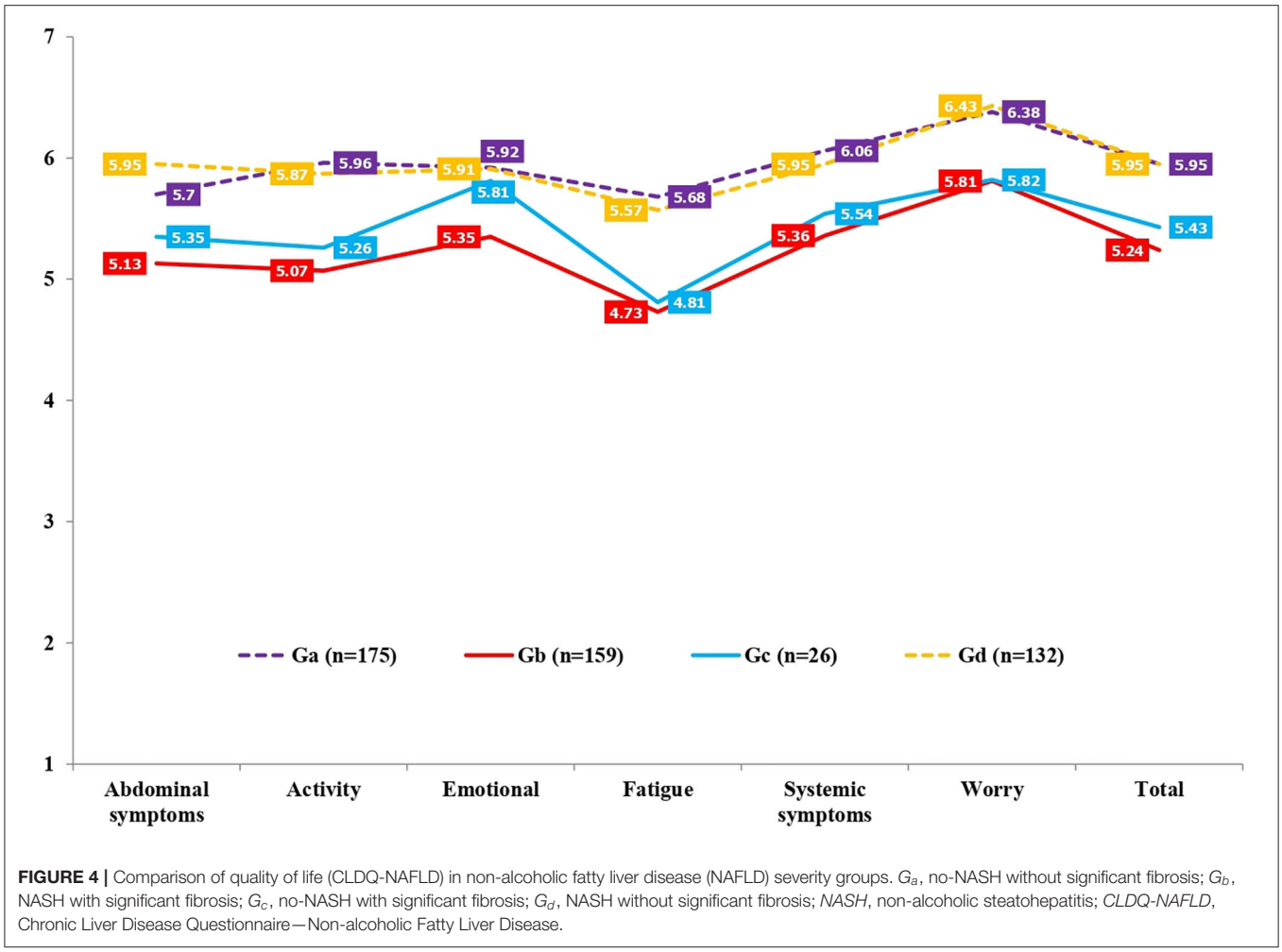


FIGURE 4 | Comparison of quality of life (CLDQ-NAFLD) in non-alcoholic fatty liver disease (NAFLD) severity groups. G_a, no-NASH without significant fibrosis; G_b, NASH with significant fibrosis; G_c, no-NASH with significant fibrosis; G_d, NASH without significant fibrosis; *NASH*, non-alcoholic steatohepatitis; *CLDQ-NAFLD*, Chronic Liver Disease Questionnaire—Non-alcoholic Fatty Liver Disease.

significant fibrosis) scored lower in disengagement than groups G_c (no-NASH with significant fibrosis; $p = 0.21$, $d = 0.511$) or G_b (NASH with significant fibrosis; $p < 0.001$, $d = 0.589$). G_d (NASH without significant fibrosis) also scored higher than G_b (NASH with significant fibrosis) in active coping ($p < 0.001$, $d = -0.567$) and acceptance ($p < 0.001$, $d = -0.586$) (**Table 10**).

Histological and Biopsychosocial Predictors of QoL in NAFLD Patients

A binary logistic regression was performed to evaluate the effect of the histological (steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis) and biopsychosocial (sex, age, obesity, physical and mental health-related QoL, anxiety and depressive symptoms, coping strategies, and perceived social support) variables on the disease-specific QoL of NAFLD patients. The logistic regression model was statistically significant ($\chi^2 = 367.256$, $p < 0.001$). The model explained 70.1% (Nagelkerke's R^2) of the variance in QoL, with an accuracy index of 0.852. Sensitivity was 86.6% and specificity was 83.7%, while the positive and negative predictive values were 0.841 and 0.861, respectively. Of all predictor variables, only fibrosis, sex, total depression (BDI-II), PCS (SF-12), total anxiety (HADS), and MCS (SF-12) were independently associated with total CLDQ-NAFLD. On the one hand, a significant inverse association was found between significant fibrosis (OR = 0.500, 95% CI = 0.253–0.987, $p = 0.04$), female sex (OR = 0.500, 95% CI = 0.254–0.981, $p = 0.04$), total depression (OR = 0.758, 95% CI = 0.661–0.869, $p < 0.001$), and total anxiety (OR = 0.858, 95% CI = 0.758–0.971, $p = 0.01$) and QoL (**Table 11**). On the other hand, a significant direct association was found between PCS (OR = 1.174, 95% CI = 1.123–1.227, $p < 0.001$) and MCS (OR = 1.073, 95% CI = 1.022–1.125, $p = 0.004$) and QoL (**Table 11**).

DISCUSSION

This study analyzed the differences in QoL, mental health, and coping strategies in NAFLD patients based on factors such as social support and the severity of liver damage (NASH and fibrosis). We also analyzed whether histological and biopsychosocial variables could predict the QoL of these patients. There were no important sociodemographic differences between the groups compared, except in the age, which was higher in patients with significant fibrosis than in those without significant fibrosis. This finding coincides with other studies (31, 32) which

TABLE 8 | Comparison of mental health (HADS and BDI-II) between non-alcoholic fatty liver disease (NAFLD) severity groups: non-alcoholic steatohepatitis (NASH; with and without significant fibrosis) and no-NASH (with and without significant fibrosis).

		No-NASH without significant fibrosis (G_a): $n = 175$	NASH with significant fibrosis (G_b): $n = 159$	No-NASH with significant fibrosis (G_c): $n = 26$	NASH without significant fibrosis (G_d): $n = 132$	Statistic	p
		M^a (SD)	M^a (SD)	M^a (SD)	M^a (SD)		
HADS	Total anxiety	3.19 (3.37)	4.39 (3.95)	3.12 (3.94)	3.14 (3.41)	$U_{(3,108.869)} = 3.72$	0.01
	Total depression	2.09 (2.94)	4.09 (4.44)	3.08 (4.49)	2.00 (2.62)	$U_{(3,106.161)} = 9.46$	<0.001
BDI–II	Total depression	5.75 (7.11)	9.89 (9.28)	8.19 (8.26)	5.08 (5.57)	$U_{(3,108.396)} = 10.73$	<0.001
		***Post hoc* comparisons**					
		G_a–G_b: p (d^b)	G_a–G_c: p (d^b)	G_a–G_d: p (d^b)	G_b–G_c: p (d^b)	G_b–G_d: p (d^b)	G_c–G_d: p (d^b)
HADS	Total anxiety	0.02 (−0.327 S)	1.00 (0.019 N)	1.00 (0.015 N)	0.43 (0.322 S)	0.02 (0.339 S)	1.00 (−0.005 N)
	Total depression	<0.001 (−0.531 M)	0.70 (−0.261 S)	0.99 (0.032 N)	1.71 (0.226 S)	<0.001 (0.573 M)	0.64 (0.284 S)
BDI-II	Total depression	<0.001 (−0.501 M)	0.49 (−0.317 S)	0.78 (0.105 N)	0.77 (0.193 N)	<0.001 (0.628 M)	0.27 (0.441 S)

A one-way ANOVA (Welch's U) with Games–Howell post hoc pairwise analysis were applied.
HADS, Hospital Anxiety and Depression Scale; BDI-II, Beck Depression Inventory—II.
[a]*Higher scores show worse mental health.*
[b]*Effect sizes: N, null; S, small; M, medium.*

had already noted the relationship between older age and the presence of significant or advanced fibrosis.

An interaction between NASH and social support was found in vitality, activity, anxiety, and denial. Among patients with NASH, those who reported low perceived social support showed less vitality and activity, greater anxiety, and more use of denial. This coincides with the results of a previous study done in patients with hepatitis C, which found a relationship between low levels of social support and more anxiety symptoms, as well as worse physical QoL (9). However, there were hardly any differences depending on social support in patients without NASH, except in vitality, which was higher in participants with high perceived social support.

When patients with low and high social support were compared, regardless of whether they had NASH or not, the first had poorer QoL and higher scores in anxiety and depressive symptoms, and more maladaptive coping, due to less use of strategies such as active coping, planning, using support, positive reframing, or acceptance. This ratifies the role of social support as a modulating agent of QoL, mental health, and coping strategies (33). Furthermore, low social support could be considered a major risk factor in NAFLD, especially when the disease progresses toward NASH and fibrosis. Therefore, it is fundamental to promote the creation of support networks like self-help groups because of their positive results in patient health, as already demonstrated in cancer (34), multiple sclerosis (35), or liver transplant candidate groups (36).

Moreover, patients with significant fibrosis had worse QoL in comparison with those without significant fibrosis and with the Spanish general population. This finding coincides with the study by David et al. (13), as it confirms the significant effect of fibrosis on QoL. In agreement with previous studies, the impact on QoL was mainly physical (3–6). Patients with significant fibrosis had particularly more impairment in physical functioning, role-physical, PCS, activity, emotional, fatigue, systemic symptoms, worry, and total CLDQ-NAFLD. This may be partially explained by the symptomatology associated with NAFLD, as problems affecting the patient's functionality, such as fatigue (3), daytime somnolence (37), and cognitive dysfunction (38), especially in the more advanced stages. These results agree with other studies comparing the QoL of NAFLD patients with that of the healthy population (3, 4, 13). We therefore suggest fibrosis as a determining factor in these differences, a conclusion confirmed by the results of the binary logistic regression analysis. Of all the variables analyzed for liver histology, fibrosis was the only one independently associated with QoL.

The relevance of sex in the QoL of NAFLD patients was also analyzed, with the results showing that female sex, along with the presence of significant fibrosis, was the main independent predictor of a worse QoL in these patients. Therefore, our study coincides with previous research, highlighting the greater vulnerability of the female sex to the impact caused by NAFLD

TABLE 9 | Comparison of coping strategies (COPE-28) between non-alcoholic fatty liver disease (NAFLD) severity groups: non-alcoholic steatohepatitis (NASH; with and without significant fibrosis) and no-NASH (with and without significant fibrosis).

COPE-28	No-NASH without significant fibrosis (G_a): $n = 175$	NASH with significant fibrosis (G_b): $n = 159$	No-NASH with significant fibrosis (G_c): $n = 26$	NASH without significant fibrosis (G_d): $n = 132$	Statistic	p
	M^a (SD)	M^a (SD)	M^a (SD)	M^a (SD)		
Active coping	1.99 (0.77)	1.66 (0.87)	1.88 (0.78)	2.10 (0.67)	$U_{(3,110.482)} = 7.99$	<0.001
Planning	1.27 (1.10)	1.17 (1.05)	1.50 (1.00)	1.52 (0.99)	$F_{(3,488)} = 3.02$	0.03
Instrumental support	1.20 (0.99)	1.03 (0.99)	0.88 (0.96)	1.25 (1.05)	$F_{(3,488)} = 1.93$	0.12
Emotional support	1.05 (1.01)	0.98 (0.99)	0.94 (0.93)	1.22 (1.04)	$F_{(3,488)} = 1.53$	0.21
Self-distraction	0.68 (0.98)	0.75 (1.02)	0.58 (0.66)	0.90 (0.97)	$U_{(3,120.760)} = 1.88$	0.14
Venting	1.01 (1.02)	1.06 (1.06)	0.86 (0.98)	0.96 (1.00)	$F_{(3,488)} = 0.37$	0.77
Disengagement	0.27 (0.51)	0.45 (0.63)	0.42 (0.66)	0.15 (0.35)	$U_{(3,106.617)} = 9.24$	<0.001
Positive reframing	1.38 (1.01)	0.97 (1.06)	1.17 (1.17)	1.36 (1.00)	$F_{(3,488)} = 5.35$	0.001
Denial	0.17 (0.37)	0.34 (0.54)	0.23 (0.51)	0.14 (0.36)	$U_{(3,107.139)} = 4.74$	0.004
Acceptance	2.07 (0.75)	1.77 (0.90)	1.90 (0.81)	2.23 (0.65)	$U_{(3,109.615)} = 9.05$	<0.001
Religion	0.82 (1.13)	1.01 (1.19)	0.96 (1.08)	0.98 (1.23)	$F_{(3,488)} = 0.79$	0.50
Substance use	0.00 (0.07)	0.00 (0.04)	0.00 (0.00)	0.00 (0.00)	$F_{(3,488)} = 0.35$	0.78
Humor	1.14 (1.10)	0.83 (1.01)	0.96 (1.02)	1.18 (1.03)	$F_{(3,488)} = 3.42$	0.02
Self-blame	0.54 (0.65)	0.63 (0.76)	0.54 (0.56)	0.36 (0.63)	$U_{(3,120.760)} = 1.88$	0.14

A one-way ANOVA (Snedecor's F or Welch's U) was applied depending on whether or not they met the assumption of homoscedasticity.
COPE-28, Brief COPE.
[a]*Higher scores show more use of the coping strategy.*

(2, 18). Binary logistic regression analysis also revealed that the severity of anxiety and depressive symptoms predicted the QoL of the participants, in line with Huang et al. (39), who found that worse mental health was associated with a reduced QoL in chronic liver disease patients. Physical and mental health-related QoL, measured with the generic SF-12, also predicted disease-specific QoL measured with CLDQ-NAFLD, an instrument specific to NAFLD patients. As quality of life is one of the core goals of intervention in these patients, the model's predictive variables should be given special consideration in the future. Female patients with significant fibrosis, stronger anxiety and depressive symptoms, and worse physical and mental health-related QoL are more likely to have a greater impact on their health and well-being. These patients would therefore require closer attention in the design of multidisciplinary NAFLD management strategies. Lastly, fibrosis was also associated with worse mental health and more maladaptive coping strategies. NASH patients with significant fibrosis scored higher in depression than patients without significant fibrosis, whether or not they had NASH. Patients with significant fibrosis also employed maladaptive strategies, such as disengagement, to a greater extent in comparison with NASH patients without significant fibrosis, and fewer adaptive strategies such as active coping or acceptance. The results for mental health confirm the relationship between fibrosis and depression already noted previously by Weinstein et al. (15), Youssef et al. (7), and Tomeno et al. (16) and therefore contradict the conclusion of Kim et al. (17).

In brief, the main findings of this study verified that there are differences in the QoL, mental health, and coping strategies of NAFLD patients depending on the perceived social support and histological fibrosis and confirm that the relevant variables predicting a worse disease-specific QoL in these patients are significant fibrosis, female sex, greater anxiety and depressive symptoms, and worse physical and mental health-related QoL. These results are relevant because such patients need to follow certain interventions based on lifestyle changes including diet, physical activity, and exercise to promote NASH resolution and fibrosis regression when losing weight. However, the probability of successful adherence to these guidelines is certainly low (40).

TABLE 10 | *Post hoc* comparison of coping strategies (COPE-28) between non-alcoholic fatty liver disease (NAFLD) severity groups: non-alcoholic steatohepatitis (NASH; with and without significant fibrosis) and no-NASH (with and without significant fibrosis).

COPE-28	G_a–G_b: *p* (*d*[a])	G_a–G_c: *p* (*d*[a])	G_a–G_d: *p* (*d*[a])	G_b–G_c: *p* (*d*[a])	G_b–G_d: *p* (*d*[a])	G_c–G_d: *p* (*d*[a])
Active coping	0.002 (0.402 S)	0.92 (0.142 N)	0.54 (−0.152 N)	0.56 (−0.266 S)	<0.001 (−0.567 M)	0.56 (−0.303 S)
Planning	0.82 (0.093 N)	0.74 (−0.219 S)	0.17 (−0.239 S)	0.46 (−0.322 S)	0.03 (−0.343 S)	1.00 (−0.020 N)
Instrumental support	0.41 (0.172 N)	0.45 (0.328 S)	0.97 (−0.049 N)	0.91 (0.154 N)	0.24 (−0.216 S)	0.33 (−0.368 S)
Emotional support	0.91 (0.060 N)	0.95 (0.113 N)	0.49 (−0.166 N)	1.00 (0.042 N)	0.19 (−0.236 S)	0.58 (−0.284 S)
Self-distraction	0.93 (−0.070 N)	0.89 (0.120 N)	0.22 (−0.226 S)	0.68 (0.198 N)	0.58 (−0.151 N)	0.17 (−0.386 S)
Venting	0.97 (−0.048 N)	0.91 (0.150 N)	0.98 (0.049 N)	0.81 (0.196 N)	0.87 (0.097 N)	0.97 (−0.101 N)
Disengagement	0.02 (−0.314 S)	0.68 (−0.254 S)	0.08 (0.274 S)	1.00 (0.046 N)	<0.001 (0.589 M)	0.21 (0.511 M)
Positive reframing	0.002 (0.396 S)	0.76 (0.192 N)	1.00 (0.020 N)	0.79 (−0.179 N)	0.008 (−0.378 S)	0.83 (−0.175 N)
Denial	0.008 (−0.367 S)	0.94 (−0.135 N)	0.91 (0.082 N)	0.77 (0.209 S)	0.002 (0.436 S)	0.84 (0.204 S)
Acceptance	0.005 (0.362 S)	0.75 (0.218 S)	0.19 (−0.228 S)	0.86 (−0.152 N)	<0.001 (−0.586 M)	0.22 (−0.449 S)
Religion	0.49 (−0.164 N)	0.94 (−0.127 N)	0.65 (−0.135 N)	1.00 (0.044 N)	1.00 (0.025 N)	1.00 (−0.017 N)
Substance use	0.97 (0.000 N)	0.95 (0.000 N)	0.76 (0.000 N)	0.99 (0.000 N)	0.95 (0.000 N)	1.00 (0.000 N)
Humor	0.04 (0.294 S)	0.84 (0.170 N)	0.99 (−0.037 N)	0.94 (−0.128 N)	0.03 (−0.343 S)	0.77 (−0.215 S)
Self-blame	0.07 (−0.127 N)	1.00 (0.000 N)	0.09 (0.281 S)	0.88 (0.135 N)	0.005 (0.387 S)	0.48 (0.302 S)

Tukey's honestly significant difference or Games–Howell for post hoc multiple comparisons was applied depending on whether or not they met the assumption of homoscedasticity. G_a, no-NASH without significant fibrosis; G_b, NASH with significant fibrosis; G_c, no-NASH with significant fibrosis; G_d, NASH without significant fibrosis; COPE-28, Brief COPE.
[a]Effect sizes: N, null; S, small; M, medium.

TABLE 11 | Binary logistic regression analysis with total CLDQ-NAFLD as the dependent variable.

Variables	Total CLDQ-NAFLD						
	Coefficient	SE	AUC (CI)	*P*	OR	95% CI	
						Lower	Upper
Significant fibrosis	−0.693	0.347	0.616 (0.566–0.666)	0.04	0.500	0.253	0.987
Sex	−0.694	0.344	0.614 (0.564–0.664)	0.04	0.500	0.254	0.981
Total depression BDI-II	−0.277	0.070	0.887 (0.858–0.916)	<0.001	0.758	0.661	0.869
PCS	0.160	0.023	0.789 (0.747–0.831)	<0.001	1.174	1.123	1.227
Total anxiety HADS	−0.154	0.063	0.785 (0.745–0.825)	0.01	0.858	0.758	0.971
MCS	0.070	0.02	0.768 (0.724–0.811)	0.004	1.073	1.022	1.125

SE, standard error; AUC, area under the ROC curve; OR, odds ratio; CI, confidence interval; PCS, physical component summary; MCS, mental component summary; CLDQ-NAFLD, Chronic Liver Disease Questionnaire—Non-alcoholic Fatty Liver Disease; BDI-II, Beck Depression Inventory—II; HADS, Hospital Anxiety and Depression Scale.

Indeed, in the study by Vilar-Gómez et al. (41), just 10% of patients lost 10% of body weight in spite of including behavioral meeting bimonthly. Keeping in mind the influence that variables such as QoL (42), mental health (43), coping strategies (44), or perceived social support (45) exert on therapeutic adherence, the biopsychosocial risk factors found in this study could be associated with a negative impact on adherence to intervention guidelines in NAFLD patients.

A structured psychological intervention could therefore improve therapeutic adherence and, as a consequence, the patient's clinical evolution (46), requiring special attention those patients with a low social support, significant fibrosis, or of the female sex due to their greater tendency to show a higher-risk biopsychosocial profile. We therefore recommend the inclusion of cognitive–behavioral treatment in NAFLD interventions (47) with techniques such as: psychoeducation focusing on NAFLD and how it progresses, as patients are generally unaware of their disease and the long-term consequences to their health (48); cognitive restructuring to intervene on unrealistic expectations related to weight loss, significantly linked to quitting therapy (49); problem-solving strategies to cope with obstacles to weight loss or maintenance (50); reinforcing alternative behaviors to eating without being hungry, for instance using relaxation or distraction techniques (50); using self-report questionnaires about weight, physical activity, and diet (51); setting commitments and realistic personal goals about weight loss or physical activity (49); and controlling stimuli, for example, keeping high-fat foods out of reach and placing those recommended in an accessible place at home (49).

Our study showed several limitations. Firstly, the possible collinearity of fibrosis and age. Secondly, the cross-sectional design of the current study did not allow us to analyze changes in histological features over time and their impact on the psychological profile. Thirdly, we did not analyze how self-efficacy, an important variable in chronic liver diseases, could influence the QoL and mental health of NAFLD patients (8). Lastly, analysis of the impact of other pathologies such as type 2 diabetes, arterial hypertension, hypercholesterolemia, hypertriglyceridemia, cardiovascular disease, thyroid disease, or obstructive sleep apnea syndrome on the biopsychosocial profile of NAFLD patients would be of interest for future research. Nevertheless, the large sample of consecutive patients from real clinical practice in Spain may be considered a major strength of this study.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because they may compromise the privacy of study participants and may not be shared publicly. The public availability of the data is restricted by the Ethics Committee of the Virgen del Rocío University Hospital of Seville (Spain). Data are available upon request to the authors. Requests to access the datasets should be directed to Jesús Funuyet-Salas, jfunuyet1@us.es.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Virgen del Rocío University Hospital of Seville (Spain). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JF-S, MP-S-G, AM-R, and MR-G designed and conducted research, collected, analyzed, interpreted the data, and wrote the paper. All authors provided critical revision and approval of the manuscript and contributed equally to this article.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Hypoxia and Non-alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is currently the most common chronic liver disease worldwide and comprises varied grades of intrahepatic lipid accumulation, inflammation, ballooning, and fibrosis; the most severe cases result in cirrhosis and liver failure. There is extensive clinical and experimental evidence indicating that chronic intermittent hypoxia, featuring a respiratory disorder of growing prevalence worldwide termed obstructive sleep apnea, could contribute to the progression of NAFLD from simple steatosis, also termed non-alcoholic fatty liver or hepatosteatosis, to non-alcoholic steatohepatitis; however, the molecular mechanisms by which hypoxia might contribute to hepatosteatosis setup and progression still remain to be fully elucidated. In this review, we have prepared an overview about the link between hypoxia and lipid accumulation within the liver, focusing on the impact of hypoxia on the molecular mechanisms underlying hepatosteatosis onset.

Keywords: obstructive sleep apnea, NAFLD, hypoxia, hypoxia-inducible factors (HIFs), hepatosteatosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease among adults and children around the world whose prevalence between diabetic and obese individuals is around 80% compared to 30–50% of the general population (1). While in most NAFLD patients this liver disease is usually asymptomatic, only presenting a simple accumulation of fat in the hepatocyte called hepatosteatosis, 44–59% can progress to a more advanced form of liver injury termed steatohepatitis (NASH), featured by inflammation and ballooning with varied stages of fibrosis, which in turn can lead to more severe conditions of liver disease such as cirrhosis, portal hypertension, and, ultimately, hepatocellular carcinoma (2).

Clinical and experimental evidences suggest that hypoxia may play an important role in the pathophysiology of NAFLD. In that regard, the obstructive sleep apnea syndrome (OSA), a common disorder affecting 1–4% of the general population and 25–35% of obese individuals, is characterized by recurrent apnea or hypopnea episodes during sleep, leading to nocturnal intermittent hypoxia (IH), and it has been associated with all the components of metabolic syndrome, including NAFLD (3–5). However, the molecular mechanisms by which hypoxia might contribute to NAFLD setup and progression still remain to be fully elucidated. In this review, we have prepared an overview about the link between hypoxia and lipid accumulation within the liver, focusing our attention on how hypoxia regulates hepatic lipid metabolism and how, through these metabolic effects, it could contribute to the onset of the early phase of NAFLD called hepatosteatosis.

NAFLD PATHOGENESIS

The mechanisms underlying NAFLD onset and progression are complex and multifactorial. Different theories have been formulated, leading initially to the "two hits hypothesis" (6). The appearance of hepatosteatosis, defined as the presence of fat in more than 5% of hepatocytes, is considered the first "hit." The second "hit" involves factors triggering inflammation, hepatocellular damage, and fibrosis, leading to NASH. However, nowadays, a multiple-hit hypothesis has been postulated for NAFLD pathogenesis, which recapitulates the complexity of the human NAFLD where multiple parallel factors are implicated in the development and progression of the disease (7). Considering the first "hit," it is well known that hepatosteatosis results from an imbalance between hepatic free fatty acid (FFA) uptake, *de novo* lipogenesis, lipid oxidation, and lipid export *via* very low-density lipoprotein (VLDL) particles (8). This is a crucial phase in NAFLD outcome because an excessive content of FFAs and their metabolites triggers lipotoxicity in hepatocytes, leading to progression from hepatosteatosis to the more advanced forms of NAFLD, such as NASH (9). However, the key molecular pathways driving hepatosteatosis are not completely defined, but there is a growing scientific evidence indicating that hypoxia-inducible factors contribute to hepatosteatosis onset.

MOLECULAR AND CELLULAR CONSEQUENCES OF HYPOXIA ON HEPATOSTEATOSIS SETUP

Oxygen is so highly required in the cellular machinery that its lack implicates a quick response in order to adapt the cell to this new situation. This response is mediated by the hypoxia-inducible factors (HIFs), which are composed of two subunits—HIFα and HIFβ–considering HIF1α and HIF2α as the best characterized HIFα subunits (10). In the presence of normal oxygen levels, three distinct iron-dependent enzymes called "prolyl hydroxylase domain" (PHD) proteins are able to hydroxylate two specific prolyl residues in the HIFα subunit. This hydroxylation compromised the HIFα subunit's stability since it will be recognized and ubiquitinated by the von Hippel Lindau (VHL) protein for further proteasomal degradation. When the oxygen levels decrease, PHDs become inactive, so HIFα subunits are stabilized and translocated into the nucleus, where they interact with the constitutively expressed HIFβ subunit and other factors such as CBP/p300. This transcriptional complex interacts with the hypoxia response element (HRE) and leads to gene expression induced by HIFs, which is cell- and tissue-dependent (11).

TABLE 1 | Principal clinical studies examining the impact of OSA on NAFLD.

Study	Sample size (patients/controls)	Study design	Primary endpoints	Main findings
Minville et al. (35)	226 adult OSA patients/0 controls	Cross-sectional study	NAFL and NASH by non-invasive tools and OSA by polysomnography	Tc90% was significantly associated with NAFL, but not with NASH.
Sundaram et al. (36)	25 adolescent NAFLD patients (15 with OSA/10 without OSA)	Cross-sectional study	NAFLD by liver histology and OSA by polysomnography	OSA was significantly associated with NAFL, NAS score, and fibrosis stage.
Cakmak et al. (37)	118 adult OSA patients/19 without OSA	Cross-sectional study	NAFLD by ultrasonography and OSA by polysomnography	AHI and ODI were significantly higher in NAFLD than in controls.
Benotti et al. (38)	269 obese adults with OSA/93 obese adults without OSA	Cross-sectional study	NAFLD by liver histology and OSA by polysomnography	OSA severity was associated with NAFLD only in patients without metabolic syndrome.
Jullian-Desayes et al. (39)	103 adult OSA patients treated with effective CPAP vs. sham CPAP	Randomized controlled clinical trial	NAFLD by non-invasive tools and OSA by lung function parameters	NAFLD did not improve after 6–12 weeks of effective CPAP treatment.
Trzepizur et al. (40)	1,170 adult OSA patients/115 adults without OSA	Cross-sectional study	NAFLD by non-invasive tools and OSA by respiratory recordings	OSA severity correlated with hepatosteatosis, but not with fibrosis.
Asfari et al. (41)	1,490,150 hospitalized OSA patients/29,222,374 non-OSA hospitalized patients	USA database study	OSA and NASH diagnosis by ICD-9 code in clinical records	NASH diagnosis was 3-fold more frequent among OSA patients than in non-OSA patients.
Jin et al. (42)	2,272 adult OSA patients (2007–2017)	Meta-analysis and systematic review	NAFLD by liver histology and OSA by polysomnography	OSA positively correlated with hepatosteatosis, ballooning, and fibrosis.
Kim et al. (43)	351 adult OSA patients on CPAP therapy	Institutional prospective database study	NAFLD by transaminases and APRI index and OSA by polysomnography	OSA patients with good adherence to 3 months CPAP therapy improved transaminases and APRI index (liver fibrosis).
Sundaram et al. (44)	Nine adolescent OSA patients on CPAP therapy/23 adolescent untreated OSA patients	Observational longitudinal study	NAFLD by transaminases and OSA by polysomnography	Effective 3 months CPAP therapy improved ALT.
Schwenger et al. (45)	49 obese adults with NAFLD/12 obese adults with normal liver	Cross-sectional study	NAFLD by liver histology and OSA by polysomnography	AHI positively correlated with liver inflammation.

OSA, obstructive sleep apnea; NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver or hepatosteatosis; NASH, non-alcoholic steatohepatitis; NAS, NAFLD activity score; AHI, apnea/hypopnea index; ODI, oxygen desaturation index; Tc90%, percentage of sleep time with oxygen saturation <90%; CPAP, continuous positive airway pressure; APRI, aspartate aminotransferase-to-platelet ratio index.

Different experimental approaches have been used to determine the effects of hypoxia on NAFLD development. In this regard, it has been proposed that hypoxia signaling is involved in the regulation of hepatic lipid metabolism, given to both PHDs and HIFs a major role in this process. There is convincing experimental evidence that hypoxia induces lipid accumulation in mouse livers and human hepatocytes. Regarding the role of PHDs, whole-body *Phd1* gene inactivation promoted hepatosteatosis in mice fed a low-fat diet (12), and likewise the combined genetic deficiency of PHD2 and PHD3 led to severe hepatosteatosis (13), but, interestingly, the presence of a single *Phd1* or *Phd3* allele reduced liver fat content (14). Moreover, genetic deficiency of PHD2 protected mice against diet-induced hepatosteatosis (15), whereas oral administration of a pan-PHD inhibitor improved metabolic dysfunction, but was unable to reduce hepatosteatosis in wild-type mice fed a high-fat diet (HFD) (16), suggesting that the potential beneficial effects of pharmacologic PHD inhibition on hepatosteatosis should target only PHD2. In any case, the role of PHDs on hepatosteatosis onset still remains to be fully elucidated and deserves further investigation.

Downstream of PHDs which constitute the first oxygen sensors, HIFs are the key mediators of the cellular transcriptional response to hypoxia, regulating the expression of more than 300 genes involved in many biologic processes such as angiogenesis, erythrocytosis, and glucose and lipid metabolism, among others (17). Regarding the role of HIFs on hepatosteatosis setup, some experimental studies revealed that both HIF1α and HIF2α were involved in hypoxia-induced lipid accumulation in hepatocytes, whereas other studies showed HIF2α as the major regulator of hepatic lipid metabolism because the absence of HIF2α, but not HIF1α, protected against lipid accumulation in the livers from mice lacking the *Vhl* gene (18, 19). Supporting the latter, we have just demonstrated that HIF2α induced CD36 expression and function, the major driver of FFA uptake, triggering lipid accumulation in hepatocytes *in vitro* and *in vivo*, thus contributing to the hepatosteatosis onset (20). In this line, it has been demonstrated that the development of steatosis in hypoxic HepG2 cells is a consequence of increased HIF2α, which upregulated the hepatic expression of the adipose differentiation-related protein (ADRP), also involved in FFA uptake (21). It has also been reported that hypoxia-induced HIF2α stabilization led to β-oxidation suppression *via* PPARα in fat-laden hepatocytes (22). Moreover, oxygen therapy ameliorated hepatic steatosis induced by HFD in mice by reducing hepatic HIF2α and lipogenic gene expression (23). Taken together, the results derived from these studies suggest that HIF2α increases hepatic FFA uptake and *de novo* lipogenesis as well as decreases mitochondrial β-oxidation.

Regarding HIF1α, a number of experimental studies in distinct murine models have shown that either systemic or hepatic *Hifa* genetic deletion or HIF1α antisense oligonucleotide treatment decreased hepatosteatosis, suggesting the potential of HIF1α inhibition for the treatment of NAFLD (24, 25). Conversely, other studies have revealed that HIF1α protected against alcohol or choline deprivation-induced fatty liver (26, 27), so further investigations are needed to clarify the impact of HIF1α in hepatosteatosis setup.

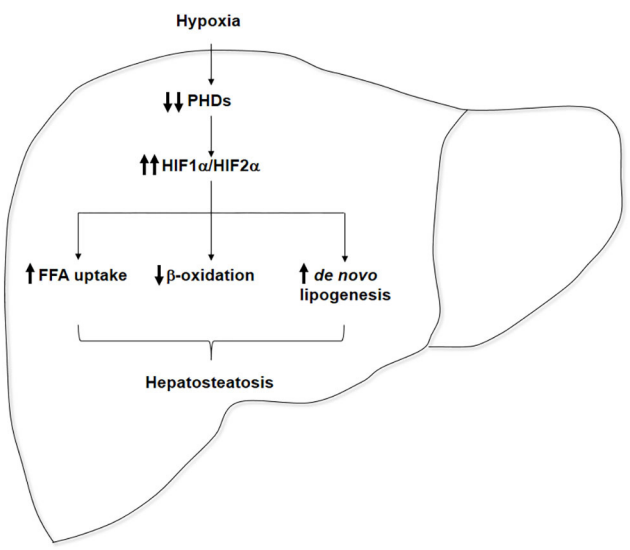

FIGURE 1 | Pathophysiological role of hypoxia in hepatosteatosis onset. Hypoxia inactivates prolyl hydroxylase domains (PHDs), enhancing hepatic HIF1α and HIF2α expressions, which could contribute to hepatosteatosis onset by the upregulation of free fatty acid (FFA) uptake, the repression of FFA β-oxidation, and the stimulation of *de novo* lipogenesis.

EXPERIMENTAL EVIDENCES LINKING INTERMITTENT HYPOXIA TO NAFLD

Besides the existence of an epidemiological relationship between OSA and NAFLD, there is emerging evidence indicating that IH featuring OSA contributes to NAFLD onset and progression, but the underlying molecular mechanisms are not fully defined. One of the main purposes of the studies published concerning this issue was to determine the gene expression profile involved in lipid metabolism under IH conditions, considering HIFs as the main drivers in this regulation. As has been stated before, HIFs are the master regulators of the cellular response to hypoxic stress (10). In this regard, it has been experimentally demonstrated that IH could be a major trigger for NAFLD. Indeed, IH directly induced hepatosteatosis through the administration of repetitive brief periods of hypoxia and reoxygenation mimicking OSA in animal models (28). Several studies from the same research group demonstrated that IH promoted hepatic lipid accumulation mainly by inducing *de novo* lipogenesis. They firstly established that IH caused dyslipidemia and hepatosteatosis by activating the SREBP1c–SCD1 signaling pathway in the liver of lean mice (29). Later on, they demonstrated that partial *Hifa*-deficient mice were protected against hepatosteatosis and hyperinsulinemia induced by IH (30). In addition, they also showed that partial HIF1α knockdown modulated SREBP1c, SREBP cleavage-activating protein (SCAP), and SCD1 expressions in mice under IH, confirming the previous hypothesis (30, 31). Regarding HIF2α, a recent study revealed that IH exacerbated hepatosteatosis in

mice fed HFD, which showed hepatic HIF2α overexpression along with a decreased β-oxidation and an enhanced *de novo* lipogenesis (22). Interestingly, silencing of *hif-2α* reduced lipid accumulation in hypoxic hepatocytes (20, 22), pointing out to HIF2α as a key driver in hepatosteatosis setup. Recently, we have observed an upregulated expression of CD36, together with an increased triglyceride content, in livers from mice exposed to IH, pointing out that IH may also modulate FFA uptake (32).

CLINICAL EVIDENCES LINKING OSA TO NAFLD

As stated above, OSA has been linked to lipid accumulation in the liver (33). In this regard, well-designed meta-analysis and systematic reviews have pointed out the relationship between OSA and NAFLD, stating that OSA is associated with an increased prevalence of hepatosteatosis, NASH, and fibrosis, independently of well-known risk factors such as age, sex, body mass index, or waist circumference (3, 34). Additional clinical studies and clinical trials have reinforced this notion, and their more relevant findings are summarized in **Table 1**. Notably, a study has demonstrated that OSA patients were three times more likely to have NASH compared with subjects without OSA (41). Moreover, clinical evidence suggests a direct relationship between OSA and NAFLD severity (42, 45). Interestingly, low O_2 saturation has been proposed as an important NAFLD risk factor in OSA patients: the lower the O_2 saturation, the higher the NAFLD severity (37, 46). Indeed, in a large study comprising 1,285 patients with suspected OSA aimed to assess the potential relationship between OSA and NAFLD, a significant positive correlation between the severity of hypoxemia and serum markers of liver injury was observed (40).

OSA is especially prevalent among obese individuals, but IH may differently affect the liver and adipose tissue in obese patients as it has been strongly associated with liver damage, whereas, apparently, it has no effect on adipocyte morphology or adipose tissue macrophage accumulation (47). Several studies examining cohorts of obese patients with sleep apnea have found that IH is closely associated with NAFLD diagnosed using non-invasive tools (35), but, even more important, with the histological features of NASH including lobular inflammation, hepatic ballooning, and hepatic fibrosis (28). Interestingly, a dose–response relationship has been observed between the severity of nocturnal hypoxia and liver injury in obese patients in the absence of metabolic syndrome (38).

OSA has been related to pediatric NAFLD as well: it affects 68% of obese and 44% of non-obese children with NAFLD. In fact, a correlation between the severity of hypoxia and the severity of pediatric NAFLD has been observed since liver tissue infiltration by leukocytes and activated macrophages as well as fibrosis and liver apoptosis are increased in these patients (36).

Taking this background into account, it is conceivable that continuous positive airway pressure (CPAP), which is the first-line therapy for OSA patients, could be useful by attenuating IH-related deleterious effects. In this regard, there are clinical evidence that arterial hypertension and elevated circulating catecholamine levels, commonly seen in OSA patients, improve after CPAP treatment (39). Regarding NAFLD, contradictory reports have been published. It has been reported that CPAP treatment appeared to have no significant effect on OSA-related liver injury as well as on lipid and glucose metabolism (48); conversely, CPAP treatment in adult and adolescent patients with OSA caused an improvement in serum aminotransferase activity as well as an apparent regression of hepatic fibrosis (43). Therefore, the potential beneficial effects of CPAP therapy on cardiovascular complications and metabolic disorders, such as insulin resistance and NAFLD, associated with OSA remain to be fully elucidated, and studies in large well-designed clinical trials assessing the impact of CPAP therapy on NAFLD in patients with OSA patients are clearly needed.

CONCLUSIONS

Emerging evidence suggests that OSA may play a role in the onset of hepatic steatosis and in the progression of NAFLD. Several cross-sectional studies showed that the severity of IH in patients with OSA predicts the severity of NAFLD on liver biopsy. Different animal models have provided insights on the potential effects of hypoxia on the molecular mechanisms underlying NAFLD pathogenesis, which are graphically represented in **Figure 1**, showing that hypoxia upregulates both HIF1α and HIF2α in the liver, which may increase hepatic steatosis by the induction of *de novo* lipogenesis and FFA uptake and by the repression of FFA β-oxidation. However, the role of HIFs in the pathogenesis of IH-induced NAFLD is yet to be fully elucidated. Thus, multiple studies point out that IH featuring OSA may contribute to the progression of NAFLD, but definitive clinical studies and experiments in validated mouse models of NAFLD have yet to be done. Nevertheless, hypoxia could be considered as another "hit" among the "multiple parallel hits" that have been proposed as responsible for NAFLD setup and progression to NASH.

AUTHOR CONTRIBUTIONS

ÁG-R and CG-M organized review structure. SI, EdP-M, LD-A, LE, ÁG-R, and CG-M participated in the bibliographic search. SI, ÁG-R, and CG-M wrote the manuscript. All authors were involved in editing the paper and had final approval of the submitted and published versions.

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Cigarette Smoking Increased Risk of Overall Mortality in Patients With Non-alcoholic Fatty Liver Disease: A Nationwide Population-Based Cohort Study

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Background: The evidence suggests a detrimental effect of cigarette smoking on the progression of chronic liver disease. However, the impact of cigarette smoking on mortality among patients with non-alcoholic fatty liver disease (NAFLD) remain unclear.

Methods: We used the National Health Examination Survey data collected during 2008–2009 to link the National Death Index to follow-up respondent survival. Diagnosis of NAFLD was based on a lipid accumulation product in participants without significant alcohol use or other liver diseases.

Results: During 64,116 person-years of follow-up, 928 of 7,529 participants with NAFLD died, and the cumulative all-cause mortality was 14.5 per 1,000 person-years. In a Cox regression model adjusted for age, body mass index, alcohol intake, exercise, comorbidities, lipid profiles, and handgrip strength, current smoking increased the risk of mortality by 109% (adjusted hazard ratio (aHR): 2.09, 95% confidence interval [CI]: 1.18–3.71) compared with never smoker status in women, but showed only a trend toward harm among men (aHR: 1.41, 95% CI: 0.96–2.08). After controlling for potential confounders, smoking ≥10 pack-years continued to show a significant harmful effect on all-cause mortality among women (aHR: 5.40, 95% CI: 2.19–13.4), but not in men. Among women who drink alcohol ≥10 grams per day, current smoking (aHR: 13.8, 95% CI: 1.66–145) and smoking ≥10 pack-years (aHR: 310, 95% CI: 78–1,296) also significantly increased risk of death.

Conclusion: This nationwide population-based study highlight a detrimental effect of cigarette smoking on mortality, with a similar but more definite association in women than in men with NAFLD.

Keywords: cigarette smoking, alcohol, mortality, non-alcoholic fatty liver disease, gender

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a growing global health problem that affects almost a quarter of the world's population (1). NAFLD-related liver complications are predicted to become the most common indication for liver transplantation within the next decade (2). Notably, a large body of clinical evidence indicates that NAFLD is also associated with an increased risk of other extrahepatic manifestations, such as cardiovascular disease and extrahepatic cancers, which are the predominant causes of mortality in patients with NAFLD (3–6). Since NAFLD causes a considerable health burden worldwide, it is important to identify modifiable risk factors and develop prevention strategies.

Cigarette smoking is a known major risk factor for developing chronic diseases, such as chronic obstructive pulmonary disease, cardiovascular disease, and several malignancies (7–9). The risk of death from these conditions increases with increasing exposure to cigarette smoking, as measured by the number of cigarettes smoked daily and the duration of smoking (10). There is also growing evidence that cigarette smoking has negative association with the prognosis of chronic liver diseases (11–16). Cigarette smoking was demonstrated to be associated with a dose-dependent relationship with the stage of liver fibrosis in patients with NAFLD via its effect on insulin resistance (11).

Patients with advanced liver fibrosis are believed to have the highest risk of progressing to end-stage liver disease. A recent meta-analysis of 4,428 NAFLD patients showed fibrosis stage to be the histological feature independently associated with liver-related events, liver-related mortality, and overall mortality (17). The relationship between fatal clinical outcomes and lifestyle risk factors, mainly moderate alcohol consumption, has been established (18). However, little is known about the effect of cigarette smoking on long-term prognosis among persons with NAFLD in the general population. Accordingly, this study was designed to evaluate the effect of cigarette smoking on overall mortality in patients with NAFLD after adjustment for important potential confounders using patient data derived from a nationwide population-based cohort.

MATERIALS AND METHODS

Study Population

Study participants were enrolled from the Fourth Thai National Health Examination Survey (NHES-IV), which is a nationally representative survey that employs a complex multistage, stratified strategy to sample Thai civilian, non-institutionalized population (19). The NHES-IV cohort included 21,960 persons aged ≥15 years that were recruited during August 2008 to March 2009. In the present study, 19,181 persons aged ≥18 years were included in the analysis.

Data Collection and Measurements

All information was collected in face-to-face interviews conducted by research nurses using standardized questions. Smoking status was classified as never smoker, former smoker, or current smoker. Participants who smoked <100 cigarettes during their lifetime were classified as never smokers. Current smokers were defined as those who currently smoke and who reported having smoked more than 100 cigarettes in their lifetime. Former smokers were defined as those who reported having smoked >100 cigarettes in their lifetime, but who no longer smoked at all at the time of the health checkup examination. Cigarette smoking was also quantified as pack-years, which was defined as the average number of packs per day multiplied by the number of years as a smoker. Alcohol consumption was calculated using self-reported questionnaire items relating to the frequency and amount of alcohol use per day over the 12-months period preceding the examination. The weekly frequency of physical activities was assessed by the Global Physical Activity Questionnaire version 2.

Blood pressure was measured using a standard automatic blood pressure monitor. Weight, height, and waist circumference (WC) were measured using standard procedures, and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Obesity was defined as BMI ≥25 kg/m^2, and central obesity was defined as a WC of >90 cm for men and >80 cm for women, following the Asian-specific criteria (20). Metabolic syndrome was defined according to the harmonizing criteria (21). Handgrip strength was measured by digital dynamometer.

Blood samples were obtained from participants in the morning after an overnight fast. The samples were transferred for determination of fasting plasma glucose using an enzymatic hexokinase method. Total cholesterol, low-density lipoprotein cholesterol (LDL-C), triglycerides, and high-density lipoprotein cholesterol (HDL-C) were measured by homogeneous enzymatic colorimetric methods. The non-HDL-C and total cholesterol to HDL-C ratio were calculated to estimate cardiovascular risk.

Definition of NAFLD

To identify patients with NAFLD, NHES participants with the following conditions were excluded: excessive alcohol consumption (defined as >210 g/week for men, and >140 g/week for women) or any other possible causes of chronic liver disease (**Figure 1**). Lipid accumulation product (LAP) was the parameter that was then used to identify NAFLD among the remaining subjects (22, 23). LAP is a non-invasive method that predicts the presence of NAFLD based on the patient's WC and triglyceride concentration that is calculated using the formula: [WC (cm) – 65] × triglyceride concentration (mmol/L)] for men, and [WC (cm) – 58] × triglyceride concentration (mmol/L)] for women. This model was reported to be a valid assessment for establishing NAFLD in general population. Using ultrasound as a reference, hepatic steatosis can be predicted by LAP with an area under the curve value of 0.843 (95% confidence interval [CI]: 0.837–0.849) in men, and 0.887 (95% CI: 0.882–0.892) in women (23). In the present study, participants were presumed to have NAFLD if they had a LAP score of ≥30.5 in men, and ≥23.0 in women (23).

Mortality Follow-Up

Follow-up and mortality data were obtained by linking the NHES data to the National Civil Registration and Vital Statistics System, Ministry of Interior, which is the most reliable database source of

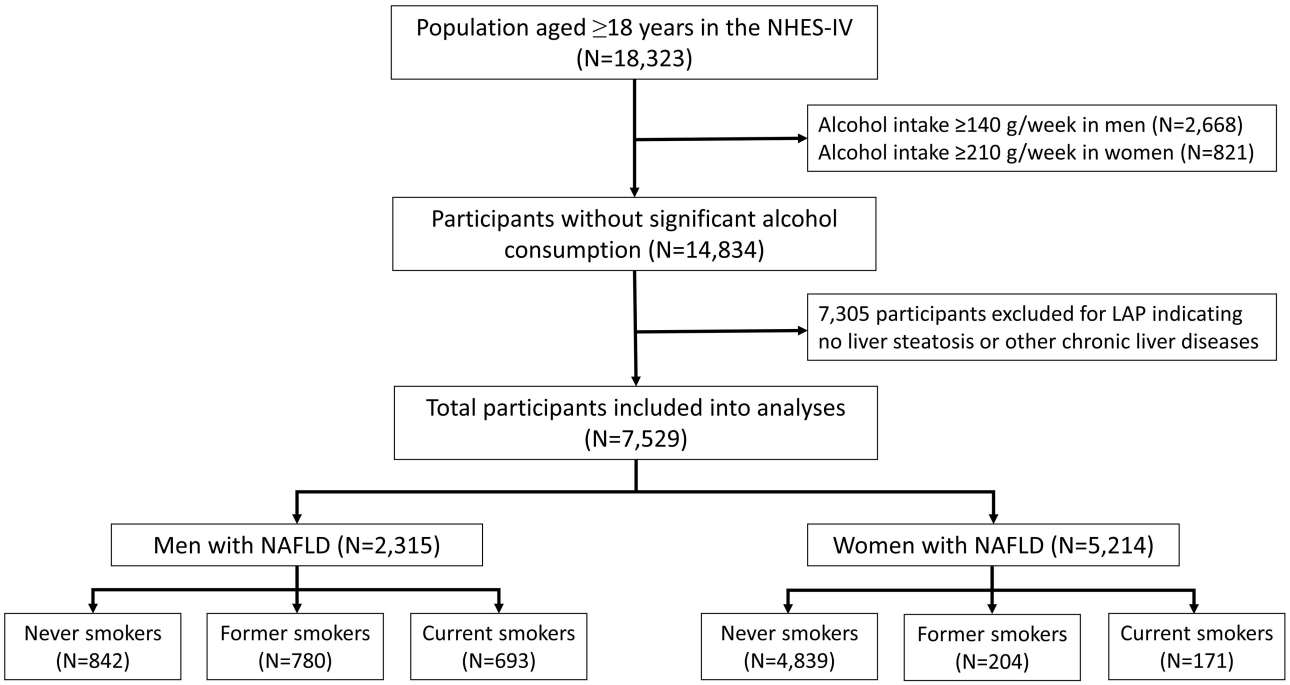

FIGURE 1 | Flow diagram of the study protocol. NHES-IV, National Health Examination Survey IV; LAP, lipid accumulation product; NAFLD, non-alcoholic fatty liver disease.

mortality data in Thailand. Information regarding vital status was obtained from the date of NHES-IV survey participation to 31 December 2019, or to the date of death. The study was carried out in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board. Written informed consent was obtained from all participants during the initial assessment.

Statistical Analysis

The analysis took into account the complex survey design, since all of the estimates were weighted according to the inverse probability of being sampled based on the registered Thai population. Chi-square test and one-way analysis of variance (ANOVA) were used to compare the baseline characteristics of study participants among the smoking status categories. *Post hoc* multiple comparison analysis was performed with Bonferroni correction. The survival analysis of participants with NAFLD was performed using unadjusted Kaplan-Meier method, and log-rank test was used to compare survival distribution between groups.

Cox proportional hazard regression models were constructed to estimate adjusted hazard ratios (aHRs) of overall mortality based on smoking status and pack-years. Models were initially adjusted for age, and then further adjusted for BMI, alcohol intake, regular exercise, and medical conditions (metabolic syndrome, diabetes, hypertension, and history of cardiovascular and cerebrovascular diseases). To account for other potential confounders, a fully adjusted analysis was performed, which included handgrip strength and LDL-C, HDL-C, and triglyceride levels. All analyses were performed using STATA version 14.0 (StataCorp LP, College Station, Texas, USA). A two-sided p-value of <0.05 was considered statistically significant.

RESULTS

Population Characteristics

After excluding participants with significant alcohol use and other possible causes of chronic liver disease, 14,834 participants were eligible for inclusion (**Figure 1**). Of those, 2,315 men (38.3%) and 5,214 (59.4%) women had a LAP score of ≥30.5 and ≥23, respectively, and those 7,529 participants were classified as having NAFLD. The proportions of never, former, and current smokers based on the self-report were 36.4, 33.7, and 29.9% in men, respectively, and 92.8, 3.9, and 3.3% in women, respectively. Among the current smokers, the median pack-year of cigarette smoking was 15 (range: 8–25.6) in men, and 8.5 (range: 4.2–15) in women.

Among men, current smokers were more likely to be younger, alcohol drinkers, have higher measurements for glucose, triglycerides, total cholesterol to HDL-C ratio, and handgrip strength and lower HDL-C, and less likely to have hypertension and history of cerebrovascular disease compared with never smokers (**Table 1**). Former smokers were more likely to be older, alcohol drinkers, have hypertension and history of cerebrovascular disease, and have lower measurements for BMI, WC, and handgrip strength compared with never smokers. In women, both former and current smokers were more likely to be older and have lower measurements for BMI and handgrip strength compared with never smokers (**Table 2**).

TABLE 1 | Baseline characteristics of male study participants with NAFLD by smoking status.

Characteristics	Smoking status				*p*-value	Multiple comparison
	Overall	Never smoker (a)	Former smoker (b)	Current smoker (c)		
Number (%)	2,315 (100%)	842 (36.4%)	780 (33.7%)	693 (29.9%)		
Age (years)	49.1 ± 13.2	47.7 ± 13.2	55.0 ± 12.5	46.5 ± 12.3	**<0.001**	a ≠ b ≠ c
Body mass index (kg/m^2)	26.6 ± 3.8	27.0 ± 3.8	26.4 ± 3.6	26.3 ± 3.8	**<0.001**	a ≠ b, a ≠ c
Waist circumference (cm)	90.7 ± 9.2	91.8 ± 9.3	91.0 ± 9.2	89.6 ± 9.0	**<0.001**	a ≠ b ≠ c
Obesity, *n* (%)	1,354 (62.8%)	541 (66.9%)	432 (60.3%)	381 (60.5%)	0.053	
Alcohol intake, *n* (%)	747 (38.7%)	219 (29.7%)	242 (41.0%)	286 (45.5%)	**<0.001**	a ≠ b, a ≠ c
Regular exercise, *n* (%)	1,729 (78.7%)	619 (77.8%)	615 (81.8%)	495 (77.6%)	0.506	
Cardiovascular disease, *n* (%)	93 (2.2%)	31 (1.5%)	44 (4.2%)	18 (1.5%)	**0.031**	a ≠ b, b ≠ c
Cerebrovascular disease, *n* (%)	83 (2.6%)	30 (4.0%)	42 (3.6%)	11 (0.6%)	**0.011**	a ≠ c, b ≠ c
Metabolic syndrome, *n* (%)	1,548 (59.2%)	582 (62.2%)	556 (60.2%)	410 (55.7%)	0.273	
Diabetes mellitus, *n* (%)	421 (11.2%)	148 (9.4%)	171 (13.2%)	102 (11.7%)	0.073	
Hypertension, *n* (%)	1,131 (33.6%)	421 (34.7%)	447 (41.6%)	263 (27.2%)	**<0.001**	a ≠ b ≠ c
Systolic blood pressure (mmHg)	130 ± 18	131 ± 19	133 ± 18	127 ± 15	**<0.001**	a ≠ c, b ≠ c
Diastolic blood pressure (mmHg)	80 ± 10	81 ± 10	81 ± 10	80 ± 10	**<0.001**	a ≠ c, b ≠ c
Glucose (mg/dL)	96 ± 31	96 ± 31	98 ± 32	95 ± 29	**<0.001**	a ≠ c, b ≠ c
Total cholesterol (mg/dL)	220 ± 44	221 ± 42	215 ± 45	223 ± 45	0.179	
LDL-C (mg/dL)	133 ± 40	135 ± 40	129 ± 38	134 ± 42	0.184	
HDL-C (mg/dL)	40 ± 8.9	40 ± 8.4	40 ± 8.3	38 ± 9.6	**<0.001**	a ≠ c, b ≠ c
Non-HDL-C (mg/dL)	180 ± 42	180 ± 39	175 ± 42	184 ± 43	**0.031**	b ≠ c
Total cholesterol to HDL-C ratio	5.7 ± 1.3	5.6 ± 1.2	5.5 ± 1.2	6.0 ± 1.4	**<0.001**	a ≠ c, b ≠ c
Triglycerides (mg/dL)	245 ± 130	226 ± 115	246 ± 132	261 ± 140	**<0.001**	a ≠ c, b ≠ c
Handgrip strength (kg)	38.6 ± 8.6	38.7 ± 9.1	36.8 ± 8.2	39.7 ± 8.1	**<0.001**	a ≠ b ≠ c

A p-value < 0.05 indicates statistical significance.

Data presented as number and percentage or mean ± standard deviation. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; NAFLD, nonalcoholic fatty liver disease.

Chi-square test and one-way analysis of variance (ANOVA) were used to compare the baseline characteristics of study participants among the smoking status categories. Post hoc multiple comparison analysis was performed with Bonferroni correction.

a ≠ b ≠ c: Statistically significant difference among never smoker (a), former smoker (b), and current smoker (c).

a ≠ b, a ≠ c: Statistically significant difference between never smoker (a) and former smoker (b), and between never smoker (a) and current smoker (c); however, there was no significant difference between former smoker (b) and current smoker (c).

b ≠ c: Statistically significant difference between former smoker (b) and current smoker (c).

a ≠ b, b ≠ c: Statistically significant difference between never smoker (a) and former smoker (b), and between former smoker (b) and current smoker (c); however, there was no significant difference between never smoker (a) and current smoker (c).

Current smokers were more likely to have a lower mean HDL-C, whereas former smokers were more likely to have metabolic syndrome and higher triglyceride concentrations compared with never smokers.

All-Cause Mortality

The mean follow-up period for the study cohort was 8.52 ± 1.43 years (range: 0.76–8.96). During the 64,116 person-years of follow-up, 928 participants with NAFLD died, and the cumulative all-cause mortality was 14.5 per 1,000 person-years. As shown in **Figure 2**, mortality from all causes was higher among male subjects with NAFLD than female subjects with NAFLD (19.2 vs. 12.5 per 1,000 person years, HR: 1.33, 95% CI: 1.17–1.52; $p < 0.001$). Compared to subjects without fatty liver and significant alcohol use, participants with NAFLD had non-significantly lower overall mortality (HR: 0.90, 95% CI: 0.68–1.20; $p = 0.46$) after adjusting for age and sex.

Smoking and Risk of All-Cause Mortality

The effect of smoking status on all-cause mortality in men and women with NAFLD is depicted in **Figure 3**. Self-reported current smoking significantly increased the risk of death in women, but not in men with NAFLD (**Table 3**). For women, after adjusting for age, BMI, alcohol intake, regular exercise, diabetes, hypertension, metabolic syndrome, and history of cardiovascular disease and cerebrovascular disease (model 1), the multivariable-adjusted HR (95%CI) for all-cause mortality comparing current smoking to never smoking was 1.99 (95% CI: 1.11–3.54). For model 2, which included LDL-C, HDL-C, triglycerides, and handgrip strength to the list of variables included in model 1, the effect of current smoking on all-cause mortality in women remained significant and was slightly increased (aHR: 2.09, 95% CI: 1.18–3.71). To investigate the combined effect of smoking and alcohol consumption, we performed stratified analysis by drinking level (<10 and ≥10 gm per day). Among women who drank <10 gm per day, former or current smoking was not

TABLE 2 | Baseline characteristics of female study participants with NAFLD by smoking status.

Characteristics	Smoking status				*p*-value	Multiple comparison
	Overall	Never smoker (a)	Former smoker (b)	Current smoker (c)		
Number (%)	5,214 (100%)	4,839 (92.8%)	204 (3.9%)	171 (3.3%)		
Age (years)	49.6 ± 13.6	49.2 ± 13.5	59.9 ± 12.5	55.7 ± 13.1	**<0.001**	a ≠ b ≠ c
Body mass index (kg/m^2)	26.8 ± 4.3	26.9 ± 4.3	25.4 ± 4.5	24.8 ± 3.7	**<0.001**	a ≠ b, a ≠ c
Waist circumference (cm)	85.9 ± 9.3	86.0 ± 9.3	84.7 ± 9.1	82.9 ± 8.8	**<0.001**	a ≠ c
Obesity, *n* (%)	3,181 (64.3%)	3,004 (65.1%)	101 (55.4%)	76 (41.6%)	**0.001**	a ≠ c
Alcohol intake, *n* (%)	619 (15.6%)	563 (15.6%)	28 (17.1%)	28 (15.1%)	0.879	
Regular exercise, *n* (%)	3,743 (81.1%)	3,490 (81.1%)	131 (75.7%)	122 (83.9%)	0.391	
Cardiovascular disease, *n* (%)	169 (2.2%)	153 (2.2%)	9 (2.4%)	7 (1.9%)	0.873	
Cerebrovascular disease, *n* (%)	127 (2.0%)	118 (2.0%)	7 (2.0%)	2 (0.6%)	0.135	
Metabolic syndrome, *n* (%)	2,228 (36.4%)	2,056 (36.4%)	106 (58.3%)	66 (37.7%)	**0.010**	a ≠ b
Diabetes mellitus, *n* (%)	917 (13.2%)	846 (13.2%)	44 (16.1%)	27 (11.4%)	0.678	
Hypertension, *n* (%)	2,330 (33.1%)	2,154 (32.7%)	104 (45.7%)	72 (35.1%)	0.171	
Systolic blood pressure (mmHg)	127 ± 19	127 ± 18	132 ± 18	125 ± 21	**0.009**	a ≠ b, b ≠ c
Diastolic blood pressure (mmHg)	77 ± 10	77 ± 10	79 ± 10	75 ± 11	0.053	
Glucose (mg/dL)	95 ± 34	95 ± 34	104 ± 38	99 ± 28	0.101	
Total cholesterol (mg/dL)	219 ± 45	219 ± 45	228 ± 49	220 ± 47	0.634	
LDL-C (mg/dL)	138 ± 41	138 ± 41	144 ± 45	140 ± 41	0.961	
HDL-C (mg/dL)	45 ± 10	46 ± 10	44 ± 10	44 ± 11	**0.023**	a ≠ c
Non-HDL-C (mg/dL)	174 ± 43	173 ± 43	185 ± 46	176 ± 44	0.432	
Total cholesterol to HDL-C ratio	5.0 ± 1.3	5.0 ± 1.3	5.4 ± 1.3	5.2 ± 1.3	0.052	
Triglycerides (mg/dL)	179 ± 101	179 ± 101	206 ± 107	180 ± 91	**0.024**	a ≠ b
Handgrip strength (kg)	24.9 ± 5.8	25.0 ± 5.7	22.2 ± 5.5	23.0 ± 5.7	**<0.001**	a ≠ b, a ≠ c

A p-value < 0.05 indicates statistical significance.

Data presented as number and percentage or mean ± standard deviation. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; NAFLD, nonalcoholic fatty liver disease.

Chi-square test and one-way analysis of variance (ANOVA) were used to compare the baseline characteristics of study participants among the smoking status categories. Post hoc multiple comparison analysis was performed with Bonferroni correction.

a ≠ b ≠ c: Statistically significant difference among never smoker (a), former smoker (b), and current smoker (c).

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associated with a significant effect on mortality compared to never smokers. Among women who drank ≥10 gm per day, former smokers (aHR: 5.91, 95% CI: 2.05–17.0) and current smokers (aHR: 13.8, 95% CI: 1.66–145) had a significant increase in the risk of death after adjusting for all potential variables.

We further explored the effect of cigarette smoking on overall mortality in patients with NAFLD based on smoking intensity (**Table 3**). Compared to never smokers, smoking ≥10 pack-years was associated with a significantly increased risk of death in women (aHR: 5.40, 95% CI: 2.19–13.4), but not in men after adjusting for age, BMI, alcohol intake, regular exercise, comorbidities, serum lipid profiles, and handgrip strength. Smoking <10 pack-years did not show a significant effect on mortality in either women or men with NAFLD. We also assessed the effects of smoking intensity on mortality in women with NAFLD according to drinking level. Among women who drank ≥10 gm per day, smoking ≥10 pack-years strongly significantly increased the risk of death (aHR: 310, 95% CI: 78–1,296) compared to never smokers after adjusting for all potential variables. Among women who drank <10 gm per day, smoking ≥10 pack-years was not associated with a significant effect on mortality compared to never smokers (aHR: 2.31, 95% CI: 0.80–6.67).

In the multivariable-adjusted model for the mortality risk, we found significant effects of sarcopenia and metabolic features, especially diabetes, on death among men and women with NAFLD (**Table 4**). To investigate the association between cigarette smoking and diabetes on mortality, we performed the stratified analysis by comorbid diabetes controlling for age, BMI, alcohol intake, regular exercise, hypertension, underlying atherosclerotic diseases, serum lipid profiles, and handgrip strength (**Table 5**). The significantly increased risk of mortality was observed with current smoking (aHR: 2.07, 95% CI: 1.07–4.03) and smoking ≥10 pack-years (aHR: 6.82, 95% CI: 2.66–17.5) in non-diabetic women but not in men. In the diabetic subjects, current smoking significantly increased the risk of

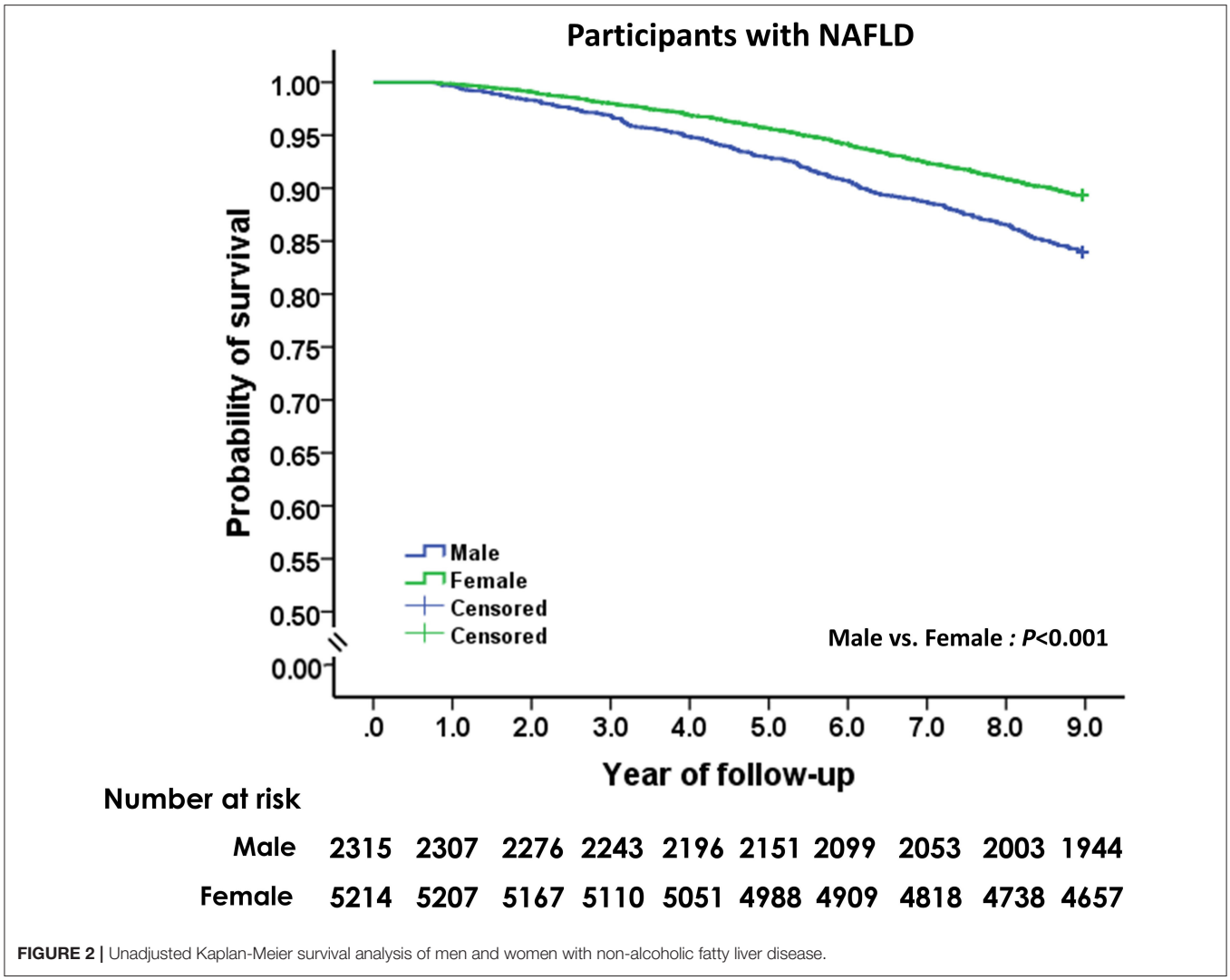


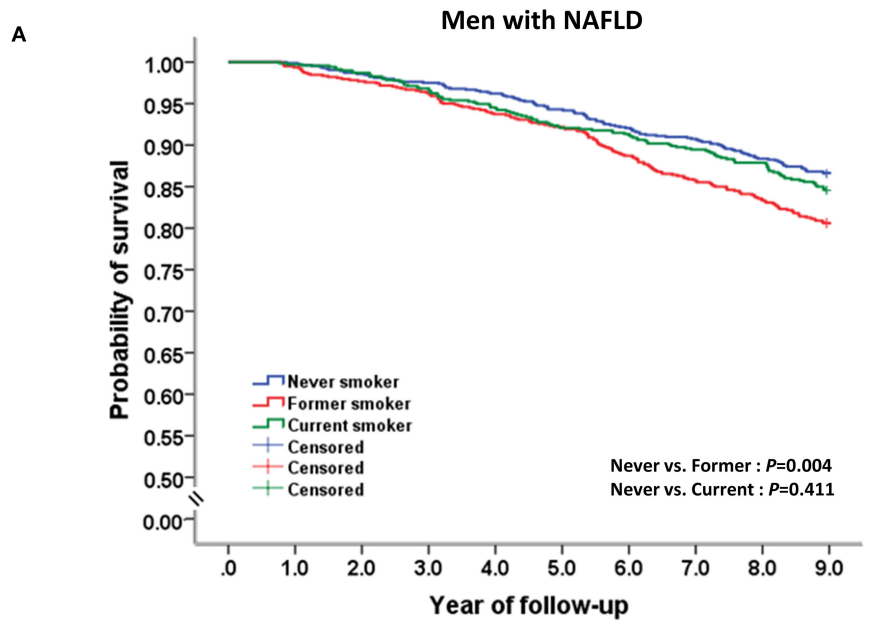
FIGURE 2 | Unadjusted Kaplan-Meier survival analysis of men and women with non-alcoholic fatty liver disease.

mortality compared with never smoking (aHR: 1.76, 95% CI: 1.09–2.84) in men, but showed only a trend toward harm among women (aHR: 1.61, 95% CI: 0.71–3.65). The potential hazard of smoking pack-years on mortality was observed in both men and women with diabetes but did not reach statistical significance.

DISCUSSION

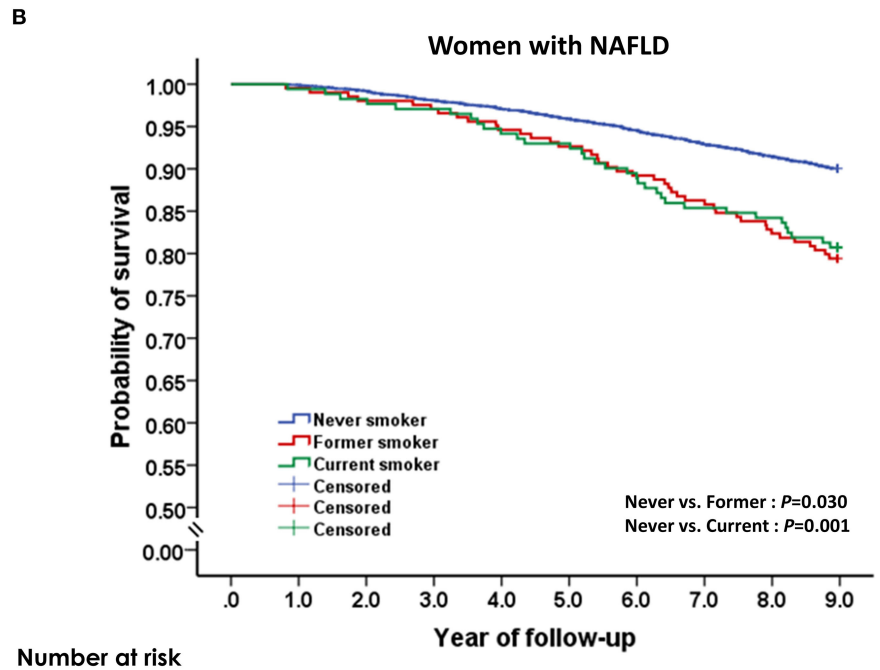
Though there is evidence suggesting potential detrimental effects of cigarette smoking in patients with NAFLD, the association between cigarette smoking and mortality has not been adequately evaluated in this population. In this nationally representative sample of 7,529 adults with NAFLD, we demonstrate that current smoking is associated with a robust and significant increase in all-cause mortality among women with NAFLD. Furthermore, smoking ≥10 pack-years is associated with an increased risk of mortality in women with NAFLD. We failed to find a significantly harmful effect of smoking in men with NAFLD. Interestingly, we found that modest alcohol consumption has a synergistic effect with cigarette smoking on all-cause mortality in women with NAFLD.

It has been suggested that cigarette smoking reduces life expectancy, primarily by increasing the risks of chronic obstructive pulmonary disease, cancer, and cardiovascular disease (10). However, epidemiological data specific to the relationship between cigarette smoking and the risk of death in NAFLD is scarce. Our study revealed current smoking to be significantly associated with a 2-fold increased risk of all-cause mortality among women with NAFLD, independent of alcohol intake, exercise, diabetes, hypertension, metabolic syndrome, underlying atherosclerotic diseases, lipid profiles, and handgrip strength. These findings are virtually identical to those from studies that evaluated the contemporary risks of smoking in Western and Asian countries, where the relative risk of all-cause mortality in current smokers compared to never smokers has been consistently reported to be 2.0 to 3.0 (24–26). Importantly, our findings indicate an elevated risk of mortality in patients with NAFLD based on the cumulative quantity of cigarettes smoked. In particular, women with NAFLD who smoked ≥10



Number at risk

Never smoker	842	841	830	821	810	794	775	764	744	729
Former smoker	780	775	762	751	731	719	692	669	650	629
Current smoker	693	691	684	671	655	638	632	620	609	586



Number at risk

Never smoker	4839	4834	4799	4746	4697	4640	4575	4496	4426	4357
Former smoker	204	203	200	198	193	189	182	176	168	162
Current smoker	171	170	168	166	161	159	152	146	144	138

FIGURE 3 | Unadjusted Kaplan-Meier survival analysis for smoking status on all-cause mortality in men **(A)** and women **(B)** with non-alcoholic fatty liver disease.

TABLE 3 | Overall mortality of study participants with NAFLD by smoking status and smoking intensity.

Variables	Person-years	Deaths	Deaths per 1,000 person-years	Age-adjusted HR (95% CI)	Multivariable-adjusted HR (95% CI)	
					Model 1	Model 2
MEN (*n* = 2,315)						
Smoking status						
Never smoker (*n* = 842)	7,131.2	113	15.8	Reference	Reference	Reference
Former smoker (*n* = 780)	6,425.9	151	23.5	1.16 (0.82–1.64)	1.27 (0.90–1.79)	1.31 (0.93–1.85)
Current smoker (*n* = 693)	5,812.4	107	18.4	1.35 (0.94–1.95)	1.52 (0.98–2.33)	1.41 (0.96–2.08)
Pack-years						
0 (*n* = 842)	7,131.2	113	15.8	Reference	Reference	Reference
<10 (*n* = 116)	982.4	12	12.2	1.79 (0.86–3.74)	2.10 (0.90–4.88)	1.46 (0.65–3.25)
10-19.9 (*n* = 93)	805.4	11	13.7	1.50 (0.54–4.19)	1.64 (0.44–6.04)	1.73 (0.53–5.64)
≥20 (*n* = 124)	1,051.7	20	19.0	1.35 (0.72–2.52)	1.20 (0.51–2.81)	1.09 (0.53–2.23)
WOMEN (*n* = 5,214)						
Smoking status						
Never smoker (*n* = 4,839)	41,646.1	482	11.6	Reference	Reference	Reference
Former smoker (*n* = 204)	1,687.9	42	24.9	1.31 (0.80–2.15)	1.34 (0.86–2.10)	1.32 (0.84–2.11)
Current smoker (*n* = 171)	1,413.3	33	23.3	1.90 (1.13–3.20)	1.99 (1.11–3.54)	2.09 (1.18–3.71)
Pack-years						
0 (*n* = 4,839)	41,646.1	482	11.6	Reference	Reference	Reference
<10 (*n* = 24)	204.2	4	19.6	0.64 (0.24–1.76)	0.17 (0.03–1.10)	0.19 (0.03–1.19)
≥10 (*n* = 21)	176.9	3	17.0	4.60 (2.31–9.15)	5.25 (2.26–12.2)	5.40 (2.19–13.4)

NAFLD, nonalcoholic fatty liver disease; HR, hazard ratio; CI, confidence interval.
Multivariable-adjusted model 1 was adjusted for age, body mass index, alcohol intake, regular exercise, diabetes, hypertension, metabolic syndrome, history of cardiovascular disease, and history of cerebrovascular disease.
Multivariable-adjusted model 2 was adjusted for the parameters in model 1 plus low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, and handgrip strength.

TABLE 4 | Effect of smoking status on all-cause mortality among men and women with NAFLD after adjusting for all variables.

	Men		Women	
	Adjusted HR (95% CI)	*p*-value	Adjusted HR (95% CI)	*p*-value
Smoking status				
Never smoke	Reference		Reference	
Former smoke	1.31 (0.93–1.85)	0.113	1.33 (0.84–2.11)	0.215
Current smoke	1.41 (0.96–2.08)	0.077	2.09 (1.18–3.71)	**0.014**
Age (years)	1.05 (1.03–1.07)	**<0.001**	1.04 (0.99–1.08)	0.058
Body mass index (kg/m^2)	1.00 (0.94–1.07)	0.875	0.99 (0.93–1.05)	0.619
Alcohol intake	0.69 (0.37–1.29)	0.227	0.77 (0.29–2.09)	0.597
Regular exercise	1.44 (0.85–2.43)	0.163	0.70 (0.43–1.14)	0.143
Cerebrovascular disease	1.43 (0.55–3.67)	0.442	0.98 (0.44–2.16)	0.948
Cardiovascular disease	1.71 (0.88–3.33)	0.107	1.67 (0.89–3.14)	0.106
Hypertension	1.52 (0.93–2.50)	0.092	1.39 (1.07–1.80)	**0.018**
Diabetes	1.65 (1.13–2.41)	**0.012**	2.09 (1.43–3.04)	**0.001**
Metabolic syndrome	0.86 (0.52–1.42)	0.545	1.06 (0.75–1.49)	0.742
LDL-C (mg/dL)	1.00 (0.99–1.01)	0.052	1.00 (0.99–1.01)	0.626
HDL-C (mg/dL)	0.97 (0.94–1.01)	0.064	0.99 (0.98–1.02)	0.874
Triglycerides (mg/dL)	1.00 (0.99–1.002)	0.423	1.001 (1.0001–1.002)	**0.039**
Handgrip strength (kg)	0.95 (0.91–0.99)	**0.018**	0.96 (0.93–0.99)	**0.024**

CI, confidence interval; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HR, hazard ratio; NAFLD, non-alcoholic fatty liver disease.

TABLE 5 | Overall mortality of study participants with NAFLD by diabetes and smoking status.

Variables	Person-years	Deaths	Deaths per 1,000 person-years	Age-adjusted HR (95% CI)	Multivariable-adjusted HR (95% CI)	
					Model 1	Model 2
MEN WITHOUT DIABETES (*n* = 1,894)						
Smoking status						
Never smoker (*n* = 694)	5938.6	75	12.6	Reference	Reference	Reference
Former smoker (*n* = 609)	5124.8	101	19.7	1.22 (0.82–1.81)	1.29 (0.90–1.86)	1.36 (0.95–1.93)
Current smoker (*n* = 59)	5027.7	75	14.9	1.32 (0.75–2.32)	1.42 (0.83–2.42)	1.30 (0.84–2.00)
Pack-years						
0 (*n* = 694)	5938.6	75	12.6	Reference	Reference	Reference
<10 (*n* = 101)	858.7	9	10.5	1.92 (0.65–5.66)	2.19 (0.75–6.36)	1.38 (0.49–3.86)
>10 (*n* = 186)	1599.6	21	13.1	1.22 (0.55–2.71)	1.14 (0.41–3.15)	1.12 (0.40–3.16)
MEN WITH DIABETES (*n* = 421)						
Smoking status						
Never smoker (*n* = 148)	1192.7	38	31.9	Reference	Reference	Reference
Former smoker (*n* = 171)	1301.1	50	38.4	1.04 (0.66–1.64)	1.13 (0.72–1.77)	1.21 (0.75–1.96)
Current smoker (*n* = 102)	784.8	32	40.8	1.48 (0.67–3.28)	1.69 (0.81–3.57)	1.76 (1.09–2.84)
Pack-years						
0 (*n* = 148)	1192.7	38	31.9	Reference	Reference	Reference
<10 (*n* = 9)	69.9	3	42.9	1.94 (0.34–11.2)	6.07 (1.06–34.9)	9.98 (1.57–63.3)
>10 (*n* = 37)	311.3	10	32.1	1.63 (0.44–6.11)	2.32 (0.38–14.2)	2.73 (0.47–15.9)
WOMEN WITHOUT DIABETES (*n* = 4,297)						
Smoking status						
Never smoker (*n* = 3,993)	34590.4	9.89	342	Reference	Reference	Reference
Former smoker (*n* = 160)	1320.9	24.98	33.0	1.31 (0.80–2.14)	1.32 (0.78–2.21)	1.33 (0.81–2.19)
Current smoker (*n* = 144)	1204.2	19.93	24.0	2.06 (1.09–3.90)	1.96 (1.01–3.82)	2.07 (1.07–4.03)
Pack-years						
0 (*n* = 3,993)	34590.4	342	9.9	Reference	Reference	Reference
<10 (*n* = 20)	173.6	3	17.3	0.49 (0.17–1.45)	0.37 (0.06–1.98)	0.44 (0.08–2.31)
>10 (*n* = 16)	136.8	2	14.6	6.11 (2.85–13.0)	6.48 (2.89–14.6)	6.84 (2.71–17.2)
WOMEN WITH DIABETES (*n* = 917)						
Smoking status						
Never smoker (*n* = 846)	7055.8	19.84	140	Reference	Reference	Reference
Former smoker (*n* = 44)	366.9	24.53	9.0	1.46 (0.69–3.09)	1.56 (0.75–3.27)	1.54 (0.73–3.26)
Current smoker (*n* = 27)	209.1	43.05	9.0	1.81 (0.93–3.52)	1.78 (0.71–4.50)	1.61 (0.71–3.65)
Pack-years						
0 (*n* = 846)	7055.8	140	19.8	Reference	Reference	Reference
<10 (*n* = 4)	30.6	1	32.7	0.66 (0.27–1.58)	NA	NA
>10 (*n* = 5)	40.1	1	24.9	1.70 (0.18–15.7)	2.23 (0.26–19.0)	1.86 (0.37–9.33)

NA, not assessed; NAFLD, nonalcoholic fatty liver disease; HR, hazard ratio; CI, confidence interval.

Multivariable-adjusted model 1 was adjusted for age, body mass index, alcohol intake, regular exercise, hypertension, history of cardiovascular disease, and history of cerebrovascular disease.

Multivariable-adjusted model 2 was adjusted for the parameters in model 1 plus low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, and handgrip strength.

pack-years had approximately 5-fold increased overall mortality compared to never smokers. This finding was adjusted for a range of potential confounders, including BMI, alcohol intake, exercise, and several comorbidities.

The influence of alcohol consumption within the accepted safe limits on the association between cigarette smoking and the risk of death by any cause was investigated in our NAFLD study. That analysis revealed an increased risk of all-cause mortality among women who smoke and drink a moderate amount alcohol, defined as consumption of equal to or more than 10 g per day. This finding is consistent with prospective studies that found even modest alcohol intake to be associated with possible disease progression (27), and more significantly, with cancer development in patients with NAFLD (28).

Gender-related differences in the effects of cigarette smoking on the risk of death are not well-established. In this NAFLD cohort, we found smoking to be more harmful in women than in men, with a larger negative impact from the same number of smoking pack-years. Since patients with NAFLD are at higher risk for cardiovascular disease compared to general population (4), active smokers with NAFLD are more likely to die from cardiovascular disease. This assertion is supported by evidence from a Danish study that showed higher cardiovascular disease risk in women who smoke than in men who smoke (29). This may be due to the fact that smoking causes downregulation of estrogen-dependent vasodilatation of the endothelial wall (30). Hence, the effect of cigarette smoking on the risk of atherothrombotic clinical events was higher in postmenopausal women (31). Cigarette smoking was also found to be a risk factor for developing extrahepatic malignancies (7). This association is clinically relevant since de novo tumors are a leading cause of death among NAFLD patients (3, 5, 6). Overall, these data indicate that female smokers with NAFLD are at higher risk for smoking-related morbidity and mortality. However, the effect of smoking on disease-specific mortality was not analyzed in our population because the NHES database could not capture the causes of death.

Although the prevalence of current smoking was somewhat higher among men than among women, we observed no significantly increased risk for overall mortality among men who currently smoke compared with never smokers. Further, we did not find a significant association between smoking $\geq$10 pack-years and mortality over the 9-year period among men with NAFLD. It is plausible that smoking-mediated diseases occur at a prolonged smoking duration and higher number of cigarettes smoked in male subjects, and it may require coincident genetic predisposition. Smoking cessation almost completely reverses the risk of cardiovascular disease due to smoking, which makes it potentially the most effective and lifesaving intervention available for those at risk for and those with existing cardiovascular disease (32). Unfortunately, we observed a non-significant increase in overall mortality among those who stopped smoking at an older age (the mean age of 55 years or older in both genders). The potentially higher risk of death in former smokers compared with never smokers might be explained by a tendency for smokers, particularly older smokers, to quit due to ill health.

Although the mechanisms by which smoking contributes to mortality among NAFLD patients remain unknown, it has been shown that smoking would accelerate atherosclerosis by inducing insulin resistance and altering lipid metabolism (33–35). However, we found that after adjusting for metabolic parameters (diabetes, hypertension, and lipid profiles), the association between current smoking and mortality risk remained significant, indicating that atherogenic risk factors did not fully explain the relationship. Another potential mechanism is that tobacco constituents induce or promote oxidative stress/inflammatory pathways, which can play a role in the pathogenesis of NAFLD (36, 37). Recently, a longitudinal cohort study demonstrated a dose-response relationship of smoking with the development and progression of NAFLD (38). Hence, it is anticipated that increased risk of steatohepatitis and advanced fibrosis possibly driven by smoking in patients with NAFLD may significantly impact their mortality. However, the NHES did not collect variables for estimating non-invasive fibrosis scores (e.g., Hepamet, Fibrosis-4, and NAFLD fibrosis scores) to identifying individuals in the NAFLD population at risk for advanced fibrosis. Given that diabetes is a major contributor to perpetual chronic injury leading to eventual steatohepatitis, advanced liver fibrosis, and hepatocellular carcinoma (39), individuals with diabetes may represent the target population with a high-risk of advanced liver disease. Thus, NAFLD patients with diabetes who continue smoking should be a subgroup with high mortality risk. Supporting this assumption, our analysis showed that current smokers with diabetes, particularly men, had a significantly greater probability of deaths than those who did not smoke. Nevertheless, a low number of current smokers in the diabetes subgroups limit the power to estimate smoking status and intensity associated with mortality, especially in women.

Sarcopenia that is defined as the loss of skeletal muscle mass and strength has been increasingly recognized in patients with NAFLD. It is a crucial indicator of adverse outcomes in patients with cirrhosis, including hepatic decompensation and premature mortality (40). In the Cox regression model, we found that weaker handgrip strength was significantly associated with an increased risk of death among NAFLD population. Thus, it may be suggested that the substantially increased risk of death may be mediated via sarcopenia among smoking women with NAFLD, who were more likely to have low measurements of handgrip strength and BMI. This finding is supported by data from a meta-analysis involving 22,515 participants reporting that cigarette smoking may contribute to the development of sarcopenia (41). Physiological mechanisms are complex, and several studies showed that cigarette smoking induced muscle protein degradation via oxidative stress and chronic inflammation (42).

This study has some limitations. Firstly, the diagnosis of NAFLD was made using LAP and not by gold standard tissue biopsy. Although a validation study showed that LAP exhibited high diagnostic accuracy for identifying NAFLD (23), some of our study participants would inevitably have been misclassified as NAFLD. Using a model based on anthropomorphic and laboratory data enabled us to study a large population-based sample and facilitate avoidance of ascertainment bias that is routinely found in clinical studies of conveniently selected patients. Secondly, the smoking assessment was based on self-reports and was not verified by objective measures of smoking (urinary cotinine and breath carbon monoxide tests). The assessment of smoking status by questionnaires may lead to inaccurate measures of smoking exposure due to smoking denial or difficulty in recalling the quantity and duration of smoking (43). This misclassification potentially leads to the underestimation of the detrimental effects of smoking exposure. However, nationwide studies in Canada and the United States revealed that smoking behavior self-reported by the participants in such surveys was highly consistent with estimates based on urinary or serum cotinine concentration (44–46). This data indicates that a low-cost questionnaire survey applicable to large samples can effectively determine the smoking status of the

general population. Lastly, the lack of a significant difference in mortality between the general population with and without NAFLD in this study should not prove that NAFLD does not lead to overall mortality. This contrasts with the findings from previous studies frequently conducted in NAFLD patients who underwent liver biopsies at specialty liver clinics (5, 6, 17). The difference between those and population-based studies is probably attributable to selection bias entailed in referral patients. Instead, we believe that it is likely a type II error, given a low number of deaths during a 9-year follow-up in our population-based sample. The study of the impact of NAFLD on mortality may require a much longer follow-up to draw a firm conclusion.

CONCLUSION

The results from this nationwide population-based cohort study suggest a detrimental effect of cigarette smoking on all-cause mortality, with a similar but more definite association in women than in men with NAFLD. In addition, our findings are consistent with previous studies showing a relationship between smoking intensity and mortality, where an increase in the quantification of cigarette smoke becomes more harmful. The results of this study can be used to improve counseling of the growing population of patients with NAFLD by discouraging smoking.

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 Ethics Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (COA no. Si 513/2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PC and WA contributed to study concept and design. PC drafted the initial manuscript. PC, KK, and WA contributed to analyze and interpret data, and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Association Between Anxiety and Depression and Nonalcoholic Fatty Liver Disease

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Backgrounds: Depression and anxiety disorder are frequently seen in patients with nonalcoholic fatty liver disease (NAFLD). However, the associations between mood disorders and NAFLD have not been fully evaluated. In this study, we investigated the relationship between NAFLD and depression or anxiety in a Korean population.

Methods: We conducted a retrospective cross-sectional study that included subjects who underwent abdominal ultrasonography and completed a symptom questionnaire for a routine health check-up. NAFLD was diagnosed and graded according to the ultrasonography findings. Depression and anxiety were assessed using the Beck Depression Inventory and State-Trait Anxiety Inventory, respectively.

Results: Among the total of 25,333 subjects, the mean age was 47 years (men, 56.2%), and the prevalence rate of NAFLD was 30.9%. In the multivariate analysis, NAFLD showed a significant association with depression [adjusted odds ratio (OR) 1.43 and 95% confidence interval (CI) 1.14–1.80, $p = 0.002$] in women. Severe NAFLD significantly correlated with state anxiety and trait anxiety (adjusted OR 1.84 and 95% CI 1.01–3.37, $p = 0.047$ and adjusted OR 2.45 and 95% CI 1.08–4.85, $p = 0.018$, respectively) in women.

Conclusions: There was a higher tendency of women with NAFLD to suffer from depression with increase in steatosis, and severe stage of steatosis was significantly associated with anxiety in the female compared to non-NAFLD. Understanding the association between NAFLD and mood disorders may have clinical implications for reducing the prevalence of comorbidities.

Keywords: hepatic steatosis, anxiety, depression, association, mood disorder

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KEY POINTS

- Depression and anxiety disorder are frequently seen in patients with nonalcoholic fatty liver disease (NAFLD). However, the associations between mood disorders and NAFLD have not been fully evaluated.
- NAFLD was significantly associated with depression, and severe stage of steatosis was significantly associated with anxiety in women compared to non-NAFLD.
- These findings provide new insight in understanding the association between NAFLD and mood disorders.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide, with increasing prevalence of up to 20–30% (1). Although NAFLD is generally a benign condition, some may progress to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (2). Hepatic steatosis is commonly associated with various metabolic conditions, including cardiovascular disease (3), diabetes (4), chronic kidney disease (5), and colorectal cancer (6). In addition, NAFLD has been associated with an increased prevalence of psychological conditions such as depression and anxiety. A previous study reported that the prevalence rates of lifetime major depressive disorder (MDD) and generalized anxiety disorder were more increased in patients with NASH with more advanced histological features compared to controls (7).

Major depression is a common, recurrent disease leading to decreased quality of life, disability (8), and mortality (9). The lifetime prevalence of major depressive episode is estimated from 3 to 29.9% (10). Depression has been associated with cardiovascular diseases (11) and metabolic syndrome (12), with increased predisposition for NAFLD, suggesting shared pathogenesis of insulin resistance (13). In particular, a recent study based on US claim data has shown that depression was independently associated with NAFLD (14). In addition, generalized anxiety disorder is one of the most common anxiety disorders (15), and anxiety disorder is associated with hyperglycemia in patients with diabetes (16). This result suggests that there is a close link between anxiety and metabolic disease.

Therefore, we hypothesized that depression and anxiety disorder would be related to NAFLD and investigated the relationship between NAFLD and anxiety or depression in Korean subjects who participated in health check-ups.

METHODS

Study Population

We performed a retrospective cross-sectional study that included subjects who underwent routine health check-up at the Seoul National University Hospital Healthcare System Gangnam Center between January 2008 and December 2011. Health examination has recently become popular in Korea because a thorough medical checkup can be performed in a few hours, and the majority of referred hospitals in Korea are now equipped with a Healthcare Center to provide such health check-ups. The subjects (age $\geq$ 20 years) voluntarily attended a general health check-up, while others were supported by their employers. They were mostly free of symptoms and voluntarily underwent examinations including abdominal ultrasonography and blood samplings and completed a symptom questionnaire on the same day. A schematic protocol of the study design is illustrated in **Figure 1**. Among the 34,147 subjects, 5,730 were excluded for potential cause of chronic liver disease, including hepatitis B virus positivity in 1,734 subjects, anti-hepatitis C virus antibody positivity in 307 subjects, and 3,689 subjects with significant alcohol consumption (>30 g/day for men and >20 g/day for women) (17). Additionally, 3,084 subjects were excluded because of missing data. The total number of eligible participants was 25,333.

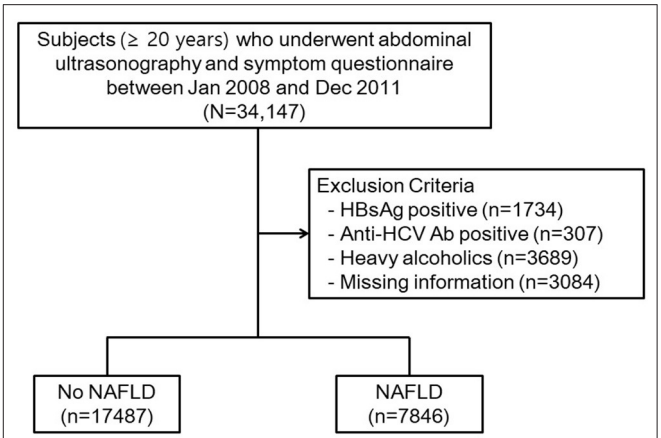

FIGURE 1 | Study population. HBsAg, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody.

The study protocol was approved by the Institutional Review Board of Seoul National University Hospital (1912–111–1089) and conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki. The requirement for informed consent from individual subjects was waived because we used de-identified secondary data.

Clinical and Biochemical Evaluations

Study data consisted of medical information based on a self-administered questionnaire and anthropometric and laboratory measurements, as described previously (18). Briefly, height and body weight were measured using a digital scale. The body mass index (BMI) was calculated as weight (kg)/height^2 (m^2). Waist circumference (WC) was measured by a well-trained person, at the midpoint between the lower costal margin and the iliac crest. Based on smoking status, subjects were categorized as smoker (former or current smoker) or never-smoker. Based on alcohol consumption, subjects were categorized as alcohol user or nonuser (does not drink any alcohol). Diabetes was defined as fasting glucose levels $\geq$126 mg/dl and/or treatment with an oral hypoglycemic agent or insulin. Systolic blood pressure and diastolic blood pressure were each measured twice, and their mean values were reported. All subjects were fasted for at least 12 h prior to blood sampling; aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol were measured.

Evaluation of the State of Anxiety and Depression

The State-Trait Anxiety Inventory (STAI) scale was used to assess the level of anxiety in all subjects (19). The STAI is a well-known psychological instrument consisting of two self-report rating scales with 20 items each, for the measurement of two types of anxiety: state anxiety (how one feels at the moment, STAI-X1) and trait anxiety (how one generally feels, STAI-X2).

Each item is rated between 1 and 4 depending on the frequency of target complaints (never, sometimes, often, and always), and overall scores are obtained by summing the ratings for the items (range, 20–80). We considered subjects showing moderate-to-severe state or trait anxiety by using a cut-off value of STAI-X1 $\geq$ 57 or STAI-X2 $\geq$ 59, respectively (18).

The depression status of subjects was evaluated using the Beck Depression Inventory (BDI) scale, which is one of the most commonly used self-report instruments designed to detect and measure the severity of depression in the general population (20). The BDI consists of 21 items describing symptoms of and attitudes regarding depression, and each item is rated from 0 (not at all) to 3 (extreme form of each symptom). The total score ranges from 0 to 63; the higher the score, the greater the degree of depression. By using the cutoff value of 15, subjects were classified as having no-to-mild depression (BDI $<$ 15) or moderate-to-severe depression (BDI $\geq$ 15) (18).

Diagnosis of NAFLD

Hepatic ultrasonography (Acuson Sequoia 512; Siemens, Mountain View, CA) was performed to diagnose fatty liver by experienced radiologists who were unaware of the clinical information of the subjects (21). Fatty liver was diagnosed based on characteristic ultrasonographic findings consistent with a "bright liver" and evident contrast between hepatic and renal parenchyma, focal sparing, vessel blurring, and narrowing of the lumen of the hepatic veins (22). We graded the stage of steatosis based on ultrasonographic findings: mild fatty liver as a slight diffuse increase in bright homogeneous echoes in the hepatic parenchyma and normal visualization of the diaphragm and hepatic and portal borders; moderate fatty liver as a diffuse increase in bright echoes in the hepatic parenchyma with slightly impaired appearance of intrahepatic vessels and the diaphragm; and severe fatty liver as a marked increase in bright echoes with poor or no visualization of intrahepatic vessel borders, the diaphragm, and the posterior right lobe of the liver (22).

Statistical Analyses

Data are presented as the mean $\pm$ standard deviation for normally distributed continuous variables and as proportions for categorical variables. The Student *t* test and analysis of variance were used to analyze continuous variables, and the differences between nominal variables were compared with the chi-square test. Differences in anxiety or depression levels among the stages of steatosis were analyzed using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference *post hoc* analysis. A logistic regression analysis was utilized to analyze the association of NAFLD and the stage of steatosis with depression or anxiety after adjusting for potential confounders. Among variables with a p value of $<$0.05 in univariate analyses, those with clinical importance were subjected to multivariate analyses. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA) and R version 3.2.3 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.Rproject.org). p values of $<$0.05 were considered statistically significant.

RESULTS

Baseline Characteristics of the Study Population

Among the total of 25,333 subjects, the mean age was 47 years, and men comprised 56.2%. The prevalence rate of NAFLD was 30.9%. The subjects with NAFLD were divided into three groups based on their ultrasonographic findings of steatosis. To rule out the effects of gender, we stratified the population according to gender. The baseline characteristics of the male participants are shown in **Table 1**. NAFLD was more frequently observed in people who were older and smokers. Prevalence of diabetes was significantly higher in subjects with NAFLD. In addition, most of the anthropometric and laboratory variables (including BMI, WC, systolic or diastolic blood pressure, AST, ALT, total cholesterol, triglyceride, and HDL-cholesterol) were less metabolically favorable in subjects with NAFLD ($p <$ 0.001). The prevalence rates of depression and anxiety were not significantly different in the NAFLD and control groups.

In the female group, NAFLD was more frequently observed in people who were older and never-smokers. Other baseline characteristics were almost similar to those of men except for the prevalence rates of depression which was higher in the NAFLD group compared to the control group (**Table 2**). **Figure 2** shows the scores of depression according to the stage of steatosis. The BDI score was significantly higher in the severe NAFLD group compared to the no-NAFLD group (7.2 $\pm$ 5.9 vs. 6.3 $\pm$ 5.5, $p =$ 0.031). The effect sizes of the differences were provided in the **Supplementary Table 1**.

Risk of Depression and Anxiety With NAFLD

We investigated the relationship between depression or anxiety and NAFLD. In the univariate analysis, the presence of NAFLD had no association with depression. Regarding anxiety, the presence of NAFLD showed a significant association with state anxiety. NAFLD was associated with a 12% increase in the risk of state anxiety [odds ratio (OR) 1.12, 95% confidence interval (CI) 1.00–1.25]. When we adjusted for age, sex, diabetes, systolic and diastolic pressure, and smoking, the statistical significance has disappeared. In addition, the presence of NAFLD had no significant association with trait anxiety (**Table 3**).

Next, we performed stratified analysis according to gender to exclude the influence of gender. In the univariate model, the presence of NAFLD showed a significant association with depression. NAFLD was associated with a 44% increase in the risk of depression (OR 1.44, 95% CI 1.17–1.76) in women. After adjusting for age, BMI, alcohol, diabetes, and smoking, the multivariate analysis revealed that the presence of NAFLD still showed a significant association with depression, suggesting that NAFLD has an independent association with the risk for depression (OR 1.43, 95% CI, 1.14–1.80). Although there was no statistical significance, there was a trend of increasing risk of depression according to the stage of steatosis in a dose-dependent manner (OR 1.35, 95% CI, 1.00–1.78; OR 1.52, 95% CI, 1.12–2.03; and OR 1.75, 95% CI, 0.76–3.56, mild, moderate, and severe, respectively; **Table 4**).

TABLE 1 | Comparison of baseline characteristics according to nonalcoholic fatty liver disease in males.

	No NAFLD (*N* = 8,067)	NAFLD (*N* = 6,169)	Mild (*N* = 2,449)	Moderate (*N* = 3,263)	Severe (*N* = 457)	**P*-value
Age (years)	47.5 ± 11.7	48.4 ± 10.1	48.9 ± 10.2	48.3 ± 9.9	46.2 ± 10.9	<0.001
Smoking, *n* (%)	5,820 (72.1)	4,758 (77.1)	1,902 (77.7)	2,512 (77.0)	344 (75.3)	<0.001
Alcohol, *n* (%)	7,081(87.8)	5,223 (84.7)	2,106 (86.0)	2,749 (84.2)	368 (80.5%)	<0.001
Diabetes, *n* %	347 (4.3)	537 (8.7)	167 (6.8)	310 (9.5)	60 (13.1)	<0.001
BMI (kg/m^2)	23.4 ± 2.3	25.8 ± 2.6	25.1 ± 2.2	26.0 ± 2.5	27.8 ± 3.2	<0.001
WC (cm)	84.3 ± 6.6	90.7 ± 6.6	89.1 ± 5.8	91.2 ± 6.5	95.4 ± 8.5	<0.001
SBP (mmHg)	116.8± 13.1	120.7 ± 13.0	119.3 ± 12.9	121.3 ± 13.0	123.9 ± 11.9	<0.001
DBP (mmHg)	76.6 ± 10.5	79.9 ± 10.5	78.8 ± 10.3	80.3 ± 10.6	82.1 ± 10.0	<0.001
AST (IU/L)	22.4 ± 10.4	27.3 ± 13.4	24.5 ± 10.0	28.2 ± 13.1	35.8 ± 23.4	<0.001
ALT (IU/L)	23.4 ± 21.1	37.1 ± 24.2	30.2 ± 16.9	39.5 ± 24.7	56.8 ± 36.4	<0.001
Total cholesterol (mg/dL)	191.7± 32.5	200.8 ± 34.7	199.4 ± 34.0	201.3 ± 34.8	204.9 ± 37.2	<0.001
Triglyceride (mg/dL)	105.9± 61.5	155.8 ± 92.3	145.8 ± 83.6	159.4 ± 93.9	183.7± 115.4	<0.001
HDL-cholesterol(mg/dL)	52.8 ± 11.3	47.1 ± 923	48.0 ± 9.3	46.7 ± 9.3	44.9 ± 7.6	<0.001
Depression, *n* (%)	298 (3.7)	198 (3.2)	74 (3.0)	105 (3.2)	19 (4.2)	0.130
State_anxiety, *n* (%)	442 (5.5)	363 (5.9)	145 (5.9)	190 (5.8)	28 (6.1)	0.317
Trait_anxiety, *n* (%)	129 (1.6)	86 (1.4)	35 (1.4)	45 (1.4)	6 (1.3)	0.355

Data are shown as the mean ± SD.
NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipid-cholesterol.
**Comparison of subjects with absence and presence of NAFLD.*

TABLE 2 | Comparison of baseline characteristics according to nonalcoholic fatty liver disease in females.

	No NAFLD (*N* = 9420)	NAFLD (*N* = 1677)	Mild (*N* = 839)	Moderate (*N* = 759)	Severe (*N* = 79)	**P*-value
Age (years)	44.2 ± 10.8	53.1 ± 9.8	53.0 ± 9.7	53.2 ± 9.9	53.4 ± 9.8	<0.001
Smoking, *n* (%)	898 (9.5)	92 (5.5)	40 (4.8)	44 (5.8)	8 (10.1)	<0.001
Alcohol, *n* (%)	4,283 (45.5)	528 (31.5)	274 (32.7)	233 (30.7)	21 (26.6)	<0.001
Diabetes, *n* %	133 (1.4)	135 (8.1)	57 (6.8)	66 (8.7)	12 (15.2)	<0.001
BMI (kg/m^2)	21.2 ± 2.4	24.9 ± 3.1	24.4 ± 2.8	25.2 ± 3.2	27.7 ± 3.1	<0.001
WC (cm)	77.7 ± 6.8	87.2 ± 7.3	86.1 ± 6.8	87.7 ± 7.3	93.5 ± 7.3	<0.001
SBP (mmHg)	108.5 ± 13.8	119.3 ± 14.3	118.9 ± 14.6	119.2 ± 13.8	124.6 ± 14.5	<0.001
DBP (mmHg)	68.1 ± 10.3	74.6 ± 10.5	74.4 ± 10.5	74.6 ± 10.3	77.5 ± 11.9	<0.001
AST (IU/L)	19.9 ± 10.5	24.8 ± 13.1	22.7 ± 10.4	26.3 ± 15.0	31.5 ± 14.6	<0.001
ALT (IU/L)	16.6 ± 17.4	27.3 ± 18.6	23.0 ± 12.2	30.6 ± 22.2	41.8 ± 22.5	<0.001
Total cholesterol (mg/dL)	190.7 ± 33.1	205.9 ± 36.1	205.2 ± 36.4	206.9 ± 35.6	204.6 ± 38.4	<0.001
Triglyceride (mg/dL)	76.5 ± 41.9	126.7 ± 70.9	118.7 ± 63.2	131.1 ± 73.7	168.8 ± 97.8	<0.001
HDL-cholesterol(mg/dL)	62.0 ± 12.7	53.2 ± 11.0	54.0 ± 11.2	52.8 ± 10.8	49.3 ± 10.2	<0.001
Depression, *n* (%)	609 (6.5)	137 (8.2)	64 (7.6)	65 (8.6)	8 (10.1)	0.012
State_anxiety, *n* (%)	1,055 (11.2)	178 (10.6)	86 (10.3)	79 (10.4)	13 (16.5)	0.509
Trait_anxiety, *n* (%)	465 (4.9)	76 (4.5)	36 (4.3)	32 (4.2)	8 (10.1)	0.518

Data are shown as the mean ± SD.
NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipid-cholesterol.
**Comparison of subjects with absence and presence of NAFLD.*

Regarding anxiety, the presence of NAFLD had no significant association with anxiety. However, the stage of steatosis showed significant associations with both state anxiety and trait anxiety (severe NAFLD, both $p < 0.05$), and these associations remained significant after adjusting for age, BMI, smoking, alcohol, and diabetes (adjusted OR 1.84 and 95% CI 1.01–3.37, $p = 0.047$ and adjusted OR 2.45 and 95% CI 1.08–4.85, $p = 0.018$, respectively) in women. When we performed analysis in male subjects with NAFLD, the associations were not significant (data not shown).

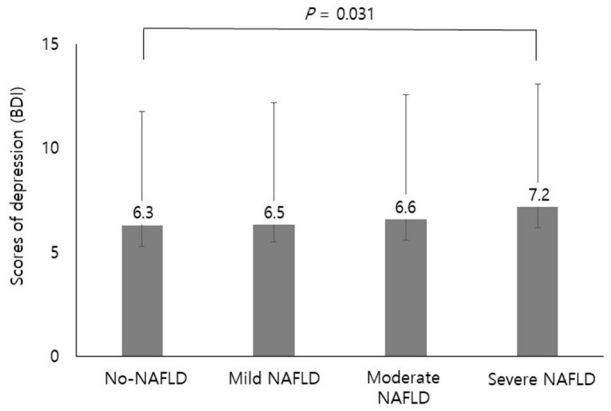

FIGURE 2 | Scores of depression according to the stage of steatosis. The BDI score was significantly higher in the severe NAFLD group compared to the no-NAFLD group (7.2 ± 5.9 vs. 6.3 ± 5.5). Differences in depression levels among the stages of steatosis were analyzed using one-way ANOVA with Tukey's honestly significant difference *post hoc* analysis. BDI, Beck Depression Inventory; NAFLD, nonalcoholic fatty liver disease.

TABLE 3 | Univariate and multivariate analyses of the risk for depression or anxiety with vs. without NAFLD.

	Univariate OR (95% CI)	*P*-value	Multivariate OR (95% CI)	*P*-value
Depression				
NAFLD vs. control	1.09 (0.95–1.26)	0.205		
Mild NAFLD vs. control	1.05 (0.86–1.26)	0.642		
Mod NAFLD vs. control	1.10 (0.92–1.31)	0.274		
Severe NAFLD vs. control	1.34 (0.88–1.96)	0.147		
State_anxiety				
NAFLD vs. control	1.12 (1.00–1.25)	0.045	1.11 (0.99–1.24)	0.069
Mild NAFLD vs. control	1.12 (0.96–1.29)	0.151		
Mod NAFLD vs. control	1.10 (0.96–1.27)	0.172		
Severe NAFLD vs. control	1.27 (0.90–1.74)	0.155		
Trait_anxiety				
NAFLD vs. control	1.00 (0.83–1.21)	0.986		
Mild NAFLD vs. control	1.00 (0.77–1.28)	0.985		
Mod NAFLD vs. control	0.96 (0.74–1.23)	0.753		
Severe NAFLD vs. control	1.36 (0.75–2.26)	0.272		

NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; CI, confidence interval. Multivariate analysis adjusted for age, sex, diabetes, systolic and diastolic blood pressure, and smoking.

DISCUSSION

In the present study, there was a trend of increasing risk of depression according to the stage of steatosis in a dose-dependent manner in women compared with the non-NAFLD group, even after adjusting for confounding factors. In addition, severe stage of steatosis was significantly associated with state and trait anxiety in women.

These findings are in agreement with previous results showing the association between NAFLD and depression. A previous study with biopsy-proven NAFLD patients showed that more severe histological steatosis and higher NAFLD activity score were found in NAFLD patients with MDD than in those without MDD (23). Another study performed in Japan showed that depression was associated with more severe hepatocyte ballooning in pathology in patients with NAFLD (24). Although these findings were strengthened based on histological diagnosis, they are limited in their small sample size. In a study based on US claim data, those with depression were 1.6- to 2.2-fold more likely to have NAFLD compared to subjects without depression (14). A recent study based on a large Korean population showed the dose-dependent pattern of the relationship between the risk for depression and the ultrasonographically graded severity of NAFLD (25). Unlike our results, the significant association was found especially in men in the previous study, and these different results may be due to the heterogeneous study population, including the mean age of the subjects (40 vs. 47 years) and the prevalence of depression (10.6 vs. 4.7%), and the different definitions regarding depression, which were based on the Center for Epidemiological Studies-Depression scale. Regarding anxiety, few studies have evaluated the association with NAFLD. A previous study based on patients with biopsy-proven NAFLD showed that anxiety tended to be associated with less hepatocyte ballooning; however, there was no significant association between anxiety and portal fibrosis (24).

Previous studies have investigated the influence of mood disorder on the therapeutic effect of liver disease. Tomeno et al. suggested that NAFLD patients with depression had poor response to standard treatment for NAFLD (23). A meta-analysis showed that psychological distress such as depression and anxiety was associated with mortality in chronic liver disease (26). Taken together, NAFLD patients with depression may experience worse clinical outcomes than those without; thus, active screening and appropriate treatment of depression may prevent the development or progression of NAFLD.

It is well known that there is gender difference in the prevalence of NAFLD and depression. While NAFLD is more common in men (1), depression is more prevalent in women (27). In our study, women had a prevalence of depression (6.7%) up to twice that of men (3.5%), whereas men had a higher prevalence of NAFLD (43.3%) than women (15.1%). Thus, we stratified the population according to gender, and the rate of individuals having both depression and NAFLD increased after adjusting for gender, resulting in a significant association between NAFLD and mood disorder only in women.

The pathogenesis underlying the association between NAFLD and depression or anxiety has not been fully elucidated. There are possible explanations for the close link between hepatic steatosis with insulin resistance and poor glycemic control, both of which have been associated with depression (28) or anxiety (16). The involvement of insulin signaling on brain mechanisms related to depression indicates that insulin resistance may be one of the main pathogenic drivers for NAFLD (29). In addition, increased inflammation markers (30) and pro-inflammatory cytokines, such as tumor necrosis factor-alpha and interleukin-6, in patients with mood disorders may offer another plausible explanation for the association of these mood disorders with

TABLE 4 | Univariate and multivariate analyses of the risk for depression or anxiety in women with vs. without NAFLD.

	Univariate OR (95% CI)	*P*-value	Multivariate OR (95% CI)	*P*-value
Depression				
NAFLD vs. control	1.44 (1.17–1.76)	<0.001	1.43 (1.14–1.80)	0.002
Mild NAFLD vs. control	1.33 (1.00–1.74)	0.041	1.35 (1.00–1.78)	0.044
Mod NAFLD vs. control	1.52 (1.14–1.98)	0.003	1.52 (1.12–2.03)	0.006
Severe NAFLD vs. control	1.83 (0.81–3.60)	0.110	1.75 (0.76–3.56)	0.151
State_anxiety				
NAFLD vs. control	1.11 (0.93–1.32)	0.263		
Mild NAFLD vs. control	1.06 (0.83–1.34)	0.625	1.07 (0.84–1.36)	0.557
Mod NAFLD vs. control	1.08 (0.84–1.38)	0.522	1.09 (1.00–1.39)	0.478
Severe NAFLD vs. control	1.85 (0.97–3.26)	0.046	1.84 (1.01–3.37)	0.047
Trait_anxiety				
NAFLD vs. control	1.05 (0.80–1.35)	0.722		
Mild NAFLD vs. control	1.00 (0.68–1.39)	0.948	1.00 (0.69∼1.41)	0.997
Mod NAFLD vs. control	0.97 (0.66–1.39)	0.890	0.98 (0.66–1.40)	0.908
Severe NAFLD vs. control	2.50 (1.10–4.95)	0.015	2.45 (1.08–4.85)	0.018

NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; CI, confidence interval.
Multivariate analysis adjusted for age, body mass index, alcohol, diabetes, and smoking.

NAFLD (31). Another line of evidence suggests the involvement of the serotonin pathway. Experimental murine models of NAFLD showed that serotonin played an important role in the pathogenesis of NASH. Additionally, the expression of monoamine oxidase-A, one of the main enzymes catalyzing monoamines such as serotonin, increased in patients with NASH (32).

The strengths of this study include the large sample size, which ensured the robustness of results, and the STAI scale, which was able to assess both types of anxiety, including trait or chronic anxiety, which reflects a person's permanent characteristics, and state or acute anxiety, which reflects a recent state (33).

There are several limitations in this study. First, due to its observational study design, the results need to be interpreted cautiously. The association between NAFLD and mood disorder may not imply causality. Further research with longitudinal study design is needed to clarify the causal relationship. Second, since depression or anxiety was defined based on the self-reporting questionnaire, there is the potential for over- or underreporting-related symptoms. Because the recommended cutoff points of BDI or STAI is not consistent among specific disease or populations (34–38), we used the cutoff values referring to previous study based on Korean population (18). Third, although liver biopsy is considered to be a gold standard for the diagnosis of NAFLD, it was assessed only by ultrasonography in this study. Thus, there may be a limitation of lack in accurate diagnoses for mild steatosis. And although the fibrosis was found to be a critical factor in mental health of NAFLD patients (39), we could not evaluate the fibrosis stage. In clinical practice, liver biopsy is not typically used in healthy subjects due to its invasiveness. Thus, radiographic techniques such as ultrasonography or magnetic resonance imaging are used for the diagnosis of NAFLD. Fourth, we could not assess the influence of the history of anti-depressant medications. Fifth, as a result of this study using the previous cohort (18), there is a limitation that there is no information on the history of hypertension. However, systolic and diastolic blood pressure were adjusted as variables in the multivariate analysis. Finally, because we could not exclude all patients that have drinking habit, there might be bias in the results.

In conclusion, NAFLD was significantly associated with depression in women, and severe NAFLD was significantly associated with anxiety in the female group compared to non-NAFLD. Understanding the association between NAFLD and mood disorders may have clinical implications for reducing the prevalence of comorbidities, and appropriate screening and active referrals for early treatment of depression may be suggested for patients with NAFLD.

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 Institutional Review Board of Seoul National University Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GC conceived the idea, determined the study design, collected the data, drafted and revised the manuscript. JC and SK

collected the data, performed the statistical analysis and revised the manuscript. MK, JYa, and JYi collected and reviewed the data, and revised the manuscript. BP performed the statistical analysis. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.585618/full#supplementary-material

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REVIEW
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Oxidative Stress in Non-alcoholic Fatty Liver Disease. An Updated Mini Review

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Non-alcoholic fatty liver disease (NAFLD) is a challenging disease caused by multiple factors, which may partly explain why it remains still orphan of an adequate therapeutic strategy. Herein we focus on the interplay between oxidative stress (OS) and the other causal pathogenetic factors. Different reactive oxygen species (ROS) generators contribute to NAFLD inflammatory and fibrotic progression, which is quite strictly linked to the lipotoxic liver injury from fatty acids and/or a wide variety of their biologically active metabolites in the context of either a two-hit or a (more recent) multiple parallel hits theory. An antioxidant defense system is usually able to protect hepatic cells from damaging effects caused by ROS, including those produced into the gastrointestinal tract, i.e., by-products generated by usual cellular metabolic processes, normal or dysbiotic microbiota, and/or diet through an enhanced gut–liver axis. Oxidative stress originating from the imbalance between ROS generation and antioxidant defenses is under the influence of individual genetic and epigenetic factors as well. Healthy diet and physical activity have been shown to be effective on NAFLD also with antioxidant mechanisms, but compliance to these lifestyles is very low. Among several considered antioxidants, vitamin E has been particularly studied; however, data are still contradictory. Some studies with natural polyphenols proposed for NAFLD prevention and treatment are encouraging. Probiotics, prebiotics, diet, or fecal microbiota transplantation represent new therapeutic approaches targeting the gut microbiota dysbiosis. In the near future, precision medicine taking into consideration genetic or environmental epigenetic risk factors will likely assist in further selecting the treatment that could work best for a specific patient.

Keywords: non-alcoholic fatty liver disease, oxidative stress, antioxidants, obstructive sleep apnea syndrome, gut microbiota, obesity, metabolic syndrome

INTRODUCTION

The term *non-alcoholic fatty liver disease* (NAFLD) was originally coined by Ludwig et al. (1). It indicated a hepatopathy similar to that of alcohol abuse without alcohol consumption history, and it is now reputed as the hepatic component of metabolic syndrome (2, 3). It affects approximately a quarter of the population, mostly obese, and has no approved drug therapy. Although NAFLD

is generally benign, ∼20–30% of patients develop liver inflammation, fibrosis/cirrhosis (non-alcoholic steatohepatitis, NASH), and, in some cases, hepatocellular carcinoma (4, 5). Moreover, patients with NAFLD are at higher risk of cardiovascular diseases. Because of the lack of valid therapies and of the obesity pandemic, NAFLD is one of rapidly growing indications for liver transplantation (6).

Most NAFLD patients are obese and present a mild systemic inflammation, which hampers insulin signaling [insulin resistance (IR)], playing a relevant role in the pathomechanism of liver damage (7, 8). Recently, in consideration of this association, an international group of experts highlighted the poor coherence of the term *non-alcoholic fatty liver disease* and proposed that of *metabolic (dysfunction)–associated fatty liver disease* (9). The reason why some patients with simple steatosis show a progression to more severe hepatic injury, whereas others do not, was in part simplified by the so-called "two-hit" model, founded on IR, and the deposits of relatively inert triglycerides (TGs) within the liver as initial damage. This first event was thought to be due to a "second hit" generated by oxidative stress (OS) or depletion of ATP (10) with the activation of an inflammatory cytokine cascade contributing to the development of NASH necroinflammation and fibrosis (10–12).

However, it has been found that hepatic lipid accumulation in NAFLD occurs mostly as relatively inert TGs droplets, and this is nowadays regarded as a protective rather than a deleterious mechanism, by impeding the storage of free fatty acids (FFAs), which are the actual harmful agents in this hepatopathy. Most recent evidences underline that inflammation may even precede fat accumulation, which would become only a response (12, 13). As schematically shown in **Figure 1**, hepatic FFAs originate from lipolysis in adipose tissue and dietary lipids. Moreover, particularly in conditions of IR, they may also be synthesized *de novo* (so-called *de novo* lipogenesis) from carbohydrates in the liver and be deposited as TG droplets (hepatic steatosis), or exported contributing to the very low-density lipoprotein pool (14).

The previous "two-hit theory" has therefore led the way to the "multiple parallel hits theory" (12), with the contribution of a number of "multiple parallel (and not sequential)" offenders acting with different combinations, at times synergistically, to generate NAFLD. These offenders include, in addition to IR (3) and OS, hormones secreted from the adipose tissue, intestinal dysbiosis, increased intestinal permeability, and also exposure to environmental agents such as endocrine disruptors (15) and particulate matter (PM) (16, 17) interacting among themselves in individuals predisposed by genetic and epigenetic factors.

Genes that modulate hepatic fat accumulation and retinol metabolism [i.e., transmembrane 6 superfamily member 2 (TM6SF2), variants of patatin-like phospholipase domain which contain protein 3 (PNPLA3), membrane-bound O-acyltransferase domain containing 7 (MBOAT7), hydroxysteroid 17β-dehydrogenase (HSD17B13), and glucokinase regulator (GCKR)] (9) and the deregulation of microRNAs are known to influence NAFLD development and progression (18).

In addition, also genetic variants involved in OS regulation play an important role in NAFLD pathogenesis. These genes include SOD2 gene, coding for the manganese-dependent superoxide dismutase (MnSOD); UCP3, coding for the uncoupling protein 3, a mitochondrial transporter that enhances the proton leak of mitochondrial inner membrane and unhooks the oxidative phosphorylation; uncoupling protein 2 (UCP2), regulating oxidative metabolism and mitochondrial lipid efflux; and MARC1 (A165T), which codes for the mitochondrial amidoxime reducing component 1, a protein involved in the neutralization of reactive oxygen species (ROS) (19, 20).

The NAFLD story is even more complex than this, as it may start even before conception and pregnancy. Epigenetic changes, comprising microRNA features, may cause fetal reprogramming during the pregnancy of an obese mother and transgenerational transmission of the susceptibility to NAFLD in childhood and progression to NASH across the lifetime. Moreover, improving obese mothers' diet reduces fetal hypoxemia and counteracts metabolic pathways able to generate OS, liver injury precursors, and lipotoxicity in non-human primates (21–23).

On the basis of the most recent literature, herein we will focus especially on OS because the understanding of a main role for OS in NAFLD development and progression can have important preventive and therapeutic implications for possible novel treatments.

OXIDATIVE STRESS AND ITS ROLE IN NAFLD PATHOLOGY

OS is caused by a discrepancy between ROS generation and antioxidant defenses, which lead to DNA and tissue damage (24, 25). It may occur both for the increasing production of pro-oxidant products and the dysfunction of the antioxidant system.

Although it is essential to tissue repair, it may conceal also negative features implying the development and/or exacerbation of several systemic diseases and conditions [e.g., mental/neurological diseases (26, 27), inflammatory bowel diseases (28), cardiovascular disease (29), and cancer (30)]. Starting from these premises, one can therefore easily predict that OS represents an important mediator triggering low-grade inflammation also in metabolic syndrome and in the progression of NAFLD into NASH (31–35).

ROS, in fact, appear tightly involved in those processes that lead to hepatic fibrosis (36). Multiple interlaced pro-oxidative triggers operate together with the mitochondrial dysfunction as a likely common denominator of OS (37). In NASH, there are more evidences of mitochondrial DNA and protein abnormalities being responsible for the increase of OS (38, 39). A decreased oxidative capacity of the electron transport chain (ETC) and mutations in complex II could also lead to a condition of "electron leakage" (40), meaning that the electron normal flow could be interrupted, binding with oxygen to produce superoxide or hydrogen peroxide. Moreover, the levels of glutathione (GSH) peroxidase, MnSOD, and catalase seem to be low in NASH, so that the capability of the mitochondria to reduce ROS levels is reduced. In NASH patients, an increased activity of CYP2E1 (41) has been also observed, an important microsomal source

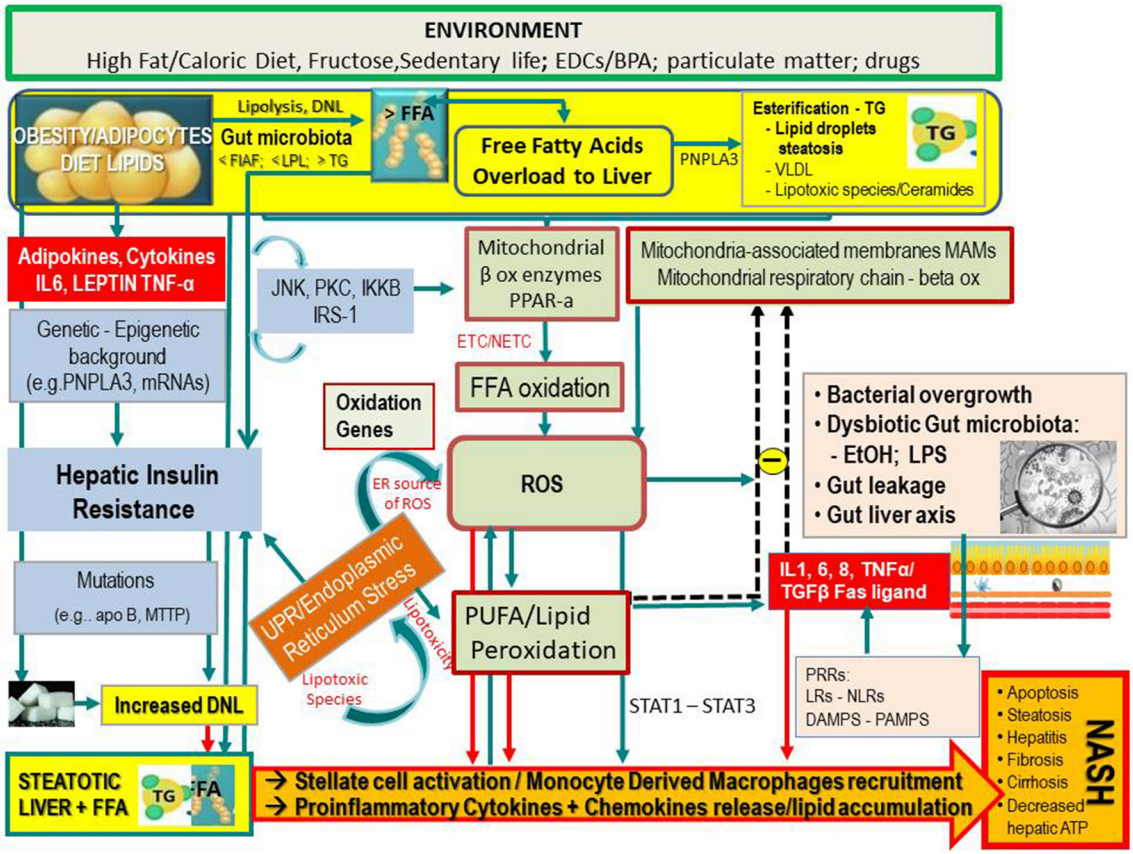

FIGURE 1 | Simplified flow of pathogenetic events in non-alcoholic fatty liver disease. The figure shows the crosstalk between systems and metabolisms in the pathogenetic events leading to fatty liver and its progression to NASH. In the upper part (yellow lane), one can note that hepatic FFAs derive from lipolysis in adipose tissue, dietary lipids, and DNL from COH in the liver. These FFAs may either be stored in the liver as TG droplets (hepatic steatosis) or be exported as VLDL to adipose tissue. FFA overload may concur in the hepatic IR (vertical light azur lane), which interplays with the JNK, PKC system where the activation of JNK1 may impair insulin signaling via serine phosphorylation of IRS1. The UPR/ER stress is a source of ROS and of lipotoxic species and plays a link between the OX stress and IR. Upon disruption of mitochondria-associated membranes (MAM) integrity, miscommunication directly or indirectly disrupts Ca^{2+} homeostasis and increases ERS (brown box) and OS, leading to defective insulin secretion and accelerated lipid droplet formation in hepatocytes. Inflammatory mediators (adipokines, cytokines) in large part arrange the progression from NAFLD to NASH (red boxes) in case of shortage of endogenous antioxidant molecules. These mediators are variously triggered by oxidative hepatic environment [ROS, lipid peroxidation] and bacterial overgrowth (pink boxes) after the infraction of the gut barrier (gut leakage) by bacterial Eth and enhanced intestinal permeability, which allows lipopolysaccharides (a) to activate PRR–LRs—NLRs–DAMPS—PAMPS and (b) to concur with ROS/PUFA in the inhibition of the mitochondrial respiratory chain. Lipotoxic lipid species lead to hepatic stress and subsequent release of extracellular vesicles, cytokines, chemokines, and DAMPs from hepatocytes. This results in enrolment of bone marrow immune cells. As shown in the lower part of the figure, liver-resident stellate/KCs are activated by several triggers (mainly ROS, gut microbiota), resulting in the release of chemokine (C-C motif) ligand 2 (CCL2) and other proinflammatory cytokines (i.e., TNF-α, IL-1, and IL-6). The oxidative hepatic environment also stimulates transcription programs (STAT-1 and STAT-3) promoting T-cell recruitment and hepatic disease progression. Overall, the scenario ultimately leads to the recruitment of bone marrow–derived monocytes and neutrophils that further contribute to the inflammatory response and a rebound ROS production. A number of genetic variants are implicated in NAFLD development, and progression is shown. BPA, bisphenol A; CYP, cytochrome; COH, carbohydrates; DAMPS, damage-associated molecular patterns; DNL, *de novo* lipogenesis; EDC, endocrine-disrupting chemicals; ETC, electron transport chain; Eth, ethanol; FFA, free fatty acids; FIAF, fasting-induced adipose factor; HNE, hydroxynonenal; IKKB, inhibitor of nuclear factor κB kinase subunit β; IL, interleukin; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; LPL, lipoprotein lipase; LPS, lipopolysaccharide; LRs, lectin receptors; MAM, mitochondria-associated membrane; MDA, malondialdehyde; mRNA, microRNA; MTTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NLRs, NOD-like receptors; NF-κB, nuclear factor κ-light-chain enhancer of activated B cells; NTC non-electron transport chain; PAMPs, pathogen-associated molecular patterns; PKC, protein kinase; PNPLA3, patatin-like phospholipase domain-containing protein 3; PRR, pattern recognition receptor; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; TG, triglyceride; TNF, tumor necrosis factor; UPR, unfolded protein response; VLDL, very low-density lipoprotein; <, decrease; >, increase.

of OS, especially together with C47T polymorphisms of SOD2 (encoding MnSOD) (41–45).

In the development of NASH, OS probably occurs not only due to the saturation of the antioxidant machinery secondary to the increased pro-oxidant species production and its direct insult. In the liver, actually, these conditions trigger lipid peroxidation by specific polyunsaturated fatty acids (PUFAs), along with the formation of highly reactive aldehyde products [e.g., malondialdehyde (MDA) and 4-hydroxy-2-non-enal (4-HNE)]. Overall, these events appear involved in the diffusion of

ROS and reactive nitrogen species (RNS) into the extracellular space, perpetuating intracellular and tissue damage. Moreover, hepatic OS may result from gut microbiota (GM)–related inflammation and the disturbance in the normal functions of endoplasmic reticulum [so-called ER stress (ERS)] (see below) [(37, 46); **Figure 1**].

IMPLICATION OF THE OXIDATIVE STRESS IN HEPATIC INJURY

ROS/RNS (i.e., hydrogen peroxide, superoxide anion radical, peroxynitrite, and hydroxyl radical), and not lipid peroxidation byproducts, are the responsible for cytokine elevations (47) such as tumor necrosis factor α (TNF-α), transforming growth factor β, interleukin 8 (IL-8), and Fas ligand. The sum of these events results in NAFLD development (25). The oxidative hepatic environment in obesity furthermore promotes the signal transduction and activation of transcription programs (STAT-1 and STAT-3) that promote T-cell recruitment and liver damage with disease progression up to its malignant transformation [(48); **Figure 1**].

OS and Hepatic Injury: Possible Implications in NAFLD Progression

Hepatocyte damage involves a cascade of events leading to NAFLD progression into NASH and cirrhosis: damage-associated molecular patterns, discharged from damaged hepatocytes, lead to the release of chemokines and cytokines from Kupffer cells (KCs) and the recruitment of monocyte-derived macrophages. ROS directly and indirectly contribute to stellate cell activation and to chronic inflammatory response with up-regulation of proinflammatory cytokines (TNF-α, IL-6, and IL-1), apoptosis, and development of hepatic fibrosis [(49–51); **Figure 1**].

In conditions of progressive NAFLD, OS can also result from increased ROS generation due to impairment of mitochondria caused by an overload of FFAs and an increase of their metabolism, lipotoxicity, and hypoxia, as well as ROS production through NADPH-oxidase isoforms associated to ligand–receptor link or by activated inflammatory cells (49).

Evidences suggest that lipotoxicity mediated by FFAs (52) may induce disruption of ER homeostasis, known as "unfolded protein response," an intracellular signaling activated by the accumulation of unfolded/misfolded proteins. Thanks to it, ER can communicate the folding status of its proteins to the rest of the cell, particularly to the nucleus, and so activate genes transcription. As a result, the ERS, a term that includes also several other mechanisms conducing to ROS generation, occurs (37), and this leads to

- increased endoplasmic reticulum oxidoreduction-1 (ERO-1) activity, the enzyme that catalyzes disulfide bond formation (53) with H_2O_2 production;
- upregulation of CCAAT/enhancer-binding protein homologous protein (Chop), a proapoptotic mechanism (54);
- calcium leakage from ER, which increases its flow through mitochondrial membranes leading to proapoptotic mitochondrial membrane permeabilization (55);
- GSH depletion (56), altering GSH–oxidized glutathione balance, which is essential to redox homeostasis; and
- inhibition of nuclear factor, erythroid 2–related factor 2, a factor encoding for antioxidant proteins (57).

The cross talk between ERS and ROS (**Figure 1**) appears relevant in the pathogenesis of NAFLD (58). Mitochondria-associated membranes (MAMs) represent a physical junction between ER and mitochondria, allowing Ca^{2+}, lipids, and ROS exchange. Because normal communication between mitochondria and ER depends on MAM structural and functional integrity, lack of calcium homeostasis may lead to ERS and OS increase, defective insulin secretion, and accelerated lipid droplet formation in hepatocytes. The steps involve apoB misfolding, impaired lipoprotein secretion, and lipogenesis stimulation. On these bases, protecting the ER via the administration of antioxidants or activation of peroxisome proliferator-activated receptor (PPAR) has been suggested as promising avenues against hepatic steatosis (59, 60).

Studies in rodents show the existence of a link between ERS and regulation of hepatic iron metabolism both in ASH and NASH models mainly due to the capacity of ferrous iron to catalyze the production of hydroxyl radical (OH^-) from H_2O_2, deriving by peroxisomal β-oxidation (52, 61, 62). Interestingly, iron deficiency too may reduce the cell antioxidant capability by inhibiting heme oxygenase-1 by Bach1 (63). Beyond doubt, it is not always simple to study the progression of a disease, especially in humans *in vivo*, and establish if a certain factor is exactly the cause or the effect of NASH. Moreover, a disagreement often happens between animal models and clinical studies due to several factors such as gut microflora differences and patient inclusion criteria/ethnicity–related predisposition, respectively (37).

In order to assess the redox state in NAFLD/NASH, some markers of OS and antioxidants have been studied in NAFLD and NASH models, both clinical and experimental. OS biomarkers include nitric oxide, lipid damage products (lipid peroxides, thiobarbituric acid reactive substances/MDA), hydroperoxides, 8-isoprostane, 4-HNE, DNA oxidation product [CYP2E1 and 8-hydroxydeoxyguanosine (8-OH-dG)], and protein oxidation products (nitrotyrosine, protein carbonyl). All these had increased activities in most NAFLD/NASH clinical models evaluated. On the contrary, antioxidant markers (superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione) measured in rodent models showed decreased activities mainly in NASH (64).

GUT MICROBIOTA AND INTESTINE PERMEABILITY AS A CAUSE OF OXIDATIVE STRESS

Gut Microbiota as a Source of ROS

Human commensal microbiota (**Figure 1**) generates physiological ROS levels in intestinal human epithelial

cells. Basically, aerobic cell systems are exposed to oxygen free radicals (65, 66), and their damaging role relies on their concentrations. When the levels of ROS exceed antioxidant defenses, harmful effects on cells may occur, conducing to uncontrolled proliferation, inflammation, and/or apoptosis (67, 68). This is what happens also in obesity and its related hepatometabolic comorbidities, including NAFLD progression to NASH (see below). ROS can also operate as second messengers in intracellular signaling stimulated by proinflammatory cytokines and growth factors and by the quick and reversible oxidative inactivation of proteins having thiol groups sensitive to oxidants (69). In inflammation and obesity, ROS generation is probably strictly related with activation of nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) and degradation of NF-κB inhibitor (IκB), making NF-κB more transcriptionally active (70–72). As shown in **Figure 1**, a quantitative or qualitative (in term of dysbiosis) bacterial alteration (small intestine bacterial overgrowth) is also concatenated with OS through the inhibition of mitochondrial respiratory chain.

Interaction Between Gut Microbiota, OS, and Intestinal Permeability in NAFLD

Gut mucosal barrier separates, functionally and physically, the luminal content from the underlying compartment that, in addition to gut epithelia, includes immune, vascular, and structural elements in the lamina propria. The intestinal mucosa is constantly exposed to oxidants and carcinogens taken in from diet and/or bacteria, whose chronic exposure may cause production of free radicals leading to redox imbalance and subsequent DNA damage, disturbing the intestinal metabolic equilibrium (73).

GM plays an important role in different processes (metabolic, nutritional, physiological, and immunological) involved in maintaining a healthy status (69, 74). Its qualitative and quantitative composition differs in the distinct parts of gastrointestinal (GI) tract because of the influences by different conditions [e.g., age, dietary habits, ethnicity, delivery mode, exposure to therapies, pathogens, and contact with several environmental stimuli (75–78)]. Perturbation of GM composition, called "dysbiosis," has been recognized in diseases associated not only with the GI tract [e.g., inflammatory bowel disease (79)] but also with systemic conditions such as obesity, diabetes mellitus, autism, depression, and NAFLD (80). While a quite clear causal role of a specific GM has been demonstrated in murine models of NAFLD (e.g., unhealthy diet dependent shift from Bacteroidetes to Firmicutes), recognition of a corresponding human microbiome signature is more difficult. In fact, it may be hindered by the components of associated metabolic syndrome and several other confounding factors. Anyway, Gram-negative harmful bacteria release lipopolysaccharide (LPS), lipoteichoic acid, flagellin, lipoprotein, or other toxins recognized by the pattern recognition receptors (PRRs) expressed on the surface of innate immune system cells. Similarly, structurally conserved motifs present on the surface of different types of pathogens (pathogen-associated molecular patterns) are recognized and bound by PRRs, inducing mitochondrial ROS production and nuclear gene expression.

PRR classes sensitive to the microbiota's factors are Toll-like receptor (TLR), Rig-1-like receptor, Nod-like receptor, and C-type lectin receptor. They induce the NF-κB pathway activation and enhance the inflammatory response when proinflammatory cytokines and antibacterial factors are released (81, 82). Differently, small formylated peptides produced and released by commensal bacteria are recognized by another kind of receptor, known as formylated peptide receptors. These are G-proteins linked to surface receptors of neutrophils and macrophages, stimulating ROS synthesis in phagocytes and epithelial cells (83). In particular, their activation stimulates superoxide anion production by NADPH oxidase 1, increasing ROS levels in cell cytoplasm that lead to an inflammatory response and increase of cell OS (84). As a consequence of cell stress, mitochondrial and bacterial DNA may be integrated in the nuclear genome causing the alteration of cellular gene expression.

Intestinal mucosa permeability has an important role in modulating how GM can influence also other parts of the body. An alteration of its barrier function consents to the GM and its endotoxins to cross the intestinal epithelium and the endothelial barrier (85) traveling into systemic circulation and reaching different target organs (75, 86).

There are many evidences according to which gut bacteria are involved in the pathogenesis of liver injury induced by alcohol, and gut leakiness promotes proinflammatory bacterial products reaching the liver, thus initiating the proinflammatory cascade that causes alcoholic steatohepatitis (ASH). Alcohol impairs intestinal epithelial cell permeability *in vitro* through a mechanism mediated by OS (87), supporting therefore the idea that OS may be the main cause of alcohol-induced intestinal leakage (88, 89).

In NAFLD, with a quite similar mechanism, endogenous ethanol produced by some microbial species [e.g., *Escherichia* genus members of the Proteobacteria phylum induced by high-fat diet; (90)] is able to induce the formation of ROS by HSC cells and impair intestinal integrity. The latter allows LPS to reach hepatic TLRs activating and further enhancing oxidative, inflammatory, and fibrogenetic mechanisms (75, 90, 91, 91–94).

GM seems to mediate the progression from simple steatosis to NASH. In particular, increased Gram-negative bacteria expose KCs to an elevated amount of LPS and upregulation of PRRs (37). It has been hypothesized that the endocytosis of LPS by KC could induce upregulation of cytokine receptors, especially the TNF-α receptor, which seems to be also involved in the increased ROS production (95). Activated KCs have a role in IR, fibrosis development, and inflammation amplification.

Also, the association between obstructive sleep apnea syndrome (OSAS) and NASH severity seems to correlate with endotoxemia increase and gut barrier function alteration, conducing to increased hepatic susceptibility to endotoxemia mediated by TLR-4 (96). An alarming 60% OSAS incidence has been reported in pediatric NAFLD (97, 98). This disorder of breathing during sleep has been associated with fatty acid accumulation in the liver and inflammation caused by frequent nocturnal hypoxia (NH), IR, OS, and adipokine dysregulation

(99). Growing experimental evidences link the alternation of NH with normoxia (so-called chronic intermittent hypoxia) caused by OSAS to NAFLD development and progression (49, 100). A study that compared healthy controls and NAFLD patients (some of which with OSA/NH), identified NH as a possible source of OS in NAFLD. OSA/NH is common in pediatric patients with liver biopsy-proven NAFLD and is associated with more advanced liver injury and histological disease (97, 98). Intermittent hypoxia conduces to tissue hypoxia and can lead to OS, mitochondrial malfunction, inflammation, and sympathetic nervous system hyperactivation. As a consequence, intermittent hypoxia causes IR, impairment of hepatic lipid metabolism pathways (84), and hepatic steatosis and fibrosis, each of which is involved into NAFLD development and/or progression [(101); **Figure 2**].

THERAPEUTIC STRATEGIES

Non-enzymatic Anti-oxidants Defenses

Antioxidants are substances that inhibit the oxidation of any biomolecule (102), neutralizing the harmful effects of oxidation caused by free radicals, maintaining therefore the redox homeostasis. Antioxidants are either synthesized endogenously (e.g., GSH, superoxide dismutase) or taken from the diet. Anthocyanins, lycopene, coenzyme Q10, flavonoids, β-carotene, lipoic acid, selenium, lutein, catechins, and vitamins A, C, and E are among the many substances normally present in foods that possess a high antioxidant activity. As reported in the table, they can be also classified in two large groups on the basis of the presence/absence of their enzymatic action [(103–114); **Table 1**].

Despite the above premises, antioxidants as potential pharmacological agents have hitherto not appeared extremely effective *in vivo* as either a preventive or therapeutic tool in NAFLD (31, 117–122). Studies that have investigated the role of vitamin E as a treatment of NASH confirm that it acts against pathogenic mechanisms conducting to liver damage and NASH, thanks to its antioxidant and anti-inflammatory activity (123–126). The antioxidant power of vitamin E is due to the hydroxyl group in the tocochromanol ring, which neutralizes free radicals and ROS by donating hydrogen. The major forms of tocopherol and tocotrienol are α, β-, γ-, and δ-, with the antioxidant activity of the δ-isoform being weaker than the others, the vitamin E isoforms are also involved in many other activities (**Table 2**). Among the vitamin E isoforms, the α-tocopherol, has other different properties independently from its antioxidant ability: it can inhibit the activity of protein kinase C, reducing the proliferation of different cell types (vascular smooth muscle cells, mesangial cells, neutrophils, monocytes/macrophages, fibroblast, and various cancer cell lines) and the 5-lipoxygenase pathway, inhibiting the release of proinflammatory cytokine IL-1β.

Clinical Trials of Vitamin E for NAFLD: Vitamin E in the Clinics

Available data are still conflicting. The largest trials with vitamin E in NAFLD are the PIVENS (Pioglitazone vs. Vitamin E vs. Placebo for the Treatment of Non-diabetic Patients with Non-alcoholic Steatohepatitis) (127) and the TONIC (Treatment of Non-alcoholic Fatty Liver Disease in Children) (121) trials. The first showed that both drugs tested in adults ameliorated steatosis, lobular inflammation, and hepatocellular ballooning, but did not ameliorate fibrosis. Vitamin E but not pioglitazone induced a clinical improvement in NASH. The TONIC trial, which evaluated therapeutic intervention with vitamin E vs. metformin in children with NAFLD, showed that both improved hepatocellular ballooning and the NAFLD activity score (NAS), but neither vitamin E nor metformin decreased alanine aminotransferase (ALT) values or hepatic steatosis, inflammation, or fibrosis in NASH. The reasons of these disappointing results depend on the need of better patient selection and protocols. Interestingly, a most recent systematic review and meta-analysis (1,317 patients from 15 randomized controlled trials) concluded that vitamin E improves biochemical and histological outcomes in adults and pediatric patients, with a significant negative association between transaminases levels and vitamin E dosage—more satisfactorily ranging between 400 and 800 IU. However, while adults receiving vitamin E improved significantly transaminases, fibrosis, and NAS both at early and late follow-up, children showed more significant changes at long-term follow-up, which could partly explain the negative results obtained by certain short-term studies (128). Some still unsolved safety concerns should be considered as well. Vitamin E, in fact, has been suspected to have a dichotomous suppressive and promoting activity with respect to tumorigenesis [e.g., co-cancerogenic in prostate cancer; (25, 129)] possibly explainable by still poorly studied host gene–supplement interactions (130). In our opinion, further carefully designed studies are still necessary for substantiating this view and supporting optimum procedures in terms of both efficacy and safety profiles.

Results from multiple regression models showed a significant negative association between ALT, AST levels, and vitamin E dosage—more favorably between 400 and 800 IU.

A quite large number of other nutraceutical antioxidants that seem to improve NASH through more than one pathway (**Table 2** and **Figure 3**) include but are not limited to the following:

- Curcumin (37, 103), with effects on different amino acids, bile acids, tricarboxylic acid cycle, and GM (131, 132), although only few human clinical trials are available (132–134).
- PUFAs of omega-3 series (PUFA omega-3), which may act as an antioxidant, have a role in modulating OS improving the defense capacity against an increased oxidative burden (135, 136).

Thanks to them, the cellular metabolism switch from lipogenesis and triacylglycerol accumulation to fatty acid oxidation thus plays a role in decreasing fatty liver. Furthermore, they have anti-inflammatory activity and enhance insulin sensitivity (103). Clinical trials evaluating the efficacy of n-3 PUFA (including docosahexaenoic acid and eicosapentaenoic acid) on systemic OS in NAFLD and NASH have shown controversial results. While n-3 PUFA supplementation appears useful in NAFLD early stages (137), unfortunately, total (enzymatic and non-enzymatic) antioxidant capacity is not enough to attenuate the hepatic damage (35). Interestingly, dietary antioxidant

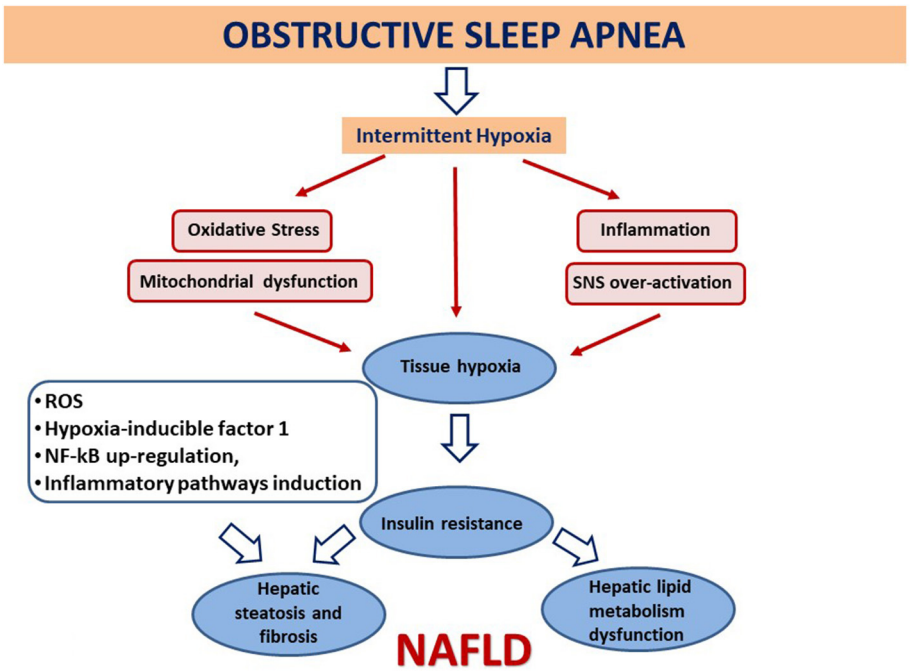

FIGURE 2 | Association between obstructive sleep apnea and the development and evolution of non-alcoholic fatty liver disease. Intermittent hypoxia leads to tissue hypoxia, OS, mitochondrial dysfunction, inflammation, and overactivation of the sympathetic nervous system (SNS). Generated reactive O_2 species (ROS) may amplify liver injury by activating hypoxia-inducible factor 1, a transcriptional activator and master regulator of O_2 homeostasis during hypoxia, and by up-regulating nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), with subsequent downstream induction of inflammatory pathways. As a consequence, this involves insulin resistance, dysfunction of key steps in hepatic lipid metabolism, atherosclerosis, and hepatic steatosis and fibrosis, each of which is pertinent to the development and/or progression of non-alcoholic fatty liver disease (NAFLD) (98–101).

intake is significantly lesser in NASH patients than in healthy controls (138).

- Among polyphenols, blueberry leaf polyphenols appear to have a positive effect on hepatic mitochondrial dysfunction and redox homeostasis, whereas bergamot polyphenolic formulation seems to improve IR, hepatocellular ballooning, inflammation, and fibrosis (115, 116).

Probiotics and Prebiotics: Other Tools Improving Defenses Against OS

Improving defenses against OS through modulation of the GM composition and functionality offers a promising means of managing or treating metabolic disorders (74).

Probiotics are living microorganisms with beneficial health activity on the host. For example, they are able to improve GM composition and reduce LPS serum amount and liver TLR4, delaying liver disease progression (81, 98, 99). Lactobacilli and bifidobacteria are the most commonly used, usually present in dietary supplements or fermented foods such as yogurt and cultured milk (100). Changing the resident GM composition and the gut lumen, they create an anti-inflammatory environment, obtaining decreased proinflammatory bacterial products and gut barrier integrity improvement. *Lactobacillus rhamnosus* GG (LGG) is the subject of numerous studies (139–144); it has different beneficial effects on the intestinal function through dysbiotic microbiota normalization (100, 144, 145) and reducing intestinal OS (146). A recent meta-analysis found a beneficial effect of probiotics also on hepatic antioxidative capacity as mirrored by the increase of SOD and GSH-PX activities and decrease of MDA content (147). A daily LGG treatment in alcohol-fed rats significantly improves severity of ASH and gut leakiness induced by alcohol, decreases intestinal and liver OS markers and inflammation, and normalizes the gut barrier task, avoiding to trigger liver disease (148). GM regulates also the powerful antioxidant glutathione and amino acid metabolism (144). It is not surprising therefore that fecal microbiota transplantation (FMT) from control donors in steatotic rats has been found to have beneficial effects in terms of decrease of portal hypertension through insulin sensitivity improvement mediated by the endothelial nitric oxide synthase signaling pathway, a pathway clearly involved in the antioxidant mechanisms (149). A pilot study of FMT in NASH is currently undergoing to evaluate whether restoration of healthful GM through FMT from lean donors (FMT-L) ameliorates NASH (150).

Similarly, "prebiotics" are fermentable carbohydrates that selectively modulate microbiota composition and/or activity, resulting in a beneficial effect for the host (146). Finally, also synbiotics (i.e., a combination of prebiotics and probiotics) have shown a positive effect on GM and have been proposed as a support for the treatment of NAFLD (151).

Summing up, the modulation of quality and diversity of every single human microbiota appears therefore an appealing tool in the management of intestinal ROS, OS, inflammation, and some metabolic anomalies caused by dysbiosis (152). Moreover, it is suggested that changes in GM occurring upon prebiotic consumption may be due to gut bacterial functions improvement. In other words, products generated by *Lactobacillus* and metabolites derived by microbiota, such as antioxidants and fatty acids, could be employed for target medicine in the management of liver disease including NAFLD (146).

TABLE 1 | Antioxidants with and without enzymatic action.

Enzymatic antioxidants	Non-enzymatic antioxidants
Superoxide dismutase (SOD)	**Low-molecular-weight compounds** Glutathione, thioredoxin, lactoferrin
	Endogenous substances Lipoic acid, melatonin, albumin, bilirubin, uric acid, polyunsaturated fatty acids omega 3
Catalase (CAT)	**Flavonoid polyphenols** Silymarin (104, 105) Blueberry leaf, bergamot polyphenols (115, 116)
	Stilbenes Resveratrol (106, 107)
Glutathione peroxidase	**Herbs** Erchen decoction, danshen, berberine (108)
	Carotenoids β-Carotene, astaxanthin, lycopene, β-cryptoxanthin, lutein, fucoxanthin, crocetin (109, 110)
Paraoxonase 1 (PON 1)	**Phenolic compounds** Açai (111)
	Vitamins Ascorbic acid (vitamin C), α-tocopherol [vitamin E, vitamin A, vitamin D (112–114)]

OTHER THERAPEUTIC STRATEGIES

Drugs

Ursodeoxycholic acid (UDCA) remains one of the most studied drugs: in addition to exerting a possible therapeutic effect on NAFLD by modulating autophagy and apoptosis dysregulation, UDCA appears to have also antioxidant properties (153).

TABLE 2 | Activities influenced by vitamin E isoforms.

I. Regulation of the inflammatory response
II. Gene expression
III. Membrane-bound enzymes
IV. Cellular signaling
V. Cell proliferation
VI. Regulation of several enzymes involved in signal transduction: Protein kinase C (PKC), protein phosphate 2A (PP2A), 5-lipoxygenase, cyclooxygenase 2 (COX-2), and monocyte chemoattractant protein 1 (MCP-1)
VII. Regulation of several factors in the mitogen-activated protein kinase (MAPK) signal transduction pathway

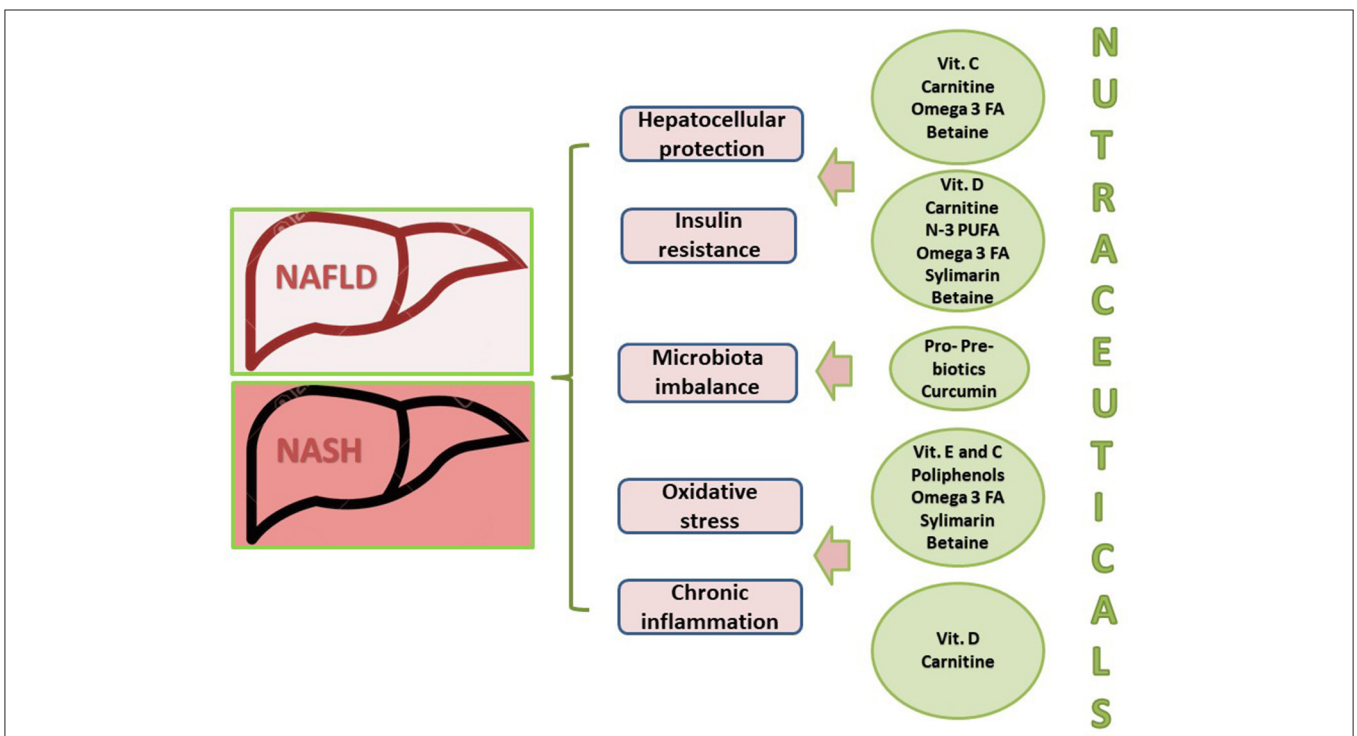

FIGURE 3 | Multiple targets of nutraceuticals for the treatment of non-alcoholic fatty liver disease. FA, fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis. Adapted and modified by Del Ben et al. (103).

A number of other drugs that have been tested for their influence on hepatic steatosis have still uncertain/elusive molecular mechanisms. There are several innovative agents currently undergoing phases II and III clinical trials with different targets (154).

Obeticholic acid, a semisynthetic bile acid analog, is an agonist of the farnesoid X receptor, which has anti-inflammatory and antioxidant activities (155).

Silymarin, a botanical product extracted from milk thistle, because of its antioxidant properties appears to improve NAFLD hypertransaminasemia and reduce liver disease progression in NASH, but at present, available results are inconclusive (156).

Cannabidiol, a chemical without psychotropic effects, has antioxidant and anti-inflammatory properties by acting on the endocannabinoid system. After stimulation of the G-protein–coupled receptors and their endogenous lipid ligands, it interferes with progression toward NASH (26, 157).

Physical Activity

Physical activity (PA) acts favorably in NAFLD primarily by reducing intrahepatic fat content with β-oxidation of fatty acids and lipogenesis regulation, enhancing the expression and activity of PPAR-γ, insulin sensitivity, and hepatoprotective autophagy, reducing hepatocyte apoptosis, and inflammation of the liver by decreasing the proinflammatory mediators. PA, moreover, has several beneficial effects on NAFLD also with the improvement of several antioxidants activity [e.g., catalase, SOD, glutathione peroxidase and reductase, glutathione-S-transferase, thioredoxin reductases, NADH cytochrome B5 reductase, and NAD(P)H quinone acceptor oxidoreductase], leading to decreased ROS production and proinflammatory cytokines (158).

CONCLUDING REMARKS

Our review exhibits that OS not counteracted by intact antioxidant defense system plays an important role in NAFLD/NASH with a number of other casual factors. Excessive FFA β-oxidation due to increased FFA fueling leads to excessive ROS formation, which, in turn, downregulates ETC, and non-ETC systems, affect insulin sensitivity, hepatic lipid metabolism, and inflammatory responses by interacting with innate immune signaling (159).

Gut dysbiosis may induce further signaling processes, which engage the epithelium and immune/inflammatory cells. In these conditions, GM may take advantage of the increased intestinal permeability and/or impairment of epithelial tight junctions. This results in an enhancement of the gut–liver axis with bacteria and endotoxin transit through the intestinal and endothelial vascular wall, ending up into hepatic and other systemic diseases as well.

The above scenario would suggest a therapeutic role of antioxidants in patients with fatty liver disease, but this approach has not been entirely translated yet in human (160), as most studies still derive from murine models with substantial differences in genetic background and in the digestive system; the need to perform more human studies appear evident (161).

Vitamin E has shown promising data but without significant benefit in fibrosis improvement (162). Several natural polyphenols and n-3 PUFA supplementation provided with a number of antioxidant, antiobesity, and anti-inflammatory effects could have potential in NAFLD prevention and treatment by acting on its multifactorial pathogenetic components, but also here data either to support or refuse their use are insufficient (115, 116).

In addition to healthy diet (e.g., a Mediterranean diet seems to reduce OS), probiotics, prebiotics, and fecal transplantation appear to be emerging strategies to modulate microbiota quality and diversity, in order to prevent and/or avoid gut damage. Avoidance of exposure to endocrine disruptors (15) and to ambient PM (16, 17) also appears strategic to add benefices to NAFLD.

Last but not least, the accurate assessment of NAFLD-associated genetic/epigenetic risk factors of diseases and likelihood of disease progression is going to aid to target individualized appropriate treatments (163).

AUTHOR CONTRIBUTIONS

FM and APDB collected literature and prepared the first draft of the manuscript. MS and MAS collected literature on specific areas and gave critical suggestions. PV gave critical suggestions, made substantial intellectual contributions to the study design, and manuscript preparation. CM provided a major intellectual input, verified/contributed to data analysis, and took over writing of the manuscript as and when required. All authors gave substantial contributions to the work and revised critically and approved the final manuscript.

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Dynamic Chronological Changes in Serum Triglycerides Are Associated With the Time Point for Non-alcoholic Fatty Liver Disease Development in the Nationwide Korean Population Cohort

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Background: We investigated the effects of anthropometric, laboratory, and lifestyle factors on the development of non-alcoholic fatty liver disease (NAFLD) in a nationwide, population-based, 4-year retrospective cohort.

Methods: The propensity score-matched study and control groups contained 1,474 subjects who had data in the Korean National Health Insurance Service-National Sample Cohort in 2009, 2011, and 2013. NAFLD was defined using medical records of a diagnosis confirmed by primary clinicians and meeting two previously validated fatty liver prediction models. Chronological changes in anthropometric variables, laboratory results, and lifestyle factors during two periods were compared between patient and control groups in order to find out parameters with consistent dynamics in pre-NAFLD stage which was defined as period just before the NAFLD development.

Results: Among the 5 anthropometric, 10 laboratory, and 3 lifestyle factors, prominent chronological decremental changes in serum triglycerides were consistently observed during the pre-NAFLD stage, although the degrees of changes were more predominant in men (−9.46 mg/dL) than women (−5.98 mg/dL). Furthermore, weight and waist circumference changes during the pre-NAFLD stage were noticeable only in women (+0.36 kg and +0.9 cm for weight and waist circumference, respectively), which suggest gender difference in NAFLD.

Conclusion: Early screening strategies for people with abrupt chronological changes in serum triglycerides to predict NAFLD development before the progression is recommended.

Keywords: non-alcoholic fatty liver disease, laboratory results, lifestyle factors, serum triglyceride, nationwide study

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), a disease complex spectrum ranging from benign hepatic steatosis to hepatic inflammation, fibrosis, and cirrhosis, is now the leading cause of chronic liver disease in developed countries (1). In Korea, the prevalence of fatty liver is estimated to be as high as 25–30% (2), and 10–15% of those affected could have steatohepatitis with inflammation. Because metabolic syndrome components such as obesity and diabetes are independent predictors of NAFLD (3), many reports emphasize comprehensive lifestyle modifications based on reduced energy intake and increased physical activity as a primary therapeutic intervention for NAFLD (4–7). Although systematic guidelines for NAFLD assessment and management proposed by the National Institute for Health and Care Excellence (NICE) recommend ultrasonography as the first-line diagnostic step and lifestyle intervention for management of NAFLD (8), few publication has previously reported data on changes in NAFLD-associated parameters from a chronological viewpoint (9), and none using real-world human clinical data.

As the early detection of NAFLD becomes more important, identification of early changes that predict future NAFLD development and chronological changes in parameters evaluated in the national health screening examination is essential to both the clinical field and national policy. Korea has systematic and comprehensive national registry data collected from the Korean National Health Insurance Service-National Sample Cohort (KNHIS-NSC) (10). The KNHIS-NSC database is a population-based sample cohort. Its purpose is to provide representative, useful health insurance and health examination data to public health researchers and policymakers. A total of 1,025,340 participants (513,258 men and 512,082 women), 2.2% of the total eligible population in the 2002 Korean nationwide health insurance database, were selected using proportionally allocated, stratified, systematic random sampling with a total medical expenses distribution within strata reflecting gender, age group, qualifications, and income quintile. Importantly, the data include laboratory results from general health examinations of cohort participants that were followed for 11-years, until 2013.

The purpose of this study was to investigate the effects of anthropometric, laboratory, and lifestyle factors recorded in the KNHIS-NSC database on the development of NAFLD in a nationwide, population-based, 4-year retrospective cohort.

MATERIALS AND METHODS

Database

In Korea, all citizens are obligated to enroll in the KNHIS. A total of 97% of the Korean population is covered by the Medical Assistance Program, and 3% of the Korean population is covered by the Medical Care for Patriots and Veterans Affairs Scheme, respectively. Thus, nearly all of the data in the health system are centralized in large databases. In Korea, patients with KNHIS pay ∼30% of their total medical expenses, and medical providers are required to submit claims for the remaining 70%. Claims are accompanied by data regarding diagnostic codes, procedures, prescription drugs, personal information about the patient, information about the hospital, the direct medical costs of both inpatient and outpatient care, and dental services. No health care records are duplicated or omitted because all Korean residents receive a unique identification number at birth for use by the Korean government for purposes related to the health care system. For diagnostic codes, the KNHIS uses the Korean Classification of Diseases (KCD), which is similar to the International Classification of Diseases (ICD).

This study adhered to the tenets of the Declaration of Helsinki, and the KNHIS-NSC 2002–2013 project was approved by the Institutional Review Board of the KNHIS. This study design was reviewed and approved by the Institutional Review Board of the National Health Insurance Service, Ilsan Hospital (IRB No. 2015-11-010). Written informed consent was waived.

Study Population

Data for this study were derived from the KNHIS-NSC 2009–2013. This database includes all medical claims and general health examination results filed from January 2009 to December 2013. The study population for this study was nationally representative subjects who have undertaken annual medical check-ups in all three of the following years: 2009, 2011, and 2013.

A flowchart for the study population is in **Figure 1A**. From a total of 108,653 Koreans who participated in medical check-ups in all 3-years (2009, 2011, and 2013), we excluded subjects with a history of liver disease before 2009 (n = 37,934) using the KNHIS-NSC 2002–2008 database. Then, we selected individuals who were newly diagnosed with NAFLD in 2011 or 2013 (The criteria for NAFLD diagnosis is explained in section Definition of NAFLD). Among the 1,775 subjects newly diagnosed with NAFLD in 2011 or 2013, we excluded those missing anthropometric, biochemical, medical history, or lifestyle variables that we examined in this study (n = 968). Thus, we analyzed a total of 737 NAFLD patients (470 men and 267 women) for this study. We derived a control group (470 men and 267 women) from the whole population without an NAFLD diagnosis (n = 46,671) using a propensity score matching algorithm for age and gender. All subjects in the control group received negative results when evaluated by the hepatic steatosis index and fatty liver index using laboratory results from 2009, 2011, and 2013.

We further divided the NAFLD group into subgroups (**Figure 1B**). Group 1 was defined as subjects who did not have a diagnosis of NAFLD in 2009 or 2011 but who had newly developed NAFLD in 2013. Group 2 was defined as subjects who did not have a diagnosis of NAFLD in 2009 but showed newly developed NAFLD in 2011 and sustained the diagnosis of NAFLD in 2013.

Study Period

Because time-dependent changes in various factors were the major end-point result, we assigned chronological differences from 2009 to 2011 as the first period and chronological differences from 2011 to 2013 as the second period (**Figure 1B**). "Pre-NAFLD stage" was defined as the period just before the NAFLD development for patient groups. Therefore, the first period was the pre-NAFLD stage for group 2, and

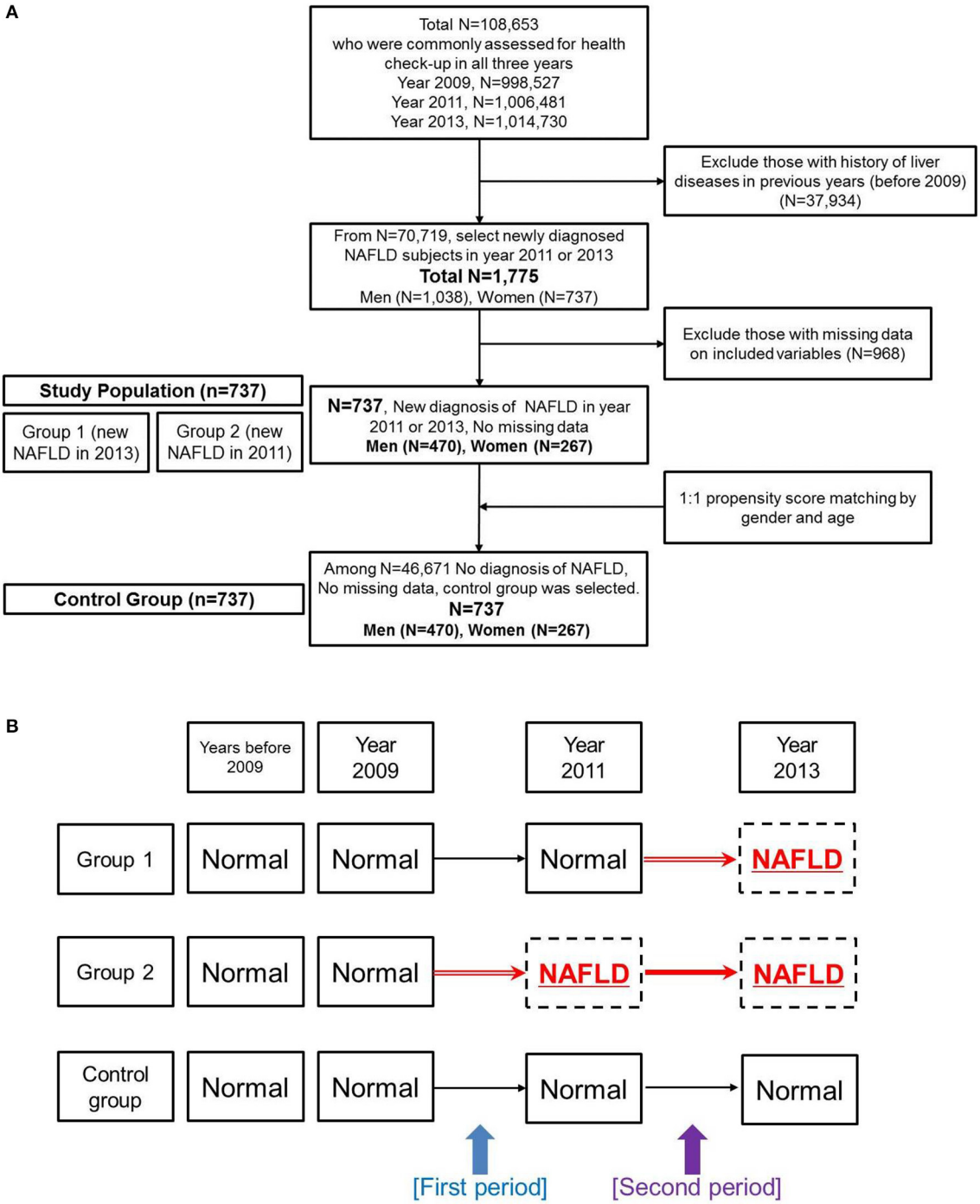


FIGURE 1 | Overview of the study design. **(A)** Flowchart of study population selection. **(B)** Designations for subgroups of the non-alcoholic fatty liver disease group and for the control group. Changes were evaluated during two periods.

the second period was the pre-NAFLD stage for group 1. Although the data did not contain the exact time when NAFLD developed in each patient, we assumed a 2-year difference for both the first and the second periods as an approximate value.

Definition of NAFLD

Each patient was tracked on the basis of his or her index dates for ambulatory and inpatient care visits over the 11-years from 2003 to 2013, to detect those patients who developed NAFLD (KCD code K76.0, corresponding to ICD-10-CM code K76.0). To rule

out the effects of liver disease with other etiologies, we excluded subjects who also had a diagnosis of alcoholic fatty liver or toxic liver (KCD codes K70–75).

To confirm the medical diagnosis of NAFLD as assessed by patients' primary physicians, we calculated two previously validated fatty liver prediction models: (1) hepatic steatosis inde× (HSI) = 8 × [alanine transferase (ALT)/aspartate transferase (AST) ratio] + body mass index (BMI) (+2, if female; +2, if diabetes mellitus) (11) and (2) fatty liver index (FLI) = [[e∧ [0.953 × loge [triglycerides (TG)] + 0.139 × BMI + 0.718 × loge [gamma-glutamyl transferase (GGT)] + 0.053 × waist circumference −15.745]]]/[[1+e∧ [0.953 × loge (TG) + 0.139 × BMI + 0.718 × loge (GGT) + 0.053 × waist circumference −15.745]]] × 100, with TG measured in mg/dL, GGT in U/L, and waist circumference in cm (12). All parameters for the equations were available in the database. We defined NAFLD as HSI of 35 or higher or FLI of 60 or higher, following a previous study (13).

Anthropometric, Laboratory, and Lifestyle Variables

We chronologically compared 18 variables in all subjects in the 3 groups, calculating absolute differences of mean values for numerical parameters and percentage differences for categorical parameters from 2009 to 2011 and from 2011 to 2013. As the time intervals were both ∼2-years, we could also compare changes between 2 time periods within each group.

For anthropometric factors, we evaluated body weight, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), and waist circumference. Body weight was measured to the nearest 0.1 kg. BMI was calculated as weight/height^2 (kg/m^2). Waist circumference was measured at the narrowest point between the lower border of the rib cage and the iliac crest.

For laboratory values, we measured a total of 10 parameters in blood samples: liver enzymes (AST, ALT, and GGT), fasting glucose, total cholesterol, TG, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, creatinine in serum samples, and hemoglobin level in whole blood sample.

For the lifestyle evaluation, we reviewed questionnaires related to smoking, alcohol drinking, and exercise status. Because all questions were answered based on each individual's experience, we objectively categorized answers into binary outcomes for smoking (current smoking or not) and exercise (no exercise at all or at least minimal physical activity). For alcohol consumption, we scored an alcohol index according to the following criteria: 0 = no consumption, 1 = alcohol fewer than or equal to 2 days per week, 2 = alcohol more than 3 days and fewer than or equal to 4 days per week, 3 = alcohol more than 5 days per week.

For the family history investigation, baseline profiling on hypertension, stroke, cardiovascular disease, and diabetes mellitus were incorporated for only available participants based on self-check questionnaires.

Statistical Methods

Participants' characteristics were compared using independent-sample Student's *t*-tests for continuous variables and Chi-square tests for categorical variables. The differences between the 2 NAFLD subgroups and control group were verified by ANOVA test. All data are presented as mean ± standard deviation for continuous variables and frequency percentages for categorical variables. $P < 0.05$ were considered statistically significant. All analyses were conducted using SAS v9.4 (SAS Institute Inc., USA).

RESULTS

Characteristics of the Study Population

The clinical features of the study population at baseline (2009) are shown in **Table 1**. Because propensity score matching was based on age group and gender, the NAFLD group and control group were the same in terms of age group distribution and gender percentages. All tested anthropometric factors were statistically significantly higher in the NAFLD group than in the control group, with the exception of height. Considering the laboratory variable measurements, all three liver enzymes showed statistically significant differences between the NAFLD group and the control group in both genders, as expected. Interestingly, serum HDL cholesterol level was lower in the NAFLD group than in the control group with statistical significance in men but without statistical significance in women. Additionally, the NAFLD group showed higher LDL cholesterol than the control group with statistical significance in women but without statistical significance in men. For the lifestyle parameters, we found no statistically significant differences in either gender for any variable (smoking, alcohol consumption, or exercise status). For the family history variables, men in the NAFLD group appeared to report more family history of hypertension and diabetes mellitus compared to the control group, which was not observed in women.

Chronological Changes in Anthropometric and Laboratory Parameters in the NAFLD Subgroups

We compared the time-dependent changes in anthropometric and laboratory parameters by absolute mean value differences among the 2 NAFLD subgroups and the control group by gender (**Figure 2**). When liver enzyme dynamics during pre-NAFLD stage were evaluated, AST, ALT, and GGT all increased in group 2 during the first period (2009–2011) with statistical significance in men, but not in women. Furthermore, during the second period (2011–2013), AST, ALT, and GGT all increased in group 1 and decreased in group 2 with statistical significance in both genders, as expected (**Figure 2**, green box).

Importantly, serum TG in both group 1 and group 2 showed the most prominent decrease during pre-NAFLD stages in both genders (**Figure 2**, red box). Interestingly, the decremental change of serum TG in men for group 2 during pre-NAFLD stage was most prominent with statistical significance ($p < 0.05$). The average degree of decrease in serum TG level among NAFLD patient groups compared to control group in pre-NAFLD stages was 8.38 mg/dL. Additionally, total cholesterol in females showed the most significant decrease in group 2 during the first period and in group 1 during the second period, although statistical significance was observed only in the first period.

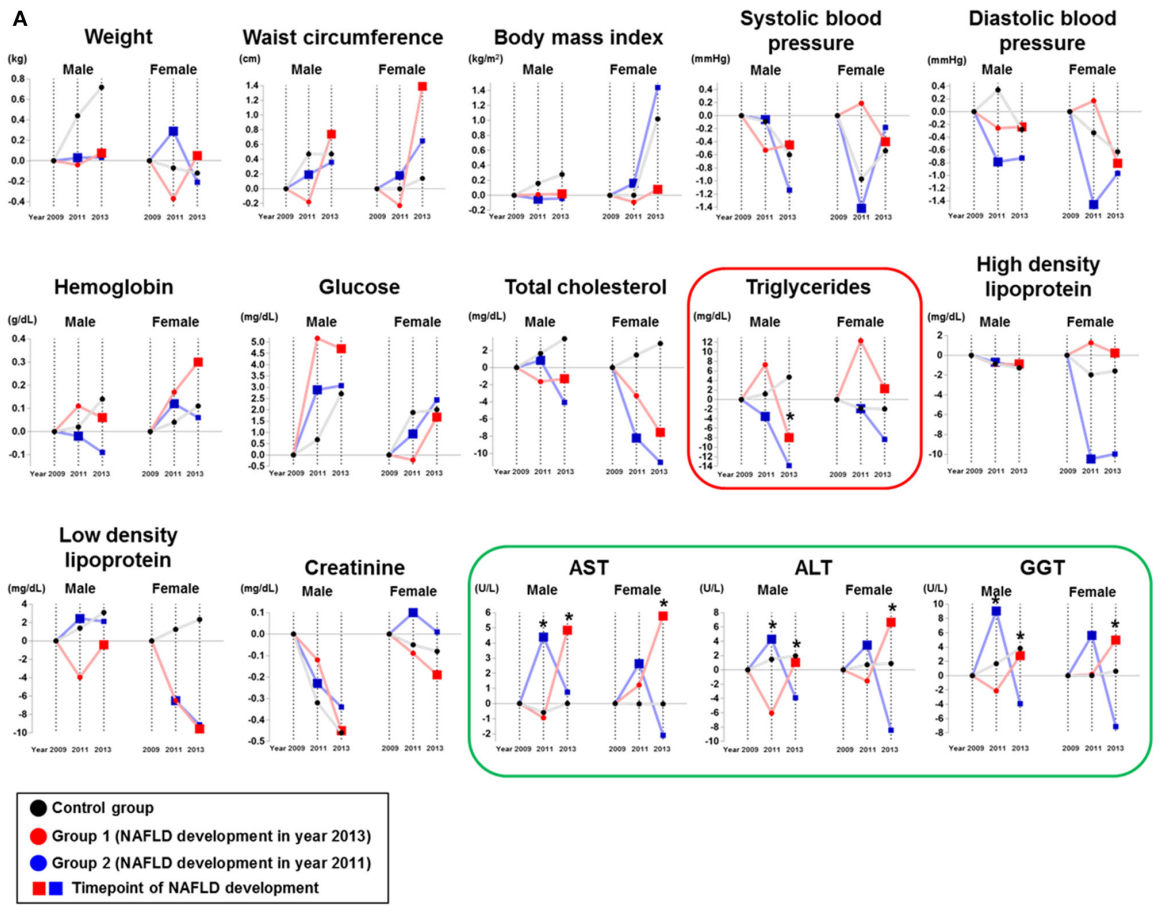
TABLE 1 | Clinical characteristics of study population measured in year 2009 as baseline characteristics.

Gender	Male			Female		
	NAFLD (*N* = 470)	Normal (*N* = 470)	*p*-value	NAFLD (*N* = 267)	Normal (*N* = 267)	*p*-value
DEMOGRAPHIC FACTORS [NUMBER, (%)]						
Age group (years)						
20–29	35 (7.4)	35 (7.4)	1.00	1 (0.4)	1 (0.4)	1.00
30–39	128 (27.2)	128 (27.2)		10 (3.7)	10 (3.7)	
40–49	152 (32.3)	152 (32.3)		56 (21.0)	56 (21.0)	
50–59	91 (19.4)	91 (19.4)		115 (43.1)	115 (43.1)	
60–69	51 (10.9)	51 (10.9)		64 (24.0)	64 (24.0)	
>70	13 (2.8)	13 (2.8)		21 (7.9)	21 (7.9)	
ANTHROPOMETRIC FACTORS [MEAN, (SD)]						
Height (cm)	170.5 (6.4)	170.0 (5.8)	0.2408	155.0 (5.6)	155.3 (5.9)	0.5550
Weight (kg)	78.6 (9.7)	66.2 (7.7)	<.0001	64.5 (7.7)	54.1 (5.4)	<.0001
Waist circumference (cm)	90.1 (7.2)	80.5 (6.1)	<.0001	84.9 (7.9)	75.0 (6.4)	<.0001
Body mass index (kg/m^2)	27.0 (2.8)	22.9 (2.1)	<.0001	26.8 (2.7)	22.5 (2.0)	<.0001
Systolic blood pressure (mmHg)	127.1 (13.3)	123.6 (13.0)	<.0001	126.8 (14.5)	122.5 (16.0)	0.0012
Diastolic blood pressure (mmHg)	80.2 (9.3)	77.2 (9.0)	<.0001	77.9 (9.2)	75.2 (9.5)	0.0008
LABORATORY RESULTS [MEAN, (SD)]						
Hemoglobin (g/dl)	15.3 (1.2)	14.9 (1.1)	<.0001	13.0 (1.3)	12.8 (1.0)	0.0302
Fasting glucose (mg/dL)	103.7 (26.7)	95.7 (20.2)	<.0001	101.1 (25.2)	93.6 (15.9)	<.0001
Total cholesterol (mg/dL)	202.3 (36.8)	192.1 (34.0)	<.0001	208.0 (41.9)	199.4 (36.9)	0.0127
Triglycerides (mg/dL)	196.4 (125.6)	134.7 (101.9)	<.0001	143.7 (81.2)	108.4 (67.8)	<.0001
HDL cholesterol (mg/dL)	49.0 (11.1)	55.1 (22.5)	<.0001	58.5 (61.9)	60.6 (43.7)	0.6575
LDL cholesterol (mg/dL)	115.1 (46.6)	111.1 (31.4)	0.1302	125.5 (37.2)	119.3 (33.9)	0.0456
Serum creatinine (mg/dL)	1.4 (2.0)	1.4 (2.0)	0.8944	0.9 (0.8)	0.9 (0.7)	0.8474
AST (U/L)	33.1 (16.3)	24.8 (19.6)	<.0001	30.2 (30.6)	23.4 (13.1)	0.001
ALT (U/L)	50.8 (32.7)	21.8 (9.7)	<.0001	37.3 (44.7)	17.4 (8.7)	<.0001
GGT (U/L)	71.0 (67.4)	36.0 (29.2)	<.0001	36.2 (53.8)	18.3 (10.2)	<.0001
FAMILY HISTORY* [NUMBER, (%)]						
Hypertension	74 (22.6)	27 (8.3)	<.0001	36 (21.6)	34 (20.7)	0.8542
Stroke	44 (13.5)	30 (9.3)	0.0917	24 (14.4)	24 (14.8)	0.9093
Cardiovascular disease	16 (4.9)	7 (2.2)	0.059	14 (8.4)	7 (4.3)	0.1318
Diabetes mellitus	59 (18.0)	31 (9.6)	0.0018	30 (18.0)	22 (13.5)	0.2655
LIFE STYLE FACTORS [NUMBER, (%)]						
Smoke			0.5538			0.0798
No	261 (55.5)	270 (57.4)		264 (98.9)	258 (96.6)	
Current smoker	209 (44.5)	200 (42.6)		3 (1.1)	9 (3.4)	
Alcohol consumption (days/a week)			0.1812			0.4846
0: 0	152 (32.3)	137 (29.1)		218 (81.6)	222 (83.1)	
1–2: 1	220 (46.8)	244 (51.9)		44 (16.5)	39 (14.6)	
3–4: 2	77 (16.4)	61 (13.0)		5 (1.9)	4 (1.5)	
>5: 3	21 (4.5)	28 (6.0)		0 (0.0)	2 (0.7)	
Exercise			0.1691			0.2552
No	90 (19.1)	74 (15.7)		73 (27.3)	85 (31.8)	
Yes (at least little physical activity)	380 (80.9)	396 (84.3)		194 (72.7)	182 (68.2)	

**Participants with family history information available were only included in the analysis.*

NAFLD, Non-alcoholic fatty liver disease; SD, standard deviation; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase.

Among the anthropometric factors, weight and waist circumference showed incremental change during pre-NAFLD stage before NAFLD was newly diagnosed (first period for group 2 and second period for group 1) but only in women. SBP and DBP decreased more in group 1 during the second period and in group 2 during the first period in women, although without



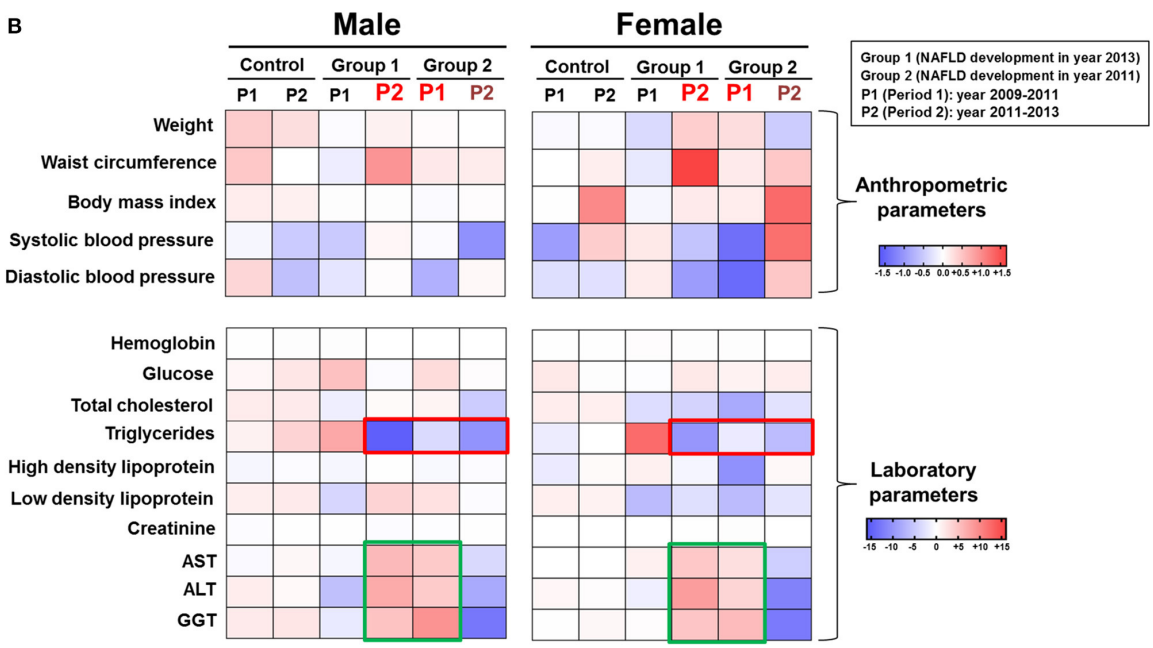


FIGURE 2 | Chronological changes of anthropometric and laboratory parameters according to NAFLD development group and gender. **(A)** Chronological changes of incremental and decremental changes are presented. **(B)** Delta difference values of each parameter during two periods are presented (Group 1: patients who newly developed NAFLD in year 2013; Group 2: patients who newly developed NAFLD in year 2011).

statistical significance. In men, parameters showed variable changes in the pre-NAFLD stage periods for the NAFLD group compared to the control group.

Chronological Changes in Lifestyle Factors in the NAFLD Subgroups

Among the three lifestyle factors considered in this study, we observed different patterns by gender (**Table 2**). In men, we found no consistent chronological change in pre-NAFLD stage periods for both groups. In contrast, the proportion of females with no exercise increased in group 2 during the first period (+1.46 %) and in group 1 during the second period (+0.77%). In other words, more NAFLD was diagnosed in female subjects who did not perform any exercise. Furthermore, the alcohol consumption scores in females decreased in greater degree in group 1 during the second period (−0.11) compared to other groups in the corresponding periods, while men did not show similar consistent trends.

DISCUSSION

In this study, we evaluated the effects of 18 anthropometric, laboratory, and lifestyle factors on the development of NAFLD by comparing chronological changes in parameters in association with new NAFLD diagnoses. Given a retrospective cohort study population, we could derive chronological changes for each subject across a 4-year period using the consistent approach of national health check-ups. While previous studies in various populations focused on finding risk factors associated with NAFLD in a cross-sectional study setting (14), we could reveal several interesting findings for associations between NAFLD and changes in measured factors over time, focusing on dynamics in pre-NAFLD stages according to gender.

Firstly, it is interesting that the serum TG level, which is normally highlighted for its important role in hyperlipidemia and metabolic syndrome when increased, is inversely associated with NAFLD, especially in men. Importantly, the decremental changes of serum TG level in pre-NAFLD stage were previously suggested as a potential biomarker to identify critical transition state for NAFLD in animal model (9). In human studies, a decrease in serum TG level is generally reported to be associated with liver cirrhosis (15). It is plausible that the dynamics of lipid species in serum and liver might be different according to disease progression, especially in pre-NAFLD stage. Although serum lipid profiles were previously studied for their associations with NAFLD (16, 17), chronological changes in liver and serum according to different NAFLD stages should be investigated using traditional parameters as well as up-to-date metabolomics approach (18).

Secondly, the relationship of weight and waist circumference with development of NAFLD appeared to be stronger in women than men. Interestingly, one Japanese population study also showed the similar results that the same degree of weight gain increased the relative risk for NAFLD more prominently in women than in men (19). Several researchers have also reported that the association between obesity and inflammatory

TABLE 2 | Chronological changes of lifestyle factors in study subgroups.

Gender	Male						Female					
Period	From 2009 to 2011 (first period)			From 2011 to 2013 (second period)			From 2009 to 2011 (first period)			From 2011 to 2013 (second period)		
Group	NAFLD		Normal	NAFLD		Normal	NAFLD		Normal	NAFLD		Normal
	Group 1 (*N* = 228)	Group 2 (*N* = 242)	(*N* = 470)	Group 1 (*N* = 228)	Group 2 (*N* = 242)	(*N* = 470)	Group 1 (*N* = 130)	Group 2 (*N* = 137)	(*N* = 267)	Group 1 (*N* = 130)	Group 2 (*N* = 137)	(*N* = 267)
LIFE STYLE FACTORS												
Current smoker, %p	−1.32	−4.58	−3.40	−8.89	−2.92	−1.92	0.00	0.00	0.00	0.77	0.00	0.00
Alcohol consumption score	0.11	0.04	−0.02	−0.10	−0.02	0.01	0.09	−0.01	0.02	−0.11	−0.01	−0.02
No exercise, %p	−8.33	1.24	4.04	0.88	1.24	−2.35	0.77	1.46	−7.52	0.77	−1.46	0.38

NAFLD, Non-alcoholic fatty liver disease.

markers is considerably stronger in women than men (20, 21), which eventually provoke increased inflammation of hepatocytes leading to NAFLD. Based on previous studies highlighting gender differences of NAFLD (22), further studies with inflammatory marker measurements would prove the hypothesis that women might be more susceptible than men to the inflammatory effects of central obesity and even development of NAFLD.

Lastly, the effects of exercise on the development of NAFLD revealed to be stronger in women than men, which is in line with the previous finding. Many researchers and the NICE guideline emphasize the importance of exercise for NAFLD management and control (8, 23). Our results revealed the greatest increases in sedentary lifestyle among females in the NAFLD patients during the time period of a new diagnosis of NAFLD, the pre-NAFLD stage. Although central obesity could be a main reason for NAFLD development, the degree of physical activity as a lifestyle risk factor for the development of NAFLD should also be emphasized (5, 6).

The fundamental limitation of our study is selection bias associated with any population-based retrospective cohort. Therefore, we were unable to discern direct causal relationships between the various factors and NAFLD. However, this is a large propensity score-matched population study, which promotes the statistical reliability of the results. Additionally, correlation analyses and comparative association quantification among parameters would validate the association of dynamic changes with NAFLD development, which was not performed in this study. Another limitation is associated with diagnosis, because we did not include the current standard diagnostic procedure of a liver biopsy which might provide NAFLD severity level for fibrosis. Even though we used several types of diagnostic parameters, such as HIS and FLI, to increase the confirmatory degree of NAFLD diagnosis, subjective diagnoses dependent on different physicians' medical decisions could still have affected the analysis, as could code input errors in the database.

In conclusion, we investigated chronological changes in anthropometric factors, laboratory results, and lifestyle factors to predict the development of NAFLD during pre-NAFLD stage. Based on our finding of inversely decreasing pattern in serum triglycerides during the pre-NAFLD stage, we suggest early screening strategies for people with abrupt chronological changes in serum triglycerides to predict NAFLD development before the progression. Furthermore, people with abrupt and prominent chronological changes in specific parameters, especially waist circumference and exercise degree for women, might require more detailed follow-up evaluations. A better understanding of the various factors related to NAFLD development will provide insight into preventive interventions to improve health and reduce the incidence of NAFLD-associated disorders.

DATA AVAILABILITY STATEMENT

As the original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board of the National Health Insurance Service, Ilsan Hospital (IRB No. 2015-11-010). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JHR conducted the research, performed the statistical analysis, and wrote the draft. TY performed the statistical analysis and collected the data. HYG and JC contributed to the literature search and proofreading of the manuscript. JY designed the study and revised the manuscript. All authors read and approved the submitted version.

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Test–Retest Reliability of the Assessment of Fatty Liver Disease Using Low-Dose Computed Tomography in Cardiac Patients

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Non-alcoholic fatty liver disease (NAFLD) is a common disorder that is associated with the risk of cardiovascular diseases. Therefore, its prevalence is high in patients with coronary artery disease. In myocardial perfusion imaging (MPI), low-dose computed tomography (CT) scans are used for attenuation correction in separate stress and rest studies. Here, the test–retest reliability of CT-based quantification of NAFLD was evaluated using these two CT scans. The study population consisted of 261 patients (156 men and 105 women, age 66 ± 10 years). Quantification of liver fat content was based on the radiodensity of the liver in Hounsfield units as well as in relation to corresponding values of the spleen. NAFLD was observed in 47 subjects (18%). CT quantification has good test–retest reliability in assessing NAFLD, with concordance correlation coefficient (CCC) ranging from 0.512 to 0.923, intraclass correlation coefficient (ICC) ranging from 0.513 to 0.923, and coefficient of variation ranging from 3.1 to 7.0%. Regarding the liver to spleen ratio, CCC for non-NAFLD patients and NAFLD patients was 0.552 and 0.911, respectively. For non-NAFLD patients ICC was 0.553 and NAFLD patients it was 0.913. The coefficient of variation for non-NAFLD and NAFLD patients was 4.9% and 3.1%, respectively. Our results suggest that low-dose CT is a feasible and well repeatable method but amount of liver fat contributes to repeatability. In NAFLD patients CCC and ICC were high reflecting excellent reliability, whereas in non-NAFLD patients test-retest reliability was moderate. Assessment of liver fat content can be used as additional information in studies where a CT scan has been done for other medical reasons, such as for low-dose attenuation correction CT along with MPI.

Keywords: computed tomography, non-alcoholic fatty liver disease, reliability, repeatability, test-retest, intraobserver, interobserver

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease in Western countries, with a global prevalence of 25.5% (1, 2). This has mainly been due to diet and lifestyle changes that have increased the prevalence of obesity and metabolic syndrome (MetS) (1, 3). In addition to NAFLD affecting the liver, it also increases the risk of type 2 diabetes mellitus, cardiovascular disease, and chronic kidney disease. Even though NAFLD is a

global epidemic, the current gold standard of diagnosis remains liver biopsy, which is invasive and expensive (4). As NAFLD is asymptomatic for most patients, highly invasive methods cannot be extensively used for screening. Non-invasive methods include ultrasound (US), magnetic resonance imaging (MRI), and computed tomography (CT), all of which have their own flaws. The reliability of US depends greatly on the operator (5), MRI is expensive and has limited availability, and in CT the patient is exposed to radiation. However, CT-based NAFLD quantification is an attractive diagnostic tool. It is widely available, has better image quality than US, and is non-invasive. CT is already used in a wide range of investigations related to a variety of clinical problems, particularly in the thoracic and abdominal regions. The same images can also be used in the quantification of NAFLD, providing a cost-effective way of diagnosis (6). According to practical guidelines, NAFLD screening should be performed in people at high risk of cardiovascular diseases (3). In myocardial perfusion imaging (MPI) and single photon emission tomography (SPECT), low-dose CT scans are part of the imaging protocol and are used for attenuation correction. NAFLD can be assessed using the same CT scans, which can be used as additional information beyond the MPI results.

Testing reliability via test–retest studies requires two CT scans, which are commonly available in MPI studies (7). The protocol for MPI is to take two low-lose CT scans for attenuation correction, one for stress imaging and the other for rest imaging. Under these circumstances, we have been able to analyze two CT images for a large number of patients without additional exposure to radiation. The goal of this study was to evaluate the test–retest reliability of low-dose CT scans in the diagnosis of NAFLD and to calculate the limits of agreement for the method. To our knowledge, this is the first study evaluating the test–retest reliability of CT-based NAFLD quantification in a large sample of cardiac patients. We also assessed intra-observer and inter-observer variability for this method.

MATERIALS AND METHODS

Study Population

The study population was formed from 586 patients who were referred for MPI during February 2010 and May 2011 at Kuopio University Hospital. We retrospectively analyzed 261 patients (156 men, 60%) who underwent both stress and rest phases of the study. The study protocol was approved by the Ethics Committee of the Northern Savo Hospital District.

Patient history (including previous diseases and laboratory values) was analyzed from the medical records. Due to retrospective design, we had no possibility to have complete data for all patients. Serum lipids were available for 227–234 subjects and plasma glucose for 231 subjects. Height and weight were measured (height to an accuracy of 1 cm and weight to an accuracy of 1 kg). Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Blood pressure was measured in the supine position from the brachial artery preceding the stress phase of the study. Blood pressure values were available for 255 subjects.

Imaging Protocol

The MPI included SPECT with attenuation correction CT (SPECT/CT). Imaging was performed using a Philips Precedence hybrid camera, which has dual-headed gamma camera and a six-slice CT scanner (Philips Medical Systems, Bothell, WA). The stress phase included pharmacological provocation (adenosine or dobutamine) and when possible in combination with low-level exercise (starting with 20 W with increments of 20 W/min twice and the highest load was 60 W for 4 min) followed by SPECT/CT imaging 45 min after the provocation. The rest phase SPECT/CT imaging was performed on the same day at least 3 h after the first imaging. The attenuation correction CT was a low-dose CT (140 kV and 20–40 mAs). Both CT studies were performed with the same image parameters. The reconstruction was done for images of 5-mm slice thickness. This forms a test–retest setting for attenuation correction CTs.

Measurements

For each patient, Hounsfield unit (HU) values of four different regions of interest (ROIs) were measured according to Kerut et al. (8), one in the spleen and three in the liver. The spherical ROIs of the liver were spread out, one in the posterior part of the right lobe, one in the anterior part of the right lobe, and one in the left lobe (**Figure 1**). All ROIs were over 100 mm^2 in size, and vasculature, cysts, and other heterogeneous areas were avoided. Measurements of HU values of selected ROIs were performed using IDS7 PACS (Sectra AB IDS7, 2019, version 21.1). The calculation of the liver to spleen ratio (L/S) was done by taking an average HU value of the two ROIs in the right lobe of the liver and dividing this value by the HU of the spleen, for example, $L/S = [(49.3 + 51.3)/2]/43.9 = 1.15$ (mean values from our results). The criterion for NAFLD was $L/S < 1$ based on average L/S of CT scan 1 and 2.

The first observer measured CT 1 scan and CT 2 scan for all 261 cases. For evaluation of inter-observer variability the second observer measured CT1 for 50 cases blinded to the initial results and repeated the measurements for evaluation of intra-observer variability.

Statistical Methods

Two groups were formed according to the L/S. Patients with $L/S < 1$ were regarded as NAFLD patients (NAFLD+), and patients with $L/S \geq 1$ were regarded as normal (NAFLD–). A Chi-square test was used to analyze the statistical significance of differences in the prevalence of obesity (BMI $\geq$ 30 kg/m^2), type 2 diabetes, dyslipidemia, hypertension, and coronary artery disease between the groups. For continuous variables, a *T*-test was used to test the statistical significance of the difference between the two groups.

The concordance correlation coefficient (CCC) (9) and the intraclass correlation coefficient (ICC) (10) were used to assess the test–retest reliability between the measurements from CT scan 1 and CT scan 2. The coefficient of variation (CV%) was calculated according to Glüer et al. (11). The 95% limits of agreement (LoaA) were calculated according to the Bland–Altman method (12) as mean difference $\pm$ 1.96*standard deviation of the differences. Furthermore, to find out possible factors which may contribute to repeatability, a *T*-test was used

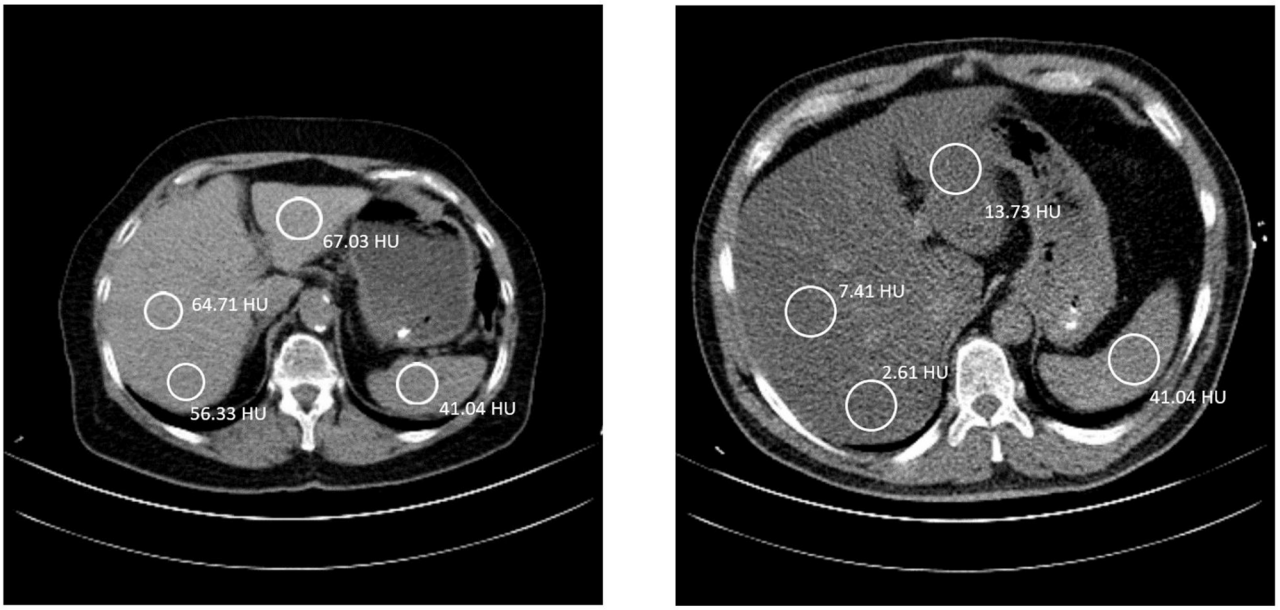

FIGURE 1 | Left: A 70-year-old female, with liver to spleen ratio (L/S) of 1.15. The coloration of the liver is clearly brighter than the spleen. She has a BMI of 26.0 kg/m^2 and has been diagnosed with hypertension, dyslipidemia, and a previous myocardial infarction. **Right**: A 50-year-old male, with L/S ratio of 0.12. The liver has a much darker color than the spleen, indicating a fatty liver. He has a BMI of 30.0 kg/m^2 and has been diagnosed with type II diabetes mellitus, hypertension, and dyslipidemia. The patient also has less subcutaneous fat and more visceral fat. In both images the table is 40 cm wide. A good indication of the size difference between the two patients.

to test statistical significance in absolute differences between measurements in CT 1 and CT 2 in which presence of NAFLD, obesity, dyslipidemia, type 2 diabetes, hypertension and coronary artery disease were used as grouping variables.

Inter-observer and intra-observer variability were expressed as CCC, ICC and LoA.

A P-value < 0.05 was considered statistically significant. SPSS software (IBM SPSS Statistics, 2013, version 22) was used to perform statistical analysis of the data.

RESULTS

Of the 261 patients enrolled in this study, 156 were men and 105 were women. The mean age was 66 years, with a range of 40–89 years. BMI for the pooled population was 29.5 kg/m^2, with a range of 19.4–56.0 kg/m^2. From the pooled population, 197 (75.5%) had been diagnosed with hypertension, 158 (60.5%) with dyslipidemia, 70 (26.8%) with type 2 diabetes, and 136 (52.1%) with coronary artery disease.

The prevalence of NAFLD according to L/S < 1 in the study population was 18%. A diagnosis of NAFLD was not mentioned in the MPI reports in any of the cases. Between the groups, NAFLD+ showed a significantly higher prevalence in obesity ($P < 0.001$) and type 2 diabetes mellitus ($P < 0.001$) (**Figure 2**). The prevalence of dyslipidemia, hypertension and coronary artery disease was not significantly different between the two groups. In addition to the prevalence, the number of patients with obesity, type 2 diabetes mellitus, dyslipidemia, hypertension and coronary artery disease in each group is reported in **Figure 2**. Compared to subjects in the NAFLD– group, those in the NAFLD+ group were statistically significantly younger; had higher weight, BMI, serum triglyceride levels, and diastolic blood pressure; and had lower serum HDL cholesterol (**Table 1**). The sex distribution was comparable in the two groups.

The mean radiodensity value according to average of CT scan 1 and 2 for the spleen ROI was 43.8 HU (SD 3.0, range 36.9–55.5 HU). The HU values for the ROIs in the liver right lobe were somewhat higher, with radiodensities of 48.9 HU (SD 9.2, range 5.3–66.3 HU) in the posterior ROI and 51.0 HU (SD 9.6, range 6.9–66.1 HU) in the anterior ROI. For the left lobe ROI, the equivalent values were 56.5 HU (SD 10.1, range 12.0–73.4 HU). The calculated mean L/S ratio for the pooled population was 1.15 (SD 0.23, range 0.14–1.57), while for the NAFLD- group the mean was 1.23 (SD 0.11, range 1.00–1.57) and for the NAFLD+ group the mean was 0.76 (SD 0.21, range 0.14–0.99). In **Table 2** these values are reported separately in CT scan 1 and 2.

The low dose CT showed moderate to excellent repeatability for all the measurements, with CCC ranging from 0.512 to 0.923. The CCC of L/S values for the NAFLD– group was lower than that for the NAFLD+ group at 0.552 compared to 0.911. Correspondingly ICC ranged from 0.513 to 0.923. For L/S it was 0.553 in NAFLD– group and 0.913 in NAFLD+ group. The CV% of L/S for the NAFLD– group was 4.9% and was 3.1% for the NAFLD+ group, both being significantly lower than the 7.0% for the pooled population. The LoA were −6.9 to 6.4 HU, −8.1 to 6.3 HU, −8.1 to 6.7 HU, and −8.1 to 8.8 HU for the spleen,

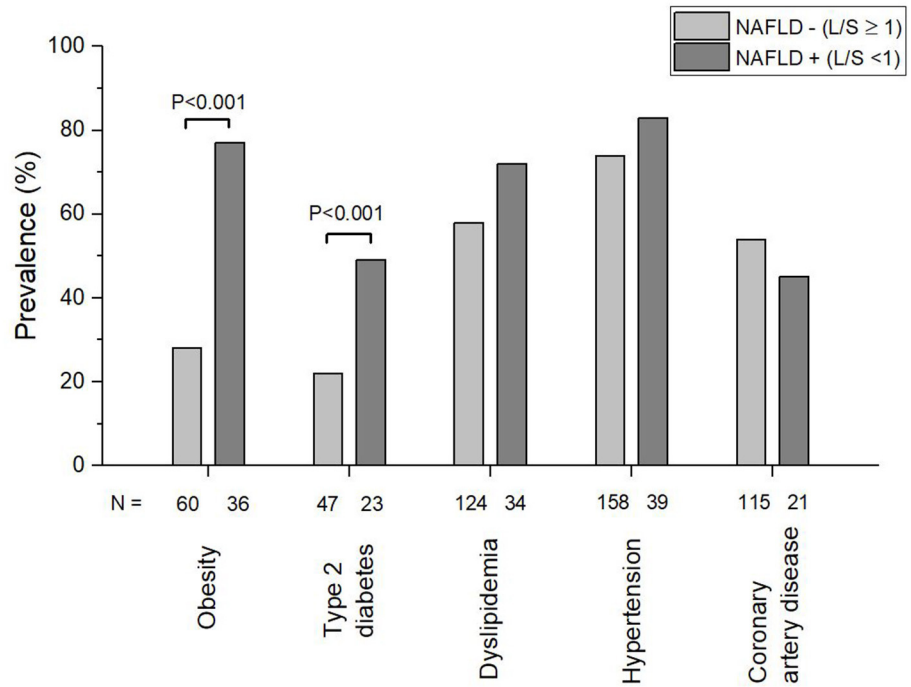

FIGURE 2 | Prevalence of obesity, Type 2 diabetes, dyslipidemia, hypertension, and coronary artery disease in subjects with non-alcoholic fatty liver disease (NAFLD+) and without non-alcoholic fatty liver disease (NAFLD–). NAFLD was diagnosed using cut off value 1 for liver to spleen ratio (L/S) based on radiodensity values in low dose computed tomography scans.

TABLE 1 | Clinical characteristics of the study population.

	Pooled population (n = 261) Mean (SD)	**NAFLD– (n = 214) Mean (SD)**	**NAFLD+ (n = 47) Mean (SD)**	***P*-value (NAFLD– vs. NAFLD+)**
Age (years)	66 (10)	67 (10)	63 (9)	0.004
Height (cm)	168 (10)	168 (10)	169 (9)	0.63
Weight (kg)	84 (19)	80 (16)	100 (24)	<0.001
BMI (kg/m^2)	29.5 (6.3)	28.2 (5.1)	35.1 (8.0)	<0.001
Total cholesterol (mmol/l)	4.35 (1.09)	4.33 (1.04)	4.44 (1.31)	0.64
HDL cholesterol (mmol/l)	1.31 (0.40)	1.34 (0.41)	1.13 (0.29)	0.002
LDL cholesterol (mmol/l)	2.40 (0.93)	2.38 (0.90)	2.51 (1.04)	0.47
Triglycerides (mmol/l)	1.57 (0.98)	1.43 (0.76)	2.20 (1.50)	0.003
Glucose (mmol/l)	6.64 (1.81)	6.49 (1.64)	7.33 (2.31)	0.029
Systolic BP (supine, mmHg)	145 (23)	145 (23)	147 (21)	0.50
Diastolic BP (supine, mmHg)	76 (11)	75 (10)	80 (11)	0.004

NAFLD–, subjects without non-alcoholic fatty liver disease; NAFLD+, subjects with non-alcoholic fatty liver disease; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BP, blood pressure.

the right lobe posterior ROI, the anterior ROI, and the left lobe, respectively. For the pooled population, the NAFLD– group, and the NAFLD+ group, the LoA of L/S ratios were −0.24 to 0.22, −0.25 to 0.23, and −0.19 to 0.17, respectively (**Table 2**).

Bland-Altman plot (**Figure 3**) shows that that the LoA are narrower in the NAFLD+ than in the NAFLD– group. NAFLD was the only factor of the clinical characteristics that contributed to the test–retest reliability. In the NAFLD+ absolute difference in L/S was 0.07 ± 0.05, whereas in the NAFLD- group it was 0.09 ± 0.07 (P = 0.037). The average absolute difference in L/S between CT scan 1 and CT scan 2 was comparable in subjects with and without type 2 diabetes (0.09 ± 0.07 vs. 0.09 ± 0.08, NS), in subjects with and without hypertension (0.09 ± 0.08 vs. 0.08 ± 0.07, NS), in subjects with and without coronary artery disease (0.09 ± 0.08 vs. 0.09 ± 0.07, NS), in subjects with and without dyslipidemia (0.09 ± 0.07 vs. 0.08 ± 0.08, NS), as well as in subjects with and without obesity (0.08 ± 0.07 vs. 0.09 ± 0.08, NS).

In patients with L/S below 1.00 in the CT-scan 1 (n = 47) CT scan 2 showed discordant result in 9 cases (19%) (**Figure 4**).

TABLE 2 | Repeatability (test-retest) of parameters defined from the low-dose computed tomography.

	CT scan 1 Mean (SD)	CT scan 2 Mean (SD)	CCC	ICC (95% CI)	CV%	95% limits of agreement	CT scan 2 - 1 difference Mean (SD)
Spleen (HU)	43.9 (3.3)	43.7 (3.5)	0.512	0.513* (0.418–0.597)	5.3	−6.9 to 6.4	−0.2 (3.4)
Liver right lobe (posterior) (HU)	49.3 (9.5)	48.4 (9.3)	0.920	0.920* (0.895–0.939)	5.3	−8.1 to 6.3	−0.9 (3.7)
Liver right lobe (anterior) (HU)	51.3 (9.7)	50.6 (9.8)	0.923	0.923* (0.902–0.940)	5.1	−8.1 to 6.7	−0.7 (3.8)
Liver left lobe (HU)	56.3 (10.4)	56.7 (10.3)	0.911	0.912* (0.889–0.930)	5.3	−8.1 to 8.8	0.4 (4.3)
L/S (pooled population)	1.15 (0.24)	1.14 (0.24)	0.877	0.878* (0.847–0.903)	7.0	−0.24 to 0.22	−0.01 (0.12)
L/S (NAFLD–)	1.24 (0.13)	1.23 (0.13)	0.552	0.553* (0.453–0.640)	4.9	−0.25 to 0.23	−0.01 (0.12)
L/S (NAFLD+)	0.76 (0.22)	0.75 (0.21)	0.911	0.913* (0.849–0.950)	3.1	−0.19 to 0.17	−0.01 (0.09)

*CT, computed tomography; CCC, Concordance correlation coefficient; ICC, intraclass correlation coefficient; 95% CI, 95% confidence interval; CV%, coefficient of variation; HU, Hounsfield unit; L/S, average HU value of the liver right lobe/HU value of the spleen; NAFLD–, subjects without non-alcoholic fatty liver disease; NAFLD+, subjects with non-alcoholic fatty liver disease. Significances: *$P < 0.001$.*

However, totally 92% of cases were classified concordantly in the CT scan 1 and 2.

According to intra-observer variability analysis ICC was 0.805 for the spleen, and 0.966 for posterior part of the right lobe of liver, 0.967 for the anterior part of the right lobe, 0.957 for the left lobe and 0.949 for the L/S. In intra-observer LoA were −4.5 to 4.9 HU for the spleen, and −3.7 to 4.8 HU for the posterior part of the right lobe of liver, −3.7 to 5.3 HU for the anterior part of the right lobe, −4.9 to 5.5 HU for the left lobe and −0.14 to 0.16 for the L/S.

In the inter-observer variability analysis ICC was 0.710 for the spleen, and 0.928 for posterior part of the right lobe of liver, 0.924 for the anterior part of the right lobe, 0.899 for the left lobe and 0.906 for the L/S. In inter-observer LoA were −6.0 to 5.3 for the spleen, and −6.6 to 5.7 for the posterior part of the right lobe of liver, −4.2 to 8.0 for the anterior part of the right lobe, −8.4 to 7.8 for the left lobe and −0.16 to 0.22 for the L/S.

DISCUSSION

According to our analysis, CT has a good test–retest reliability in the assessment of NAFLD. This makes CT a reliable tool in NAFLD quantification. The small CV% (range 3.1–7.0%) also suggest that CT can be used in monitoring the progression of NAFLD. Clinical characteristics other than NAFLD, such as obesity, dyslipidemia, diabetes, hypertension and coronary artery disease, did not have a significant effect on the repeatability. Therefore, assessment of NAFLD using CT can be used widely in patients without clinical characteristics affecting the reliability. As the test–retest reliability was higher for patients with NAFLD than for patients without NAFLD, CT works well in monitoring patients who already have NAFLD. However, especially in borderline cases, diagnosis of NAFLD needs confirmation with alternative methods.

As we included subjects who were referred for MPI due to their clinical evaluation in relation to coronary artery disease and who already had all the CT images used in the study, we were able to analyze a large population ($n = 261$) without exposing study participants to excess radiation. Repeated low-dose CT scans had been taken of the patients due to the protocol used in MPI. This allowed us an opportunity to study the test–retest reliability. To our knowledge, no previous studies on CT-based test–retest reliability have been done due to the ethical problems of exposing patients to unnecessary radiation. Having a large population allows us to take into account possible confounding factors. Test –retest setting was obtained using the 1-day stress-rest protocol of MPI which includes two low-dose CT imaging primarily for attenuation correction for SPECT images. The interval between these was 3 h. Although the first imaging was done in stress phase we consider the two CT imaging situations to be well comparable regarding the assessment of liver fat content. The stress protocol which is used in myocardial perfusion imaging included adenosine infusion for 6 min and in most cases, additional low-level exercise on bicycle ergometer was used. The half time of adenosine is <10 s and due to the ultra-short half-life of adenosine its effects were fully expired before SPECT/CT imaging. Furthermore, exercise was mild and short lasting. Highest load was 60 W, which corresponds to walking outside for 4 min. After this stress procedure, subjects were sitting in the waiting room for 45 min. Because SPECT/CT was done 45 min after cessation of adenosine infusion and mild physical exercise, such post-stress situation is quite well comparable with the rest phase as regards to the abdominal CT analyses. This is supported by our finding that in stress and rest phases average differences (CT scan 2 – CT scan 1, **Table 2**, **Figure 3**) were near zero. Because there was only minor consistent difference, between the CT scan 1 and CT scan 2, these were true replicates of the measurements.

We decided to use a cut-off point of L/S < 1 to determine NAFLD based on a Japanese study, which showed sensitivity and specificity of 86.3 and 81.4%, respectively (13). Using reasonably small ROIs (over 100 mm^2) allowed us to use homogenous liver tissue and to avoid the influence of other structures that would have affected the radiodensity values. By using this method, we found an NAFLD prevalence of 18%. This is almost the same as the prevalence of fatty liver in Finland of 18.5% (14) but is somewhat lower than the global prevalence of 25.5% and the previously reported European prevalence of 23.7% (2).

Compared to other test–retest repeatability studies on MRI- and US-based diagnosis, we see CT as a viable option. For MRI-based diagnosis (corrected T1 and MR elastography), the CV%

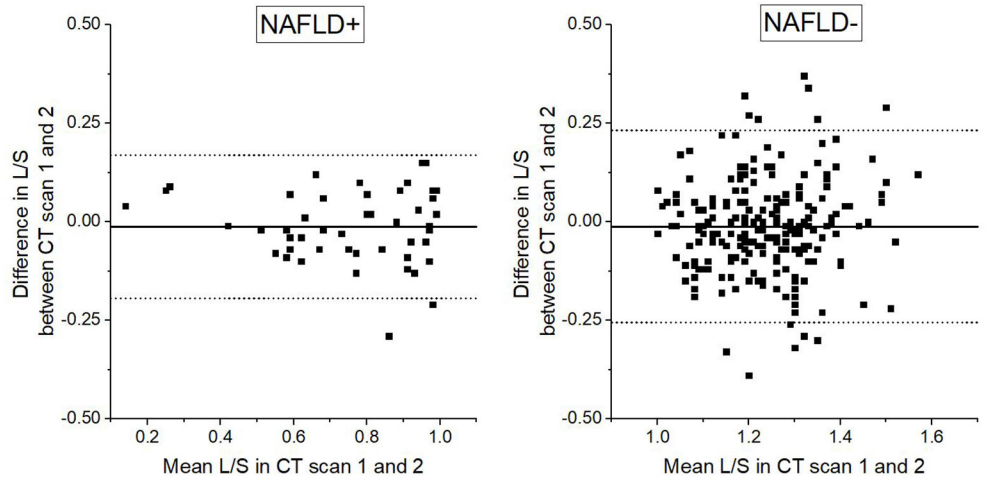

FIGURE 3 | Bland-Altman plot demonstrating differences in liver to spleen ratio (L/S) based on radiodensity values in repeated low dose computed tomography scans in relation to mean of the two L/S assessments. Straight horizontal line represents mean difference, dotted horizontal lines represent upper and lower 95% limits of agreement. L/S cut of value of 1 is used in division of study population to subjects with non-alcoholic fatty liver disease (NAFLD+) and subjects without non-alcoholic fatty liver disease (NAFLD–).

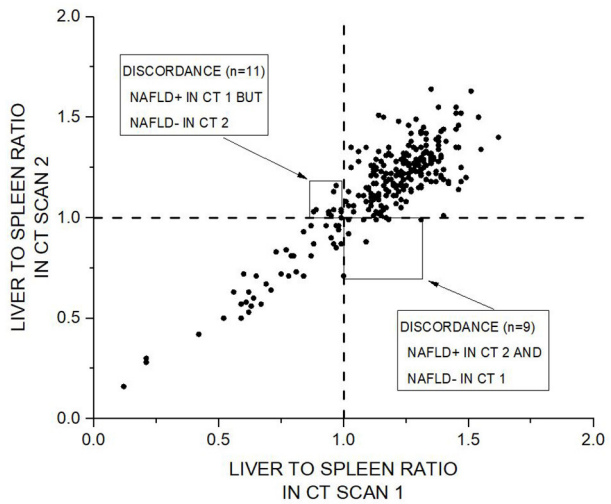

FIGURE 4 | Scatter plot of liver to spleen ratio (L/S) in CT scan 1 vs. CT scan 2 demonstrating concordance for L/S based classification in repeated low dose computed tomography scans. Dashed vertical and horizontal lines represent L/S cut of value of 1, which is used in division of study population to subjects with non-alcoholic fatty liver disease (NAFLD+) and subjects without non-alcoholic fatty liver disease (NAFLD–). Totally 241 cases (92%) were classified concordantly in the CT scan 1 and CT scan 2. Twenty of cases (8%) were discordantly classified into NAFLD– or NAFLD+.

ranged from 1.7 to 11% (15, 16) and for shear-wave ultrasonic elastography the CV% was 40% (16). Our calculations of the CV% for CT ranged between 3.1 and 7.0%. In comparing US and CT test–retest reliability, the ICC for US ranged from 0.58 to 0.82 (17) and for CT it ranged from 0.513 to 0.923.

Zeb et al. studied the inter- and intra-observer variability of CT-based NAFLD diagnosis using the same technique of calculating L/S ratios (18). Their inter-observer LoA for the liver and spleen were −5.63 to 5.25 and −5.68 to 7.15, respectively, and the intra-observer LoA for the liver and spleen were −2.79 to 2.51 and −3.94 to 4.42, respectively. In our study inter-observer and intra-observer variabilities were well in line with the previous study. As regards to two separate images (test – retest setting) LoA were somewhat higher at −6.30 to 8.10 and −6.41 to 6.86 for the liver and spleen, respectively, compared to LoA in intra-observer variability analysis. It is obvious that in repeated measurements variation results both from intra-observer variability in analyses as well as from physiological and technical differences in two separate imaging sessions. Interpretation of LoA should be based on a clinical context (12). Our results regarding LoA tell, which is the difference that will be exceeded of pairs of repeated measurements. This information can be useful in clinical situation when considering how reliable is CT based diagnosis and classification into NAFLD+ and NAFLD-, especially in borderline cases. This kind of uncertainty is also highlighted by our finding that in those patients with L/S below 1.00 in the CT-scan 1, the CT scan 2 showed discordant result in 9 cases (19%). On the other hand, totally 92% of cases were classified concordantly in the CT scan 1 and 2 (**Figure 4**).

We found that in test-retest analysis LoA were wider in the NAFLD– than in the NAFLD+ group. It can be speculated that repeatability is better in patients with NAFLD and the use of low dose CT is not as reliable in assessment of liver fat content in subjects without NAFLD. However, our observation may also be related to the phenomenon in which divergence depends on magnitude, i.e., when the differences increase in size proportionally to the size of measurement. In our study, for all parameters differences between CT scan 1 and CT scan 2 increased when magnitude of the measurements became higher. Thus, assumption of constant differences throughout the range was not met.

ICC and CCC were higher in measurements of the liver than in the spleen. Therefore, much of variability seems to be explained due to inconsistency in values of the spleen. We speculate that in the follow-up of liver disease progression, measurements of liver ROIs could be useful in addition to L/S.

We can also speculate that NAFLD would be a significant risk factor for cardiovascular diseases. As all the patients were admitted for MPI, they either had suspected or previously known coronary artery disease. Even though both groups had a similar prevalence of hypertension and coronary artery disease, for the NAFLD– group the mean age was 67 years, while for the NAFLD+ group the mean age was 63 years. This suggests that patients with NAFLD may have cardiovascular disease at a younger age than patients without NAFLD. The association between NAFLD and development of cardiovascular diseases was not analyzed thoroughly, and this needs attention in further studies.

From the results we can also see the association between NAFLD and MetS. The mean height was the same for both groups, but the mean weight was 20 kg higher for the NAFLD+ group. Consequently, the group also had an increased BMI of 35.1 kg/m^2 compared to 28.2 kg/m^2 for the NAFLD- group. Patients with an L/S ratio of <1 had a significantly higher prevalence of obesity, and type 2 diabetes mellitus, which are part of the International Diabetes Foundation MetS definition (19). In addition to a decreased liver radiodensity and a higher prevalence of some clinical problems, the NAFLD+ group also showed other characteristics of NAFLD and MetS, such as increased triglyceride levels, higher diastolic blood pressure, and decreased HDL levels compared to the NAFLD– group.

Even though the European Association of Nuclear Medicine (EANM) MPI guideline recommends screening images for extracardiac findings (7), none of the patients with an L/S ratio <1 had any mention of a fatty liver in their medical report. This highlights the under diagnosis of NAFLD in clinical practice. All these cases could have been diagnosed as a free by-product of the MPI SPECT/CT. As thoracic and abdominal CT scans are fairly common, if the image area contains the liver and spleen they could also be used to diagnose NAFLD as a by-product. For the patients with an indication of a fatty liver, further diagnosis should be performed. European practical guidelines highlight the need for NAFLD screening in people at high risk of cardiovascular diseases (3). Lifestyle changes toward a healthy diet, weight loss, and habitual physical activity are particularly emphasized in association with NAFLD (3).

Lack of information about alcohol consumption is an important limitation in the present study. Due to retrospective nature in our study design we were not able to collect exact such information and we had to rely on medical records in which this information is often inaccurate. We cannot exclude the possibility that some of our patients may have had fatty liver disease related to excessive alcohol consumption. However, the focus of this article is in repeatability of assessment of liver fat, and etiology of liver steatosis may not be crucial as regards to the main focus of our study. Additionally, none of the cases defined as NAFLD+ have been biopsy-proven. Comparative analysis with gold standard would have been valuable. Due to retrospective nature in our study design we were not able to choose to use reference methods. However, our study evaluating test – retest reliability gives a little different viewpoint to this issue. Variation due to technical or physiological reasons may partly limit the diagnostic performance, which however, seems to be good according to a previous study (13). Therefore, we focused on studying repeatability and reproducibility of the measurement to understand better the methodological variation.

In our study, the population has a high mean age of 66 years and a high prevalence of chronic diseases and therefore might not represent a general population. However, we want to encourage the assessment of NAFLD in patients undergoing MPI or in other situations where abdominal CT is available and NAFLD could be an additional finding. Furthermore, in clinical work screening for NAFLD can be performed using liver enzymes and/or US (3).

In conclusion, CT-based quantification of NAFLD has good test–retest reliability, and clinical characteristics other than NAFLD did not affect CT scan repeatability. Compared to MRI, CT shows similar reliability, but compared to US the test–retest reliability of CT seems to be even higher. Therefore, CT-based assessment of NAFLD and quantification could be used for diagnostic purposes. As the test–retest reliability was higher for patients with NAFLD than for patients without NAFLD, CT works well in monitoring patients who already have NAFLD. However, especially in borderline cases, diagnosis of NAFLD needs confirmation with alternative methods.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because permission to use this data is restricted by the General Data Protection Regulation (EU) 2016/679 and the Finnish authority (FINDATA) have defined the users and limited operating environment (THL/5431/14.02.00/2020). Output of Statistical analyses are available by request to the corresponding author (Tomi.Laitinen@kuh.fi).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the Northern Savo Hospital District. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AH: methodology, investigation, and drafting the initial manuscript. HH: investigation and review or editing of the

manuscript. TML: conceptualization/design, methodology, formal analysis, and review or editing of the manuscript. TPL: conceptualization/design, methodology, investigation, formal analysis, supervision/oversight, funding acquisition, resources, and review or editing of the manuscript. All authors contributed to the article and approved the submitted version.

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De novo Portal Vein Thrombosis in Non-Cirrhotic Non-Alcoholic Fatty Liver Disease: A 9-Year Prospective Cohort Study

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Background and Aims: Approximately 30–40% of portal vein thrombosis (PVT) remains of unknown origin. The association between non-alcoholic fatty liver disease (NAFLD) and PVT is a matter of debate. This study aimed to investigate the association between PVT and NAFLD.

Methods: We included 94 out of 105 consecutive NAFLD patients in this prospective cohort study in addition to 94 from the healthy control group. We evaluated biochemical, clinical, immunological, and histopathological parameters; waist circumference (WC); leptin; adiponectin; and leptin/adiponectin ratio (LAR) for all participants at baseline and every 3 years thereafter. We described the characteristics of participants at baseline and showed individual WC, LAR, and PVT characteristics. Potential parameters to predict PVT development within 9 years were determined.

Results: PVT developed in eight (8.5%) patients, mainly in the portal trunk. Univariate analysis showed three PVT-associated factors: diabetes mellitus ($P = 0.013$), WC ($P < 0.001$), and LAR ($P = 0.002$). After adjusting multiple confounding variables, the multivariate model showed that the only significant variables were WC and LAR. By applying the receiver operating characteristic curve, WC had 98.8% specificity, 87.5% sensitivity, and 0.894 area under the curve (AUC) for prediction of PVT ($P < 0.001$) at cutoff values of >105 cm. In comparison, LAR had 60.5% specificity, 87.5% sensitivity, and 0.805 AUC for PVT prediction ($P < 0.001$) at cutoff values of >7.5.

Conclusions: This study suggests that increased central obesity and LAR were independently associated with PVT development in non-cirrhotic NAFLD patients, and they should be considered risk factors that may participate in PVT multifactorial pathogenesis.

Keywords: adiponectin, leptin, leptin/adiponectin ratio, NAFLD, portal vein thrombosis, waist circumference

INTRODUCTION

Cirrhosis and malignancy-free portal vein thrombosis (PVT) is a rare disease with serious complications. The acute form may predispose to intestinal ischemia with an up to 50% mortality rate (1). In developed countries, the first etiology of non-cirrhotic portal hypertension is the chronic form (2). Searching for a precipitating factor is mandatory where systemic or local factors are observed in 70 and 30% of the PVT etiologies, respectively (3, 4). The causes of PVT are multifactorial in ∼30% of cases (5). However, 30–40% of cases remain of unknown etiology (6). The pathogenesis of PVT may include one or more risk factors of Virchow's triad: hypercoagulable state, reduced blood flow in the portal vein, or injury in the vascular endothelium (7, 8).

In the United States and other parts of the world, non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder, with a histopathological spectrum extending from hepatic steatosis to non-alcoholic steatohepatitis (NASH). However, not all patients progress through the full hepatological spectrum of NAFLD (9). It is the most common etiology of elevated liver enzymes and is diagnosed after ruling out other steatosis causes, especially alcohol abuse and infectious hepatitis. NAFLD is the hepatic manifestation of metabolic syndrome (10). Dramatically, the prevalence of obesity and metabolic syndrome has been increased and is considered a public health challenge (11). Waist–hip ratio and body mass index (BMI) are used to evaluate obesity. Increased waist circumference (WC) is a cornerstone in the association between venous thromboembolism (VTE) and obesity (12–14). According to visceral fat distribution, abdominal obesity is the leading risk factor for VTE. In cirrhotic patients, obesity is an independent risk factor for PVT (15). PVT's significance is recently starting to be comprehended entirely because its presence may be associated with complications negatively affecting the quality of life, clinical deterioration, hepatic decompensation, and increased mortality after liver transplantation (16).

This work aims to study the clinical and biochemical markers of non-cirrhotic NAFLD patients to identify high-risk factors for PVT development in patients attending our department.

MATERIALS AND METHODS

Patient Characteristics

According to autopsy findings, the incidence of PVT is ∼1% (17), and we hypothesized that this might reach higher levels (11–12%) in our patients within the follow-up period. We calculated the sample size at power 99% with a type 1 error = 0.01; the minimal number of cases in each group was 91.

This longitudinal prospective cohort study was conducted on 105 consecutive NAFLD patients admitted to the Mansoura University Hospital, Tropical Medicine Department (Mansoura, Egypt) from April 2006 to December 2018. All patients underwent a complete clinical examination, biochemical appraisal, radiological evaluation, histopathological characteristics, and history taking. All patients with persistently increased liver enzymes in the absence of any etiologies of elevated aminotransferases and hepatosteatosis on ultrasonography were enrolled.

The control group also included 94 healthy sex- and age-matched individuals (female/male = 64/30) who are clinically, biochemically, and radiologically free of NAFLD criteria and have no risk factors predisposing to it. We used hepatic steatosis index and NAFLD-liver fat score to characterize NAFLD's presence or absence in the control group (18, 19).

The patients were enrolled after the exclusion of all possible etiologies of chronic and acute hepatic disorders. Patients who were diagnosed with autoimmune hepatitis, viral hepatitis, human immunodeficiency virus (HIV), hemochromatosis, Wilson's disease, primary biliary cirrhosis, α1-antitrypsin deficiency, sclerosing cholangitis, alcoholic liver disease, NASH in liver biopsy, schistosomiasis (Bilharziasis), or malignancy (especially HCC) were excluded. Patients with collagen vascular diseases, bone marrow depression, chronic renal diseases, acute or chronic pancreatitis, septicemia, postoperative abdominal surgery, liver abscess, diverticulitis, personal or family history of deep vein thrombosis, usage of hepatotoxic, NSAIDs, anticoagulant or antiplatelet therapy, and oral contraceptive drugs were also excluded from this study.

Patients who had patent paraumbilical vein, reversed portal blood flow, inherited coagulation abnormalities, myeloproliferative disorders, peripheral vascular disease, heart failure, hemostatic disorders, clinically overt hyperthyroidism/hypothyroidism, Budd–Chiari syndrome, or liver transplantation were ruled out of this work.

The participants were followed up for 9 years and were assessed at baseline and every 3 years after that by BMI, liver function tests, complete blood count (CBC), C-reactive protein (CRP), activated partial thromboplastin time (APTT), fibrinogen, lipid profile, HOMA-IR, WC, leptin, adiponectin, leptin/adiponectin ratio (LAR), and abdominal Doppler US.

Based on PVT development during follow-up, we evaluated the patients' demographic and clinical characteristics at baseline for their prognostic significance. We analyzed gender, age, BMI, liver function tests, APTT, CRP, HOMA-IR, protein C, protein S, Antithrombin III, serum homocysteine, D-dimer, anti-nuclear antibody (ANA), anti-cardiolipin IgG antibodies (ACA-IgG), anti-double-stranded DNA (anti-dsDNA), WC, leptin, adiponectin, and LAR between PVT and non-PVT patients.

We carried an etiological evaluation for each patient, including antiphospholipid syndrome, myeloproliferative disorders, protein C, protein S and antithrombin III deficiency, paroxysmal nocturnal hemoglobinuria, serum homocysteine, connective-tissue diseases, local risk factors, prothrombin gene mutation, JAK2 V617F mutation, and factor V Leiden especially for patients who developed PVT during the follow-up period (3).

Clinical Assessments

We interviewed the patients to assess smoking habits, gender, age, and replacement/hormonal therapy and calculated BMI as

Abbreviations: NAFLD, non-alcoholic fatty liver disease; PVT, portal vein thrombosis; VTE, venous thromboembolism; LAR, leptin/adiponectin ratio; WC, waist circumference; HCC, Hepatocellular carcinoma; HOMA-IR, Homeostatic model assessment—insulin resistance; BMI, body mass index.

weight in kilograms divided by height in meters squared. Health experts use the WC, a measurement taken around the abdomen at the umbilicus level, to screen patients for possible weight-related health problems. WC appears to be a better indicator than the waist-to-hip ratio and BMI. WC measurement is convenient, and it is more strongly associated with cardiovascular risk factors and intra-abdominal fat content (20). Measurement of WC was done according to Ma et al. (21), who reported that WC-midabdominal (WC-mid) is a better measurement to define central obesity than WC-Iliac crest (WC-IC). WC-mid was more closely related to metabolic variables and abdominal visceral fat area and had better results for predicting and identifying metabolic diseases.

Biochemical Assessments

Serum fibrinogen levels, APTT, and prothrombin time (PT) were measured using kits from Siemens Healthcare Diagnostic Inc. (Erlangen, Germany). Serum triglycerides (TG) and cholesterol were measured using kits from Spinreact [Sant Esteve De Bas (GI), Spain]. CRP was measured on COBAS c111 Chemistry Analyzer (Roche Diagnostics, Basel, Switzerland) using commercially available reagents. Serum insulin was measured using RayBio Human Insulin ELISA Kit (3607 Parkway Lane, Suite 100 Norcross, GA 30092). The homeostasis model assessment (HOMA) method (22) was assessed as follows: Insulin resistance (HOMA-IR) = fasting glucose (mmol/L) × fasting insulin (μU/ml)/22.5. Anti-dsDNA, ANA, and ACA-IgG were measured by enzyme-linked immunosorbent assay (ELISA) from Orgentec Diagnostic (Mainz, Germany). Protein C and protein S antigens were measured using ELISA from Corgenix, Inc. (11575 Main Street, SUlte400, Broomfield, CO 80020 USA). Antithrombin III concentration was measured by rate nephelometry from Beckman Coulter, Inc., (Kraemer Blvd., Brea, CA 92821, USA). Semiquantitative evaluation of D-dimer was measured by Tulip Diagnostics Private Ltd. (Alto Santacruz, India). Human Coagulation Factor VIII ELISA Kit was made by MyBioSource, Inc. (San Diego, CA 92195-3308 USA). Plasminogen Activator Inhibitor-1 (PAI-1) ELISA kit was made by R&D Systems (614 McKinley Place NE Minneapolis, MN 55413). Serum homocysteine was measured using an ELISA kit made by DRG International Inc. (841 Mountain Avenue, Springfield, New Jersey 07081, USA). Serum leptin was measured by ELISA from Ray Biotech (Norcross, Georgia, USA). Serum adiponectin was measured by ELISA from Société de Pharmacologie et d'Immunologie–BIO (SPI-BIO) (Montigny le Bretonneux, France). The leptin/adiponectin ratio (LAR) was calculated.

Radiological Assessments

In all participants, abdominal ultrasound (using standardized criteria) was carried out using a convex probe with a 3.5–5 MHz frequency (SonoAce X6 Ultrasound System; Medison Electronics, Seoul, Korea).

After overnight fasting, examination using a color Doppler ultrasonography was performed on all participants using the same previously mentioned machine. The portal vein (PV) was assessed according to the current guidelines that diminish interobserver variability to non-significant levels (23). The portal flow velocity and PV diameter were measured automatically by the instrument. Doppler examinations were performed on all participants by two different experienced sonographers blinded to the biochemical and clinical data.

The presence of a filling defect of color Doppler US or a grayscale endoluminal material in the main trunk of the PV or its branches may suspect PVT. Computed tomography portal angiography was done for all cases with PVT, which differentiated the complete and partial obstructive thrombosis and extension to the superior mesenteric vein (SMV) and the splenic vein precisely.

PVT was categorized according to Yerdel et al. into four grades. Grade 1: partial PVT (<50% of the lumen) with or without minimal extension into the SMV. Grade 2: >50% occlusion with or without minimal extension into the SMV. Grade 3: complete thrombosis of both proximal SMV and PV with open distal SMV. Grade 4: complete thrombosis of the proximal and distal SMV and PV (24).

Histopathology Assessments

Patients underwent ultrasound-guided liver biopsy between May 2006 to September 2008, and then they were followed up regularly until December 2018. Hepatic tissues were evaluated by a single pathologist (blinded to clinical and biochemical data), and the histopathological diagnosis was assessed utilizing hematoxylin and eosin stain and Masson trichrome stains of formalin-fixed, paraffin-embedded hepatic tissue. NASH was diagnosed according to Brunt's criteria (25). Based on the NAFLD scoring system, histopathological characteristics were proposed and categorized by the National Institute of Diabetes and Digestive and Kidney Diseases NASH Clinical Research Network (26). NAFLD Activity Score (NAS) provides a composite score based on lobular inflammation, degree of steatosis, and hepatocyte ballooning. A score of 0–2 is simple steatosis, a 3 or 4 is borderline NASH, and ≥5 is likely to represent NASH (26).

NAFLD fibrosis score (NFS) (27) and Fibrosis-4 (FIB-4) score (28) have been developed as alternatives to liver biopsy and have been used as non-invasive tools to detect progression of fibrosis during the entire follow-up period.

Therapeutic Assessment

Patients received treatment for NAFLD according to the guidelines, and they were all controlled regarding DM and other comorbidities.

All patients with PVT received enoxaparin at a dose of 1 mg/kg SC/12 h for a total duration of 6 months. The schedule and dose of low-molecular-weight heparin (LMWH) varied according to the clinical status and patient's general condition. If thrombosis recurred or remained, the treatment with LMWH could be resumed or continued, or other anticoagulation agents could be used (29).

We carried out a follow-up CT angiography for response evaluations every 90 days or when clinically pertinent events happened. Therapeutic responsiveness was classified as follows: complete recanalization (complete disappearance of the intravenous thrombus), partial recanalization (decreased but remaining thrombus at >25% based on the cross-section

of the vessel), stable disease (no change or reduction of the thrombus volume of <25% of the cross-section of the vessel), or progressive status (increased thrombus size). The overall recanalization rate was defined as the sum of the fraction of patients who had partial or complete recanalization (29).

We evaluated and analyzed data on therapeutic outcomes, details of the anticoagulation therapy, and biochemical data at the beginning and the end of the anticoagulation therapy, and overall outcomes were collected through a survey of medical records and the possible adverse events during the LMWH therapy. Based on the International Society on Thrombosis and Hemostasis (ISTH)

TABLE 1 | Biochemical, clinical, and demographic characteristics of enrolled patients and control group at the baseline of the study.

Parameters	Patient group ($n = 94$)	Control group ($n = 94$)	*P*-value
Age (years)	48 (39–58)	48 (39–57)	0.772
Sex (female/male)	65/29	64/30	0.96
Smoking habits			
Current smoker	42 (45)	40 (43)	0.783
Ex-smoker	18 (19)	19 (20)	0.863
Never smoked	34 (36)	35 (37)	0.887
Hypertension	23 (24)	–	–
DM	32 (34)	–	–
BMI (kg/m^2)	27.3 ± 3.5	23.8 ± 1.1	<0.001
ALT (U/L)	68 (47–95)	29 (20–36)	<0.001
AST (U/L)	54 (46–75)	21 (18–32)	<0.001
γ-GT (IU/L)	39 (30–54)	34 (30–50)	0.216
ALP (IU/L)	96 (83–110)	89 (78–101)	0.024
Albumin (g/dl)	4.4 ± 1.3	4.3 ± 1.1	0.57
Bilirubin (mg/dl)	1.15 ± 0.3	1 ± 0.2	0.18
PT (s)	12 ± 0.8	11.8 ± 0.7	0.07
APTT (s)	35.1 ± 5.1	34 ± 4.8	0.13
Serum creatinine (mg/dl)	0.8 (0.6–1.39)	0.74 (0.6–1.2)	0.502
Waist circumference (cm)	91.6 ± 4.3	82.2 ± 1.9	<0.001
Waist circumference, cm men >102, women >88	54 (57)	–	-
Serum total cholesterol (mg/dl)	197 ± 62	185 ± 22	0.079
Serum triglyceride (mg/dl)	139 (90–184)	93 (80–113)	<0.001
Fibrinogen levels (mg/dl)	269 (227–347)	182 (141–214)	<0.001
Protein C (IU/dl)	82.5 ± 8	80.5 ± 7.8	0.084
Protein S (IU/dl)	85.9 ± 7.5	83.8 ± 7.2	0.052
Antithrombin III	82.5 ± 5.4	79.1 ± 5.1	<0.001
Factor VIII (ng/ml)	101.7 ± 9.5	99.5 ± 8.8	0.101
PAI-1 (ng/ml)	22.4 ± 3.7	10.5 ± 2.3	<0.001
Homocysteine (μmol/L)	13.3 ± 0.7	12.9 ± 0.6	<0.001
D-dimer (ng/ml)	656 (370–826)	425 (244–624)	<0.001
ANA positive (*n*) (positive ≥1.2)	3	–	–
Anti-dsDNA positive (*n*) (positive >20 IU/ml)	0	–	–
ACA-IgG positive (*n*) (positive ≥10 U/ml)	0	–	–
CRP (mg/L)	66 (40–79)	9.0 (4.7–11.6)	<0.001
HOMA-IR	3.2 (1.4–4.1)	1.8 (1.2–2.4)	<0.001
Leptin (ng/ml)	130.2 ± 8.1	95.4 ± 5.3	<0.001
Adiponectin (μg/ml)	20.56 ± 1.2	25.6 ± 1.8	<0.001
LAR	6.3 ± 0.43	3.8 ± 0.51	<0.001
Portal how velocity (cm/s)	23.8 ± 4.4	25 ± 4.5	0.066

The statistics presented are means ± SD, N (%), or median and interquartile range. DM, diabetes mellitus; BMI, basal metabolic index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; PT, prothrombin time; APTT, activated partial thromboplastin time; ANA, anti-nuclear antibody; Anti-dsDNA, anti-double-strand DNA; ACA-IgG, anti-cardiolipin IgG antibody; CRP, C-reactive protein; HOMA-IR, homeostasis model assessment-insulin resistance; LAR, leptin/adiponectin ratio; NAS, non-alcoholic fatty liver disease activity score; NASH, non-alcoholic steatohepatitis.

definition published in 2005, major hemorrhagic adverse events were also defined (30). All the patients had upper gastrointestinal (GI) endoscopy (Olympus GIF-Q200, Olympus Optical Co. Ltd., Tokyo, Japan) before starting anticoagulant treatment.

Ethics

This study's protocol was approved by the Mansoura Faculty of Medicine Institutional Research Board "MFM-IRB" (Approval no. R/17.11.84), and all methods were performed following relevant guidelines and regulations. Informed consent was obtained from all participants.

Statistical Analysis

The results were achieved by the Social Package of Statistical Science (SPSS) software version 20 (SPSS Inc., Chicago, IL, USA). Quantitative and non-normally distributed continuous data are described as mean ± SD and (interquartile) range, respectively. We used the Kolmogorov–Smirnov test to determine the compatibility of normally distributed data, Student *t*-test for normally distributed data, Mann–Whitney *U*-test for non-normally distributed continuous data, and Chi-square test for categorical data. Spearman's correlation analysis was carried out between PVT development and other variables. A scatter plot matrix showing bivariate relationships between combinations of different variables was carried out. At univariate analysis, variables with a $P < 0.05$ were enrolled in the multivariable Cox regression analysis. Univariate and multivariable Cox regression models were assessed to identify the independent variables that can be utilized to predict PVT. The receiver operating characteristic curve (ROC) and area under the curve (AUC) were performed, and the best cutoff values were calculated to predict the development of PVT. A two-tailed $P < 0.05$ was considered significant.

RESULTS

Patient Characteristics

A total of 105 patients who met the inclusion criteria were enrolled in this study. Of these, 94 patients have completed the study. Baseline biochemical, clinical, and demographic parameters of the enrolled patients are listed in **Table 1**, and histopathological characteristics and non-invasive fibrosis scores (NFS and FIB-4) are shown in **Table 2**. Patients showed a statistically significant increase in BMI, ALT, AST, ALP, WC, TG, fibrinogen, antithrombin III, PAI-1, homocysteine, D-dimer, CRP, HOMA-IR, leptin, and LAR compared to that of the control group (all $P < 0.05$). Also, patients showed a statistically significant decrease in adiponectin levels compared to that of the control group ($P < 0.001$).

The participants were followed up every 3 years for 9 years after the initial assessment at baseline by hematological and biochemical blood tests and the abdominal Doppler US.

Ninety-four of the 105 patients were included to complete this work; 10 patients were missed during the follow-up period. One patient died in a motor car accident. Out of all the patients examined during the follow-up period ($n = 94$), eight patients (8.5%) developed *de-novo* PVT, as shown in **Figure 1**. Individual

TABLE 2 | Histopathological characteristics and fibrosis scores of enrolled patients at the baseline of the study.

Histopathological characteristics	
Degree of inflammation	
No (minimal)/mild/moderate/severe	0/12/52/30
Grades of steatosis	
Non/Grade 1/Grade 2/Grade 3	0/24/43/27
Ballooning	
None/Few/Many	0/63/31
Stages of fibrosis	
0/1/2/3/4	3/9/50/32/0
NAS (non-alcoholic fatty liver disease activity score)	
0–2 (simple steatosis)	21
3–4 (borderline NASH)	29
5–8 (NASH)	44
Non-invasive fibrosis scores	
NAFLD fibrosis score (NFS)	0.18 (−1.45 to 0.37)
Fibrosis-4 (FIB-4)	2 (1.38–3.36)

WC, LAR, and non-invasive fibrosis scores (NFS and FIB-4) of patients with PVT are shown in **Table 3**, while WC and LAR changes in patients without PVT are displayed in **Figure 2**. None of the control group ($n = 94$) showed any abnormality regarding PVT diagnostic criteria when they were enrolled in the study or later on till the end of the follow-up period.

The control group did not show any statistically significant changes in LAR levels between the baseline and 3-year follow up, 6-year follow-up, or at the end of study (3.8 ± 0.51 vs. 3.85 ± 0.52, $P = 0.61$; 3.8 ± 0.51 vs. 3.9 ± 0.52, $P = 0.18$; and 3.8 ± 0.51 vs. 3.95 ± 0.54, $P = 0.052$) correspondingly. There were no statistically significant changes in WC between the baseline and 3-year follow up, 6-year follow-up, or at the end of study (82.2 ± 1.9 vs. 81.9 ± 1.8, $P = 0.27$; 82.2 ± 1.9 vs. 82.5 ± 2, $P = 0.29$; and 82.2 ± 1.9 vs. 82.6 ± 2.1, $P = 0.17$), respectively. There were no statistically significant changes in all variables between the baseline and at the end of study (all $P > 0.05$) (data not shown).

The main clinical complaints in patients who develop PVT were abdominal pain (seven patients, 87.5%) and asymptomatic (one patient, 12.5%). However, the signs varied from one case to another, irrespective of PVT sites.

Correlation Between WC and LAR With Clinical, Biochemical, and Histopathological Patterns of the Studied Patients

Actually, in Spearman correlation analysis, there were significant positive correlations between WC and age, serum fibrinogen, CRP, degree of steatosis, grades of inflammation, fibrosis scores, ballooning, and NAFLD activity score (NAS) ($rho = 0.61$, $P = 0.001$; $rho = 0.57$, $P = 0.019$; $rho = 0.78$, $P < 0.001$; $rho = 0.72$, $P < 0.001$; $rho = 0.69$, $P < 0.001$; $rho = 0.76$, $P < 0.001$; $rho = 0.78$, $P < 0.001$; and $rho = 0.73$, $P < 0.001$, respectively).

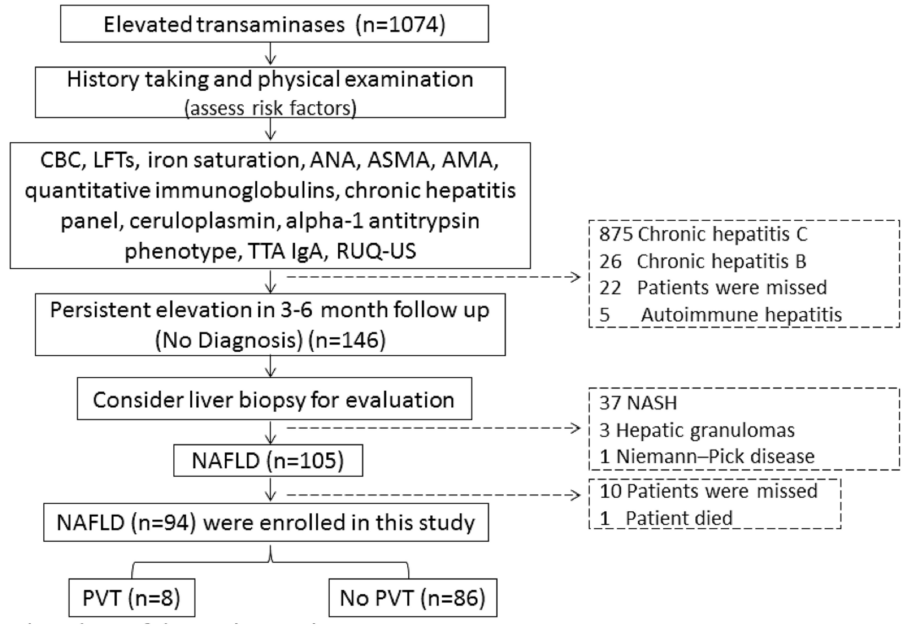

FIGURE 1 | Flow chart of the study population. CBC, complete blood picture; LFTs, liver function tests, ANA, antinuclear antibody; ASMA, anti-smooth muscle antibody; AMA, antimitochondrial antibodies; TTA IgA, tissue transglutaminase IgA; RUQ-US, right upper quadrant abdominal-ultrasonography; NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver diseases; PVT, portal vein thrombosis.

Moreover, in Spearman correlation analysis, there were significant positive correlations between LAR values and age, serum fibrinogen, serum homocysteine, CRP, degree of steatosis, grades of inflammation, fibrosis scores, ballooning, and NAS ($rho = 0.63$, $P = 0.001$; $rho = 0.59$, $P = 0.016$; $rho = 0.55$, $P < 0.001$; $rho = 0.79$, $P < 0.001$; $rho = 0.74$, $P < 0.001$; $rho = 0.71$, $P < 0.001$; $rho = 0.77$, $P < 0.001$; $rho = 0.79$, $P < 0.001$; and $rho = 0.75$, $P < 0.001$, respectively).

Also, there was a significant positive correlation between WC and LAR ($rho = 0.71$, $P < 0.001$).

Univariate and Multivariable Cox Regression Models Predicting PVT Within 9 Years Follow-Up

The biochemical, demographic, and clinical parameters of patients with and without PVT and characteristics of PVT are listed in **Supplementary Tables 1**, **2**.

Regarding sex, age, hypertension, BMI, smoking habits, ALT, AST, γ-GT, ALP, serum albumin, serum bilirubin, PT, APTT, serum creatinine, serum triglyceride, fibrinogen levels, serum CRP, HOMA-IR, protein C, protein S, Antithrombin III, Factor VIII, PAI-1, serum homocysteine, D-dimer, ANA, ACA-IgG, and anti-dsDNA, the difference was not significant between the two groups (all $P > 0.05$).

Univariate Cox regression analysis revealed that diabetes mellitus, increased WC, and LAR are significant predictors of PVT (all $P < 0.05$) in **Table 4**.

After adjusting multiple confounders, the multivariable Cox regression analysis model was re-evaluated using the formerly described baseline parameters related to PVT's occurrence during the 9 years follow-up period. This analysis revealed that WC and LAR are independent factors associated with PVT development (**Table 4**).

Using the ROC curve analysis, at a cutoff value of >105 cm and 7.5, WC and LAR had (98.8%, 60.5%) specificity, (87.5%, 87.5%) sensitivity, (0.894, 0.805) AUC, (87.5%, 17.1%) positive predictive value (PPV), and (98.8%, 98.1%) negative predictive value (NPV) correspondingly for prediction of PVT ($P < 0.001$). Combined WC and LAR were identified as the best discriminating markers in the prediction of PVT with 96.6% specificity, 100% sensitivity, 72.7% PPV, 100% NPV, and 0.993 AUC ($P < 0.001$), as shown in **Figure 3**.

Therapeutic Findings

Enoxaparin was administered to all patients who developed PVT. All patients had a complete response after 6 months of follow-up. The median duration of the anticoagulation therapy was 3.31 ± 1.6 months (range, 1–6 months). The average interval from the onset of therapy to the first CT was 87 days. The overall recanalization rate was 100% (eight patients). Complete recanalization was achieved in seven patients (87.5%), and partial recanalization was reported in one patient (12.5%). No one showed any criteria for stable and/or progressive disease during LMWH therapy. In all patients showing complete and/or partial recanalization, none of them showed PVT relapse or progression after the end of anticoagulation therapy.

TABLE 3 | WC, LAR, and fibrosis scores in patients who developed PVT within 9 years of observation.

Patient No.	WC				LAR			
	Baseline	**3 years**	**6 years**	**9 years**	**Baseline**	**3 years**	**6 years**	**9 years**
4	90	91	93	98	6.8	7.1	7.6	8.6
11	115	117	118	121	6.9	7	7.1	7.4
27	115	116	117	122	7.3	7.5	7.9	8.4
32	114	116	118	120	7.4	7.6	7.8	8.1
49	119	120	121	125	7.5	7.7	8.1	8.5
63	117	119	120	125	6.9	7.1	7.2	7.6
74	123	125	126	130	6.5	6.8	6.9	7.6
85	111	113	115	118	7.1	7.3	7.5	8
Patients with PVT (*n* = 8)	**113 ± 10**	**115 ± 10.2**	**116 ± 9.9**	**120 ± 9.6**	**7.1 ± 0.34**	**7.3 ± 0.32**	**7.5 ± 0.42**	**8 ± 0.5**
		P1 = 0.7	*P2* = 0.85	*P3* = 0.43		*P1* = 0.25	*P2* = 0.3	*P3* = 0.048
				P4 = 0.18				*P4* < 0.001
Patients without PVT (*n* = 86)	**87.6 ± 4.1**	**93.9 ± 4.8**	**95.1 ± 5.2**	**98.8 ± 5.86**	**6.3 ± 0.43**	**6.6 ± 0.5**	**6.9 ± 0.55**	**7.3 ± 0.6**
		*P1** < 0.001	*P2** = 0.12	*P3** < 0.001		*P1** < 0.001	*P2** < 0.001	*P3** < 0.001
				*P4** < 0.001				*P4** < 0.001

Non-invasive fibrosis scores					
	Baseline	3 years	6 years	9 years	
NFS in *PVT*	**0.22 (−1.45–0.55)**	**0.23 (−1.47–0.56)**	**0.24 (−1.49–0.59)**	**0.27 (−1.51–0.62)**	*P1, P2, P3*, and *P4* > 0.05
FIB-4 in *PVT*	**2.45 (1.23–3.33)**	**2.47 (1.26–3.36)**	**2.51 (1.28–3.39)**	**2.52 (1.31–3.42)**	*P1, P2, P3* and *P4* > 0.05
NFS in *non-PVT*	**0.175 (−1.45–0.37)**	**0.182 (−1.41–0.38)**	**0.184 (−1.38–0.39)**	**0.191 (−1.35–0.41)**	*P1*, P2*,P3**, and *P4** > 0.05
FIB-4 in *non-PVT*	**1.88 (1.4–3.36)**	**1.92 (1.4–3.38)**	**1.94 (1.5–3.41)**	**1.95 (1.5–3.41)**	*P1*, P2*, P3**, and *P4** > 0.05

WC, Waist circumference; LAR, leptin/adiponectin ratio; PVT, portal vein thrombosis; NFS, NAFLD fibrosis score; FIB-4, Fibrosis-4.
P1: 3 years vs. baseline in patients with PVT. P1: 3 years vs. baseline in patients without PVT.*
P2: 6 years vs. 3 years in patients with PVT. P2: 6 years vs. 3 years in patients without PVT.*
P3: 9 years vs. 6 years in patients with PVT. P3: 9 years vs. 6 years in patients without PVT.*
P4: 9 years vs. baseline in patients with PVT. P4: 9 years vs. baseline in patients without PVT.*

At the time of the analysis, all patients had completed the treatment with LMWH; 8 patients (100%) completed a 6-month treatment regimen. No clinically relevant bleeding was reported.

All included laboratory values and metabolic parameters are not significantly changed before and after the LMWH treatment (data not shown). All patients that reached the endpoint are still followed regularly in our outpatient clinic till the present day.

In addition, there is no bleeding or major complications reported during the LMWH therapy.

DISCUSSION

The relationship between a hypercoagulable state and NAFLD is a perpetually extending field of research. NAFLD is the hepatic manifestation of a metabolic syndrome often associated with thrombosis and hypercoagulability with NAFLD's natural history (9).

The current study reported that *de novo* PVT incidence within NAFLD patients during a 9-year follow-up was 8.5%.

Generally, the possible explanation may be that NAFLD's continuous and chronic inflammation leads to lipid-based oxidative injury, necrosis, and apoptosis (10, 31–33). This clarifies the stimulation of the coagulation cascade and the resultant hypercoagulable condition because procoagulant levels of factor VIII and plasminogen activator inhibitor-1 (PAI-1) have been increased, while anticoagulant levels of protein C are diminished in the late stage of NAFLD patients (34). Contrary to expectations, this study did not find a significant difference between procoagulant levels of factor VIII and PAI-1 as well as the anticoagulant level of protein C in patients with or without PVT.

In this study, multivariable Cox regression analysis revealed that increased WC and LAR were independently associated with PVT development in non-cirrhotic NAFLD patients.

It is well-known that one of the biological variations noted in metabolic syndrome is an increase in coagulation factors. Many factors known to be related to thrombosis and fat mass risk could be included, such as serum leptin or PAI-1 (35). Besides that, adipose tissue may produce an excess amount of proteins

that behave in a paracrine, autocrine, or endocrine manner. The lipid accumulation is correlated with the production of inflammatory cytokines and increased macrophage infiltration of adipose tissue. In obese individuals, the inflammatory changes present in adipose tissue may induce liver inflammation and hemostatic abnormalities.

Moreover, overweight is a well-known deep vein thrombosis risk factor (12, 13). Increased WC may be considered a landmark of metabolic syndrome and obesity (36). Abdominal obesity was implicated in VTE and coronary heart disease (37, 38) through hypercoagulability via the decreased fibrinolytic process and elevated levels of factor VIII and fibrinogen (39, 40). Abdominal obesity often has a pro-inflammatory state, characterized by elevated acute-phase reactants such as fibrinogen and CRP and a pro-thrombotic condition due to increased levels of PAI-1, clotting factors, and fibrinogen (41, 42). Both pro-thrombotic and pro-inflammatory states are closely linked to VTE development. These results clinically support the hypercoagulable state of abdominal obesity related to PVT in NAFLD patients. This observation is in accordance with Bureau et al., who reported that central obesity is related to PVT and could become one of the critical risk variables for gastrointestinal thrombosis (43).

Our findings declared that increased LAR was an independent factor associated with the development of PVT in NAFLD. As we know, in diabetes mellitus, obesity, and other metabolic syndromes, the concurrence of hyperleptinemia and hypoadiponectinemia is observed. Different studies highlighted the association between LAR and cardiovascular disease markers, including pulse wave velocity and carotid intima-media thickness (44, 45). LAR correlated with HOMA-IR, WC, BMI, and TG better than any adipokine (44). In NAFLD patients, higher levels of LAR were observed. As adiponectin

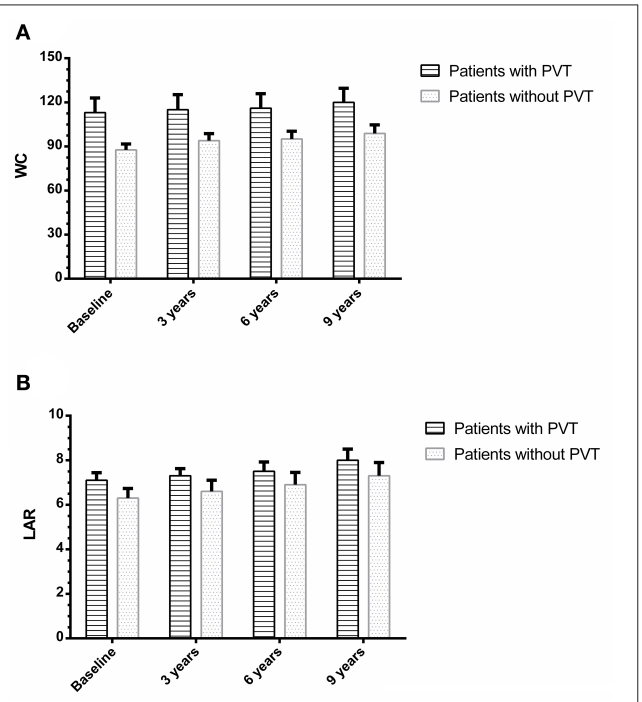

FIGURE 2 | Waist circumference **(A)** and leptin/adiponectin ratio **(B)** changes in patients with and without portal vein thrombosis.

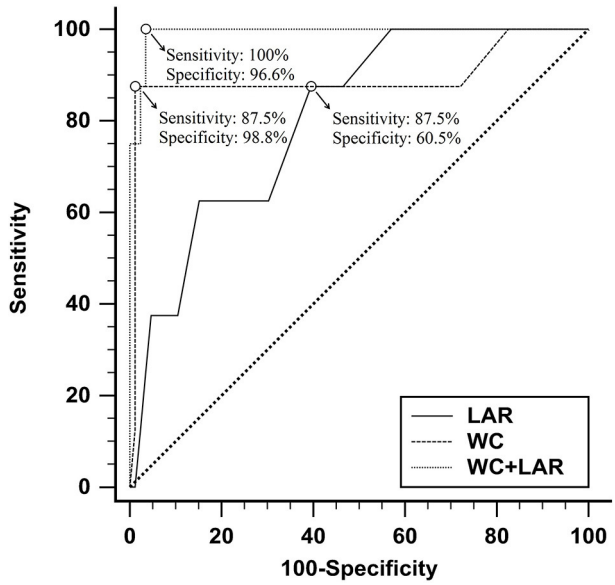

FIGURE 3 | The receiver operating characteristic curves of waist circumference and leptin/adiponectin ratio in predicting portal vein thrombosis in non-cirrhotic NAFLD.

TABLE 4 | The risk of portal vein thrombosis development in the univariate and multivariable Cox regression models in the studied patients.

Parameters	Univariate Cox regression		Multivariable Cox regression	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
DM	6.34 (1.29–31.19)	0.013	1.23 (0.15–10.32)	0.85
Waist circumference	1.16 (1.09–1.24)	<0.001	1.18 (1.09–1.28)	<0.001
LAR	7.44 (1.93–28.87)	0.002	8.04 (1.62–39.9)	0.011
NFS	0.97 (0.45–2.09)	0.93	–	-
FIB-4	1.03 (0.52–2.050)	0.92	–	-

DM, diabetes mellitus; LAR, leptin/adiponectin ratio; CI, confidence interval; HR, hazard ratio; NFS, NAFLD fibrosis score; FIB-4, Fibrosis-4.

and leptin generally show opposite variations, leptin appeared to upregulate different vascular inflammation mediators such as ROS, IL-2, IL-6, TNF-α, MCP-1, TGF-α, and Th1-type cytokines from peripheral blood mononuclear cells and endothelial cells (46–48). Experimentally, leptin prompted increments of cellular adhesion molecules and tissue factor expression in human coronary endothelial cells, though NF-κB leads to increased leukocyte adhesion and procoagulant activity (49). These results in patients with metabolic syndrome exhibit a strong relationship with increased platelet activity and circulating leptin (50–52). The leptin receptor is expressed on endothelial cells and platelets and could enhance the formation of thrombus by inhibiting vasodilatation, stimulating platelet activation, and increasing oxidative stress (53).

It is well-known that diabetes, obesity, and lower adiponectin levels are related to the stimulation of an inflammatory signaling cascade, prompting the early development of atherosclerosis in the metabolic syndrome (54). Adiponectin suppresses the endothelium's vascular inflammatory effect to TNF-α-induced stimulation of NF-κB and enhanced expression of adhesion molecules, intercellular adhesion molecules, vascular cell adhesion molecules, and endothelial selectin. Experimentally, reversed microvascular inflammatory changes may be induced by adiponectin replacement therapy (55). Vascular effects induced by pro-inflammatory cytokines like TNF-α and different interactions with adipokines (adiponectin) greatly enhance vascular thromboembolism (56). Bureau et al. observed that PVT is associated with increased abdominal obesity and could get to be distinctly one of the critical risk factors for gastrointestinal thrombosis (43). According to these findings, we hypothesized that the LAR ratio plays a pivotal role in developing PVT in our patients.

LMWH has no impact on coagulation tests' consequences and offers the advantages of dosages proportional to body weight. Therefore, it is easy to prescribe and does not require monitoring. The risk of bleeding is the primary concern related to anticoagulation therapy for PVT. In this study, the overall recanalization rate was 100%, and all treated patients did not reveal any bleeding or significant complications. All patients that reached the endpoint are still followed up regularly.

Central obesity and NAFLD are two sides of a single coin. In addition to endothelial dysfunction and liver inflammation, it is challenging to speculate each variable's role in PVT development. However, the authors' conviction is that central obesity may play a fundamental role in PVT development through hormonal imbalance, cytokine production, and hepatic affection.

To the best of our knowledge, this study is the first to measure the incidence of PVT in patients with non-cirrhotic NAFLD.

This study had several limitations. First, it is a single-center study. Second, the follow-up period was only 9 years. Third, using non-invasive techniques like NFS and FIB-4 score cannot replace the liver biopsy, but we thought it would be unethical to subject our patients to a technique as invasive as the biopsy for the second time because nothing changed, which was indicative for the biopsy during the study period. The findings of this study have several important implications for future practice. Multicenter research is usually conducted to enroll larger numbers of participants and thus improve the validity and generalizability of the study findings.

Finally, we recommend that early distinguishing of risk variables, management, and prevention of the metabolic syndrome, including lifestyle modifications and treatment for adjusting the syndrome components, are real difficulties. The point is to avert obesity, type 2 diabetes, hyperlipidemia, cardiovascular disease, and PVT.

In conclusion, this work suggests that increased WC and LAR in NAFLD patients are associated independently with PVT development. We propose that these variables may be considered risk factors for PVT and may participate in PVT's multifactorial pathogenesis.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study have restrictions and so are not publicly available. Data are however available from the authors upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mansoura Faculty of Medicine Institutional Research Board MFM-IRB (Approval no. R/17.11.84). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AA-R, NM, WS, and AY designed the study and/or contributed to the concept and performed the statistical analysis. MA, MW, EH, and AT interpreted data critically, contributed to data acquisition, and revised the manuscript. AH drafted the manuscript. KZ and RElz critically recruited and followed up with patients and revised the manuscript. RElh analyzed, acquired, and interpreted data and revised the manuscript. AF, NE-W, and WE contributed to analyzing and interpreting data and performed the statistical analysis. All authors approved the final version of the article.

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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.2021.650818/full#supplementary-material

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Exploring the Protective Effects and Mechanism of Crocetin From Saffron Against NAFLD by Network Pharmacology and Experimental Validation

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Background: Non-alcoholic fatty liver disease (NAFLD) is a burgeoning health problem but no drug has been approved for its treatment. Animal experiments and clinical trials have demonstrated the beneficial of saffron on NAFLD. However, the bioactive ingredients and therapeutic targets of saffron on NAFLD are unclear.

Purpose: This study aimed to identify the bioactive ingredients of saffron responsible for its effects on NAFLD and explore its therapy targets through network pharmacology combined with experimental tests.

Methods: Various network databases were searched to identify bioactive ingredients of saffron and identify NAFLD-related targets. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment were conducted to enrich functions and molecular pathways of common targets and the STRING database was used to establish a protein-protein interaction network (PPI). The effect of crocetin (CCT) on NAFLD was evaluated in a mouse model of NAFLD by measuring the biomarkers of lipid, liver and renal function, oxidative stress, and inflammation. Liver histopathology was performed to evaluate liver injury. Nuclear factor erythroid-related factor (Nrf2) and hemeoxygenase-1 (HO-1) were examined to elucidate underlying mechanism for the protective effect of saffron against NAFLD.

Results: A total of nine bioactive ingredients of saffron, including CCT, with 206 common targets showed therapeutic effects on NAFLD. Oxidative stress and diabetes related signaling pathways were identified as the critical signaling pathways mediating the therapeutic effects of the active bioactive ingredients on NAFLD. Treatment with CCT significantly reduced the activities of aspartate aminotransferase (AST), alanine transaminase (ALT), and the levels of total cholesterol (TC), triglyceride (TG), malondialdehyde (MDA), blood urea nitrogen (BUN), creatinine (CR), and uric acid (UA). CCT significantly increased the activities of superoxide dismutase (SOD), and catalase (CAT). Histological analysis showed that CCT suppressed high-fat diet (HFD) induced fat accumulation, steatohepatitis, and renal dysfunctions. Results of ELISA assay showed

that CCT decreased the expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and increased the expression of HO-1 and Nrf2.

Conclusion: This study shows that CCT is a potential bioactive ingredient of saffron that treats NAFLD. Its mechanism of action involves suppressing of oxidative stress, mitigating inflammation, and upregulating Nrf2 and HO-1 expression.

Keywords: saffron, crocetin, NAFLD, network pharmacology, Nrf2, HO-1

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinical syndrome characterized by lipid accumulation in the hepatocyte, steatosis, cellular injury, and hepatic infiltration of inflammatory cells (1, 2). The global prevalence of NAFLD is estimated to be ∼25% (Younossi et al., 2018). In recent years, NAFLD pathogenesis has been explained using the "one-hit," "two-hit," and "multiple-hit" hypotheses (3). Traditionally, a high-fat diet (HFD) was considered an important factor in NAFLD development (4). However, growing evidence has revealed that other factors such as race, ethnicity, gender, age, and genetic pre-disposition are associated with NAFLD (5–7). Further elucidation of the molecular mechanism of NAFLD has revealed that oxidative stress, endoplasmic reticulum stress, perturbation of autophagy, mitochondrial dysfunction, hepatocellular apoptosis, and inflammatory responses cause liver damage in NAFLD (8). Among them, the role of oxidative stress in the pathogenesis of NAFLD has attracted significant attention (9). Previous studies have suggested multiple molecular pathways related to oxidative stress that is involved in NAFLD, including IRs-1/Akt, CYP2E1/JNK, SIRT1/SIRT3-FOXO3a, A(10)MPK/Nrf2, Nrf2/HO-1, and AMPK signaling pathways (10–13).

Herbal medicine has been used in NAFLD treatment, specifically on multiple mechanisms underlying lipid metabolism and inflammation (14). Saffron is one of the most expensive herbs in the world, obtained from dried stigmas of *Crocus sativus* L. (15). Islamic traditional medicine literature introduced saffron as an astringent, resolvent, and concoctive drug, with therapeutic effects on gastro-hepatoprotective, urogenital disorders, antidepressants, ocular disorders, etc. (16). The biological activity of saffron has been widely studied, specifically focusing on its immunomodulatory, anti-inflammatory, and antioxidant activities, and in the treatment of cardiovascular and depression-anxiety (17–20). Besides, recent research demonstrates a hepatoprotective effect of saffron in diabetic rats (21). Results from clinical trials have indicated that saffron supplementation has beneficial effects on the serum levels of inflammatory, oxidative stress, and adipokines biomarkers in patients with NAFLD (22). Existing research provides evidence on the effect of saffron in the treatment of NAFLD, however, further studies are needed to better elucidate the main bioactive ingredients and signaling pathways.

In the current study, network pharmacology was used to predict the main bioactive ingredients, potential therapeutic targets, and key pathways responsible for the effect of saffron in the treatment of NAFLD. Subsequently, the effects and possible signaling pathways of CCT on NAFLD treatment were evaluated in HFD fed mice. The schematic diagram of this study is shown in **Figure 1**.

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CAT, catalase; CCT, crocetin; CR, creatinine; DL, drug-likeness; DAVID, visualization and Integrated Discovery; GO, Gene ontology; H&E, haematoxylin and eosin staining; HFD, high-fat diet; HO-1, hemeoxygenase-1; IL-1β, interleukin-1β; IL-6, interleukin-6; KEGG, Kyoto Encyclopedia of Genes and Genomes; MDA, malondialdehyde; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; Nrf2, nuclear factor erythroid-related factor; OB, oral bioavailability; PPI, protein-protein interaction network; SOD, superoxide dismutase; TC, total cholesterol; TCMSP, traditional Chinese Medicine Systems Pharmacology; TCMID; traditional Chinese Medicine Integrated Database; TG, triglyceride; TNF-α, tumor necrosis factor-α; UA, uric acid.

MATERIALS AND METHODS

Herb Materials, Reagents, and Animals

Saffron samples were collected from Shenghuo Agricultural Development Co., Ltd (Hangzhou, China). CCT reference substance was purchased from Chengdu Desite Biotechnology Co., Ltd (the content of CCT ≥ 98%, Chengdu, China). The total cholesterol (TC) assay kit, Triglyceride (TG) assay kit, Urea (BUN) assay kit, Creatinine (Cr) assay kit, Uric acid (UA) assay kit, Malondialdehyde (MDA) assay kit, Catalase (CAT) assay kit, Superoxide Dismutase (SOD) assay kit, Oil Red O dye, Haematoxylin, and Eosin dye were purchased from Jiancheng Biotechnology Institute Company (Nanjing, China). RIPA lysis buffer, Bicinchoninic acid (BCA) protein determination kit, and Nuclear and Cytoplasmic Protein Extraction kit were purchased from Beyotime Company (Shanghai, China). Nuclear factor erythroid-related factor (Nrf2) transcription factor assay kit, Hemeoxygenase-1 (HO-1) ELISA kit, Tumor necrosis factor-α (TNF-α) ELISA kit, Interleukin-1β (IL-1β) ELISA kit and Interleukin-6 (IL-6) ELISA kit were purchased from Mlbio Biotechnology Company Limited (Shanghai, China). The HFD (saccharose 20%, lard 15%, cholesterol 1.2%, sodium cholate 0.2%) was purchased from Trophic Animal Feed High-Tech Co., Ltd. (Nantong, China). C57BL/6J mice (male, 5 months old, bodyweight 27 ± 2 g; Shanghai Slac Laboratory Animal CO., Ltd., SCXK 2012-0002) were housed individually in ventilated cages (five per cage) and controlled environmental conditions in a clean grade facility at Zhejiang University of Technology (temperature 22–25°C; relative humidity 60%). The mice had free access to standard laboratory chow and tap water and were maintained under normal conditions of a 12/12 h light/dark cycle. All experiments were conducted at the Animal experimental research Center of Zhejiang University

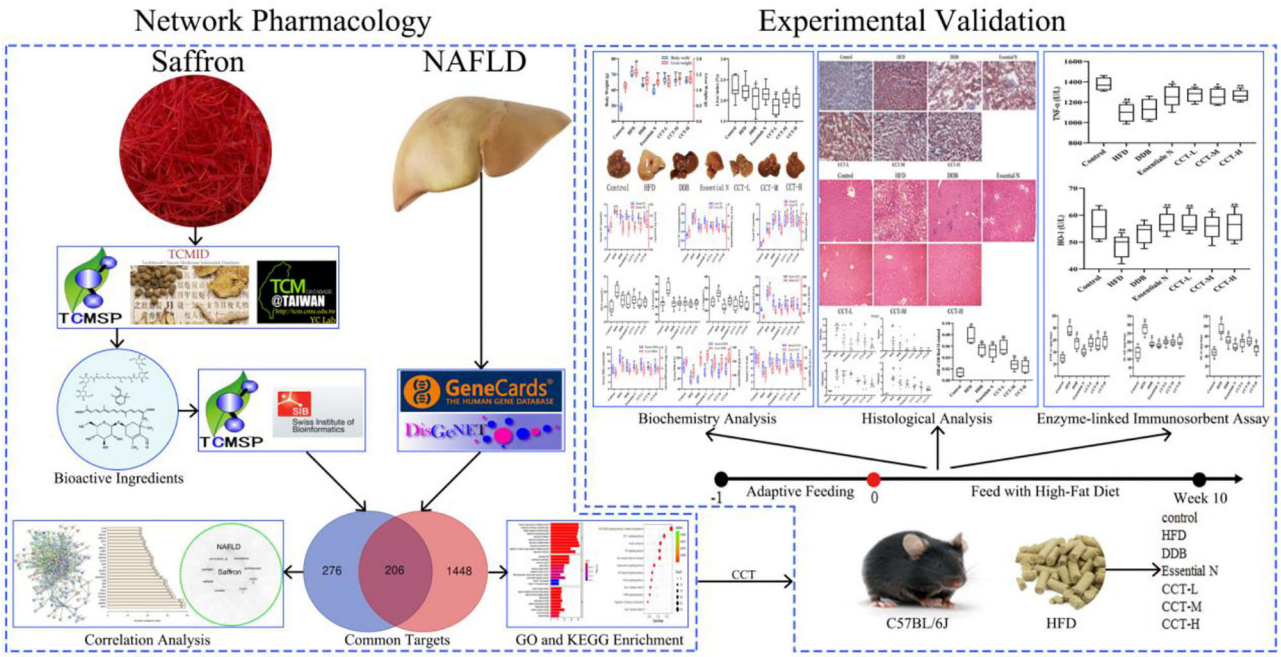

FIGURE 1 | The schematic diagram of the present study.

of Technology. The study was approved by the Animal Protection Research Ethics Committee of Zhejiang University of Technology (20190603064).

Screening for Bioactive Ingredients in Saffron

The bioactive ingredients of Saffron were obtained from the following databases: the Traditional Chinese Medicine Systems Pharmacology (TCMSP, http://tcmspw.com/tcmsp.php) (23), TCM database @taiwan (http://tcm.cmu.edu.tw) (24), and Traditional Chinese Medicine Integrated Database (TCMID, (http://www.megabionet.org/tcmid/) (25). The parameters oral bioavailability (OB) $\geq$ 30% and drug-likeness (DL) $\geq$ 0.18 were set as thresholds to identify the potential bioactive ingredients in TCMSP (26). To obtain comprehensive information of bioactive ingredients, recent literature and relevant international standards such as ISO 3632-1:2011 were reviewed (27–30).

Correlation Analysis Between Saffron and NAFLD-Related Targets

The corresponding targets of the bioactive ingredients of saffron were collected from the TCMSP database and SwissTargetPrediction database (http://www.swisstargetprediction.ch/). The GeneCards database (http://www.genecards.org/) and DisGeNET database (https://www.disgenet.org/) were searched using the terms "NAFLD" or "non-alcoholic fatty liver disease" (UMLS CUI: C0400966) to identify the potential therapeutic targets of NAFLD. The UniProt database (https://www.uniprot.org/) was used to standardize gene information, and further to remove duplicate genes and pseudogenes. The common targets of the bioactive ingredients were manually screened and the interaction network was visualized using Cytoscape 3.7.2. software. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed by using the R package "clusterProfile." The visualization and integration of enrichment results were carried out by using Enrichlot and ggplot2 R package. The above steps were completed by R software 3.6.2 (x64) (31). Data were presented using bar charts and a bubble chart. P-value < 0.05 or Q-value < 0.05 were considered statistically significant. The construction of the protein-protein interaction (PPI) network for the common targets was generated through the String database (https://string-db.org/cgi/input.pl). Subsequently, the parameters of the PPI network, including the node degree (Degree), closeness centrality (CC), and betweenness centrality (BC) were calculated using the Network Analyzer tool function of the String database, and the results presented in a bar chart and 3D scatter plot.

Extraction and Purification of CCT

The extraction and purification of CCT from saffron were performed as reported in our previous research, but with slight modifications (32). Ultrasonic technology was applied in the extraction of CCT and 65% ethanol was used as the extracting solvent. The optimal extraction parameters were as follows: extraction temperature was 50°C, a 1:14 raw material to liquid ratio and ultrasonic power of 100 W. Extraction was performed 3 times for 1 h each. The D101 macroporous resin was used in extract purification and the purification parameters were as follows: eluents were water, 25% ethanol and 60% ethanol, elution

volume of 8-bed volumes. The water and 25% ethanol eluent were discarded, while the 60% ethanol eluent was collected. NaOH solution (2 M) was used to adjust the pH of the solution to about 12, and the mixture was allowed to stand at room temperature for 12 h. H_2SO_4 solution (1 M) was used to adjust the pH to about 2, and the mixture precipitated overnight at 4°C. The sediment, which was a crude extract of CCT was collected by centrifugation. The crude extract was washed twice using methanol and 0.5% H_2SO_4 and washed twice with double-distilled water. Finally, the purified CCT was obtained by centrifugation and dried overnight in a vacuum oven at 60°C. The purity and structure of CCT were elucidated using HPLC (**Figures 2A,B**), ^{1}H-NMR (**Figure 2C**) (600 MHz) and (-) ESI-MS/MS (**Figure 2D**) as shown in **Figure 2**.

Animal Treatment and Sample Collection

After 7 days of adaptive feeding, mice were randomly assigned to seven groups ($n = 10$ in each group) as follows: (1) Mice in the control group (control) were fed normal chow diets and given 0.5% sodium carboxyl methyl cellulose (CMC-Na) daily by oral gavage; (2) The high-fat diet group (HFD) mice were fed with HFD and given 0.5% CMC-Na daily by oral gavage; (3) The biphenyldimethylesterate group (DDB) mice were fed with HFD and given 200 mg/kg of biphenyldimethylesterate daily by oral gavage; (4) The Essentiale N group (Essential N) mice were fed with HFD and given 200 mg/kg of Essentiale N daily by oral gavage; (5) The Low-dose CCT group (CCT-L) mice were fed with HFD and given 10 mg/kg of CCT daily by oral gavage; (6) The Medium-dose CCT group (CCT-M) mice were fed with HFD and given 30 mg/kg of CCT daily by oral gavage; (7) The High-dose CCT group (CCT-H) mice were fed with HFD and given 50 mg/kg of CCT daily by oral gavage. DDB and Essential N, two drugs previously shown to be effective in the treatment of NAFLD and liver injury, were used as positive controls during the validation experiment (33–35).

The body weight was recorded and blood samples were collected from the mice orbit after an overnight fast at 2, 4, 6, and 10 weeks. All blood samples were centrifuged (Thermo, American) at 3,500 rpm for 15 min and the supernatant was collected and stored at −80°C for further analysis. Mice were isolated into single cages for 4 h for collection of feces 10 weeks after the last gavage dose. The mice were sacrificed to collect the liver tissues. The liver weight was measured and the liver index calculated using the following formula: liver index = liver weight/body weight × 100% (g/g). Each of the liver samples was divided in two; one portion was frozen and stored at −80°C for further analysis and the other portion was fixed in 10% neutralized formalin for subsequent paraffin embedding and histological analysis.

Biochemistry Analysis

Fasting serum AST, ALT, TC, TG, BUN, CR, and UA were analyzed using an automatic biochemical analyzer (model 7600; Hitachi Ltd., Japan). Feces and liver samples were placed in normal saline and homogenized using a tissue homogenizer (Scientz-48, Ningbo, China) for 60 s at 60 Hz. The supernatants were obtained by centrifugation for TC and TG analysis. MDA, CAT, and SOD in serum and homogenized liver tissue were analyzed using commercial assay kits according to the manufacturer's instructions.

Histological Analysis

Haematoxylin and eosin staining (H&E) and Oil Red O staining were used to evaluate liver histopathology. Briefly, liver specimens fixed in 10% neutral formalin were embedded in paraffin, sliced at 5 μm thickness, and stained with haematoxylin and eosin. The NAFLD activity score (NAS) ranged from 0 to 8 was used to evaluate histological liver damage. More specifically, NAS includes three histological scores as follows: steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2) (36). Hepatic lipid accumulation was determined using Oil Red O staining. The images were captured using an inverted optical microscope (Olympus IX81, Japan, magnification, ×200). Isopropyl alcohol was used to elute Oil red O stain and quantification was performed at 535 nm to determine the degree of lipid deposition. Data were expressed as the fold change to control.

Inflammation Biochemical Assays

The levels of TNF-α, IL-1β, and IL-6 in the liver of each group were analyzed using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufactures' instructions. The standard curve was used to calculate the concentration.

Enzyme-linked Immunosorbent Assay of Nrf2 and HO-1Nrf2 and HO-1 were determined by an enzyme-linked immunosorbent assay as described below. Part of the liver was first taken for total protein extraction by RIPA lysis buffer and the total protein concentration was determined by BCA protein determination kit. Nuclear extracts were isolated with a Nuclear and Cytoplasmic Protein Extraction kit and further used for the determination of Nrf2 and HO-1 by using commercial kits according to the manufactures' instructions. The standard curve was used to calculate the concentration.

Statistical Analysis

All data in this study were presented as means ± SEM and analyzed using SPSS 17.0 statistical software. One-way ANOVA was used to statistically analyze the data. $P < 0.05$ was considered to be statistically significant (37).

RESULTS

Bioactive Ingredients of Saffron

A total of 70 bioactive ingredients were retrieved from TCMSP, TCM @taiwan, and TCMID databases. However, according to the screening criteria of OB (≥30%) and DL (≥0.18), 5 bioactive ingredients were identified, including quercetin, kaempferol, isorhamnetin, CCT, and n-heptanal. Besides, another four bioactive ingredients were identified from the literature and included in the final results for subsequent analysis and included, crocin I, crocin II, safranal, and picrocrocin (27–30). Detailed information on the bioactive ingredients is listed in **Table 1**. n-heptanal was not included in the follow-up analysis because of its unclear structure and chemical abstracts service (CAS) number.

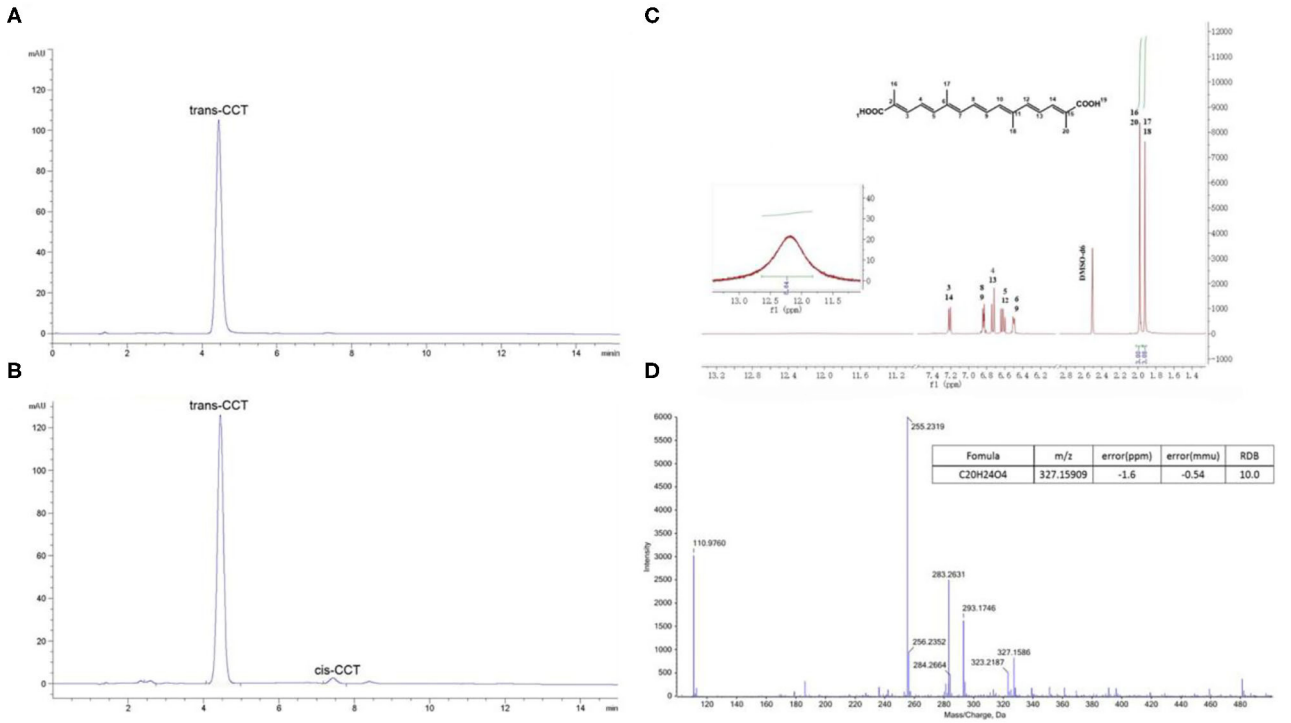

FIGURE 2 | The purity and structure analysis of CCT. **(A)** HPLC chromatogram of CCT reference substance. **(B)** HPLC chromatogram of CCT. **(C)** ^{1}H-NMR of CCT. **(D)** (-) ESI-MS/MS of CCT.

TABLE 1 | The detailed information and parameters of nine bioactive ingredients of saffron.

ID	Compound	OB(%)	DL	Molecular weight
MOL001389	n-heptanal	79.74	0.59	352.42
MOL001406	Crocetin	35.30	0.26	328.44
MOL000354	Isorhamnetin	49.60	0.31	316.28
MOL000422	Kaempferol	41.88	0.24	286.25
MOL000098	Quercetin	46.43	0.28	302.25
MOL001409	Picrocrocin	33.71	0.04	168.26
MOL001405	Crocin I	2.54	0.12	977.08
MOL001407	Crocin II	1.65	0.21	814.92
MOL000720	Safranal	39.56	0.04	150.24

NAFLD-Related and Bioactive Ingredients of Saffron Targets

A total of 167 corresponding targets of the bioactive ingredients of saffron were extracted from TCMSP. However, two similar bioactive ingredients were identified as having similar targets in the SwissTargetPrediction database, so the number of targets extracted in the SwissTargetPrediction database was up to 800. A total of 485 targets of the bioactive ingredients of saffron were identified after merging and removing the duplicates using two databases. A total of 1,058 NAFLD-related targets with a cut-off value larger than 20 were obtained from the DisGeNet database, while 1,017 targets were obtained from the Genecards database. Finally, a total of 1,654 NAFLD-related targets were identified after merging and removing the duplicate records from the two databases. After the manual screening, the common targets of the bioactive ingredients of saffron and NAFLD-related were limited to 206 as shown in **Figure 3A.** More detailed information of 206 common targets was listed in **Supplementary Table 1**. Thereafter, the bioactive ingredients targets-NAFLD-related targets network was constructed as shown in **Figure 3B**. The bioactive ingredients of saffron were found to interfere with multiple therapeutic targets related to NAFLD. The number of NAFLD-related targets associated with quercetin, kaempferol, safranal, crocin I, crocin II, and CCT was 106, 63, 49, 41, 39, and 38, respectively, while the total number of NAFLD-related targets associated with crocin I, crocin II and CCT was 118. These findings indicated that CCT and its glucosyl ester derivatives (crocin I and crocin II) play an important role in the treatment of NAFLD.

GO and KEGG Enrichment Analysis

The GO enrichment analysis of 206 common targets was shown in **Figure 4A**. The key biological processes implicated in the treatment of NAFLD, included reactive oxygen species, metabolic process, response to molecules of bacterial origin, cellular response to chemical stress, response to lipopolysaccharide, response to an antibiotic, response to a steroid hormone, etc. In terms of cellular components, they were involved in

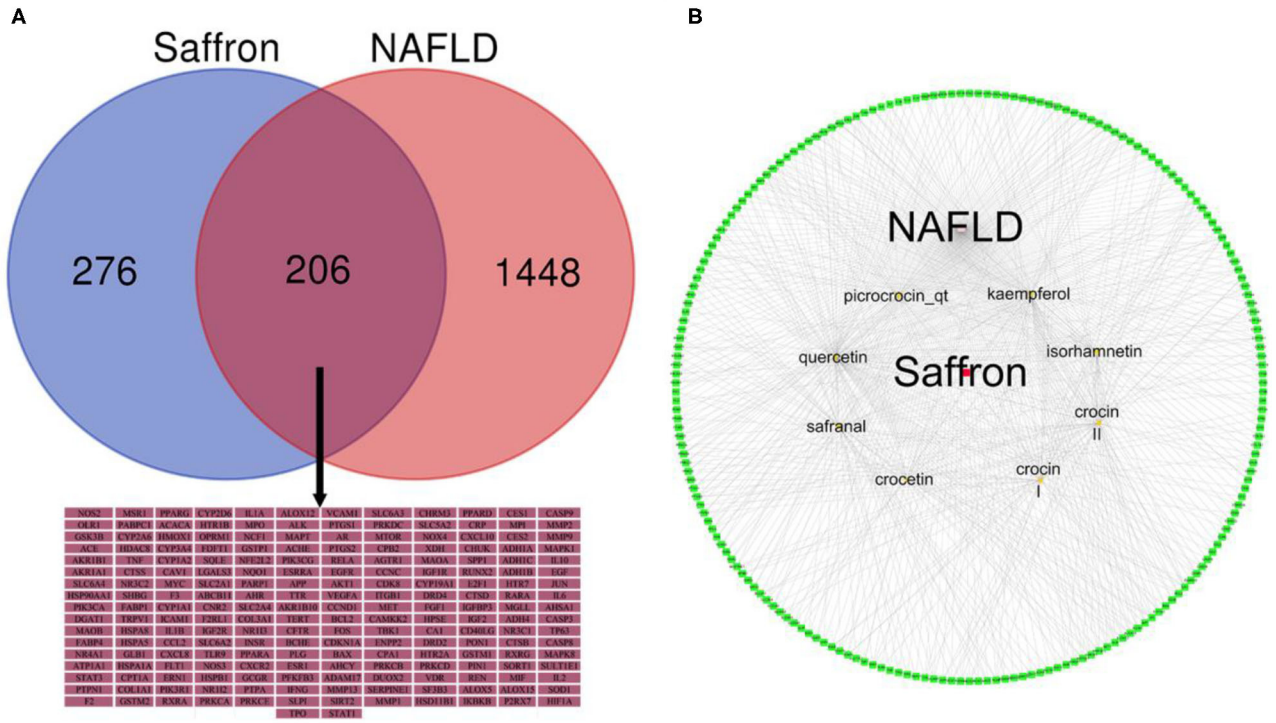

FIGURE 3 | (A) Venn diagram of the targets of Saffron in NAFLD. **(B)** A network of bioactive ingredients and NAFLD-related targets. The red node represent saffron and the eight yellow nodes represent the bioactive ingredients in saffron; the green nodes represent common targets of the bioactive ingredients of saffron and NAFLD-related. The edges denote that nodes can interact with each other.

membrane raft, membrane microdomain, membrane region, vesicle lumen, secretory granule lumen, and cytoplasmic vesicle lumen, etc. Additionally, nuclear receptor activity, ligand-activated transcription factor activity, steroid hormone receptor activity, heme binding, phosphatase binding, and protein phosphatase binding were associated with the molecular functions in the treatment of NAFLD. **Figure 4B** was a simplified bubble chart showing KEGG enrichment, which showed that the underlying mechanism of NAFLD mainly involved diabetes-related pathways, apoptosis, oxidative stress, and inflammatory pathways. For clearer presentation, the specific position and function of common targets are colored red in the signaling pathway in **Figure 4C** and the specific targets in signaling pathways were listed in **Supplementary Table 2**.

PPT Network of Common Targets

The PPI network was constructed based on the NAFLD-related and bioactive ingredients of saffron targets. As shown in **Figure 5A,** the PPI network contained 184 nodes and 1,594 edges. The light blue edges and lavender edges represent the known interactions from curated databases and experimentally determined, respectively. The green edges, red edges, and dark blue edges represent predicted interactions with the gene neighborhood, gene fusions, and gene co-occurrence, respectively. The yellow edges, black edges, and light blue edges represent predicted interactions from textmining, co-expresson, and protein homology, respectively. **Figure 5B** showed that the top 10 core targets were AKT1, MAPK1, STAT3, PIK3CA, PIK3R1, RELA, TNF, APP, JUN, and HSP90AA1. Details of the PPI network are shown in **Supplementary Table 3**.

Effects of CCT on Body Weight, Liver Weight, and Liver Index

Compared with the control group, the body weight gain of the HDF group significantly increased at week 10. CCT was found to cause a reduction in body weight gain induced by HFD. In HFD mice, the liver weight was significantly increased. Except for the Essentiale N group, the liver index in all treatment groups significantly decreased compared with the HDF group (**Figures 6A,B**). The liver of the HDF group was canary yellow and its surface had a distinct white dot shape representing hepatic steatosis. In contrast, the liver of the DDB group, CCT-M group, and CCT-H group was significantly improved as shown in **Figure 6C**.

Effects of CCT on Lipid, Liver, and Renal Function Biomarkers

As shown in **Figure 7**, the levels of serum and liver TC and TG were significantly increased in the HFD group, while were significantly decreased in all treatment groups compared with the HDF group (**Figures 7A,B**). The levels of fecal TC and TG were significantly increased in three dose groups of CCT compared

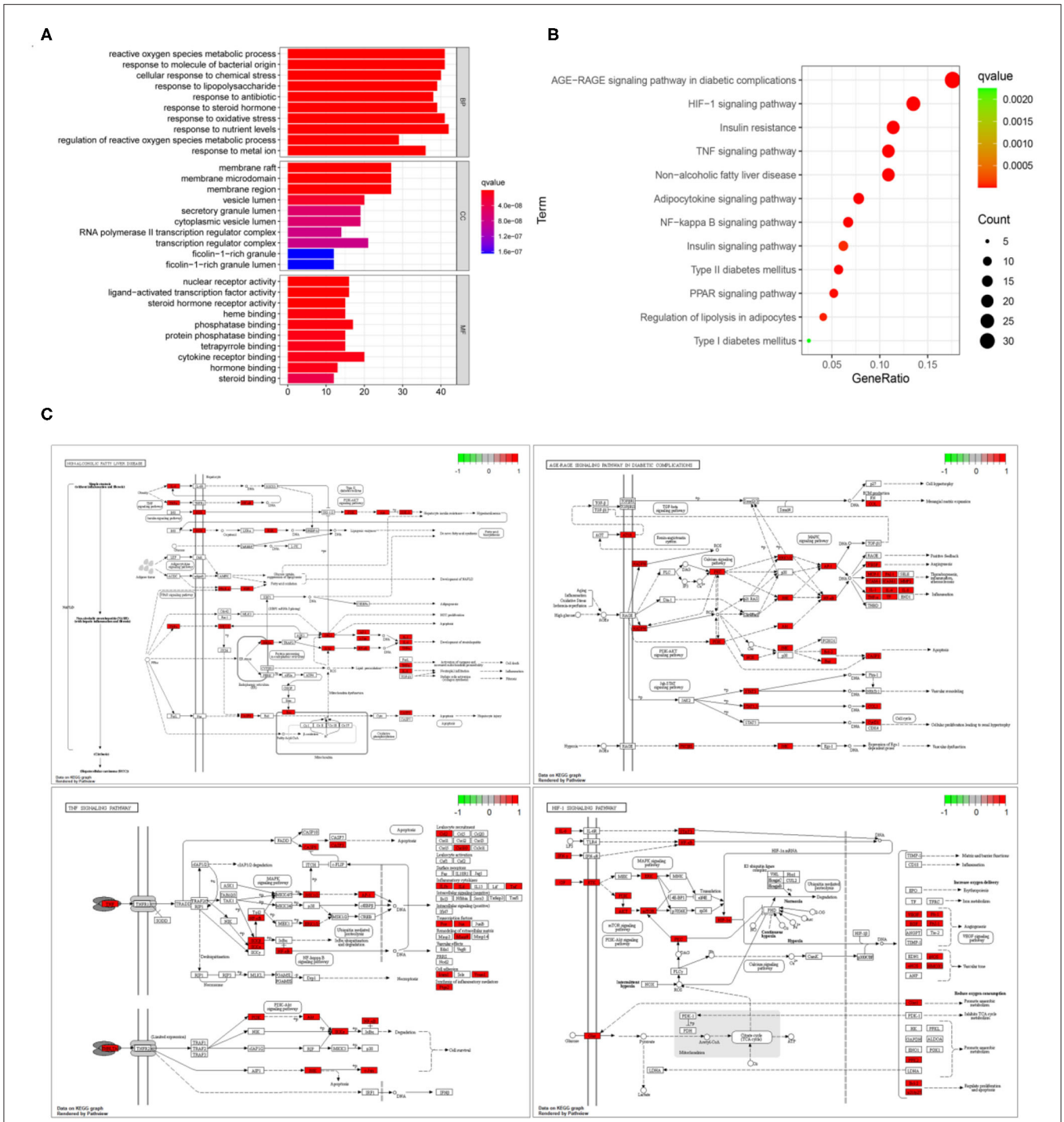

FIGURE 4 | GO and KEGG enrichment analysis. **(A)** GO enrichment analysis of the 206 common targets associated with NAFLD. The *X* -axis represents significant enrichment in the counts of these terms; The *Y*-axis showing the categories of biological process (BP), cellular component (CC), and molecular function (MF) in the GO of the target genes ($P < 0.05$). **(B)** KEGG enrichment analysis. The *X*-axis showing the counts of the target symbols in each pathway; the *Y*-axis showing the main pathways ($P < 0.05$). **(C)** The specific position and function of common targets in in signaling pathways. The red region represents the specific position of these common targets in the pathways.

with the HDF group (**Figure 7C**). These findings indicated that CCT can increase lipid excretion to alleviate lipid accumulation caused by NAFLD. The activities of ALT and AST, two markers of liver function, were also significantly increased in the HDF group compared with the control group. The effect of CCT in reducing ALT and AST activities was observed after treatment, and also in

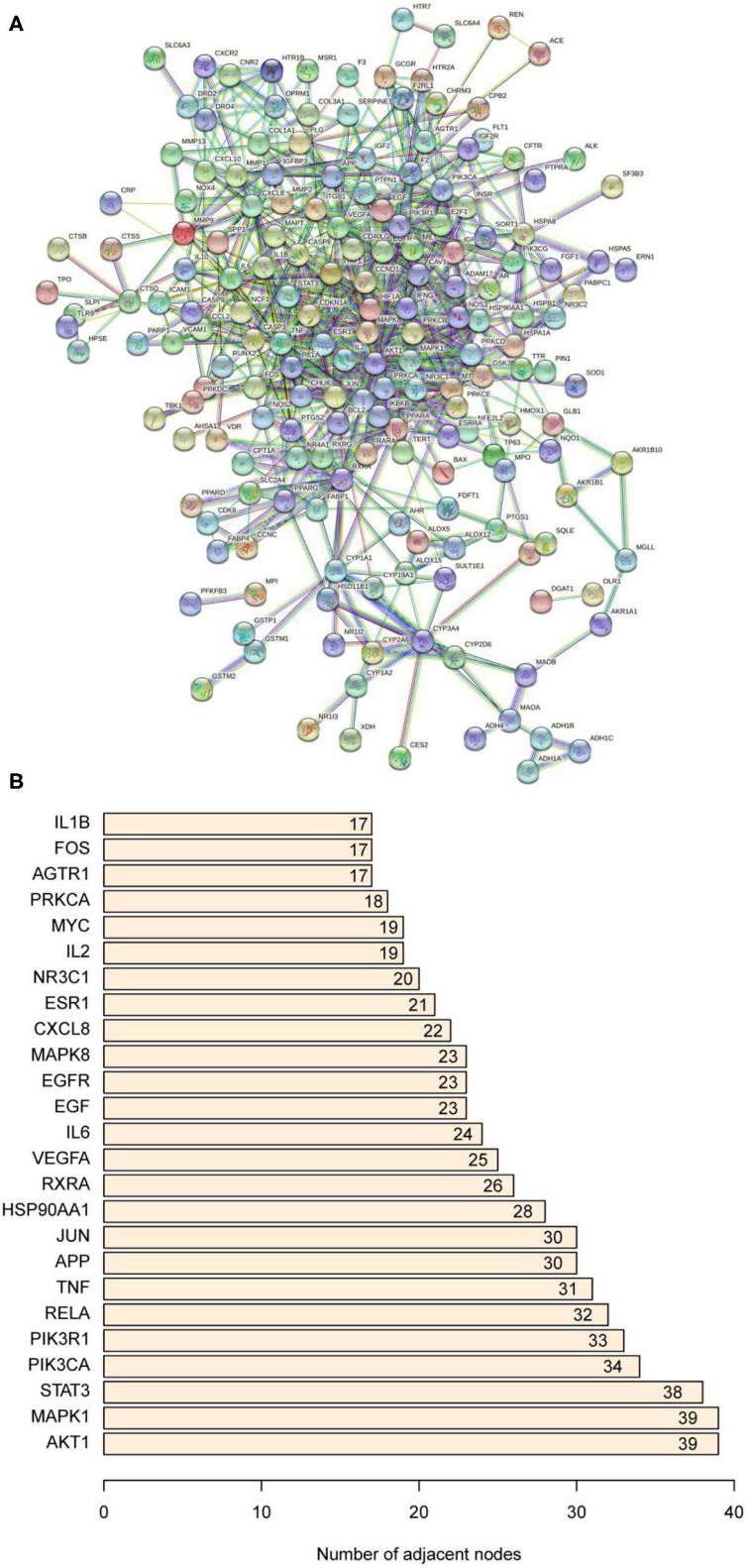

FIGURE 5 | (A) Protein-protein interaction network. **(B)** The bar plot of the protein-protein interaction network. The *X*-axis represents the number of neighboring proteins of the target protein. The *Y*-axis represents the target protein.

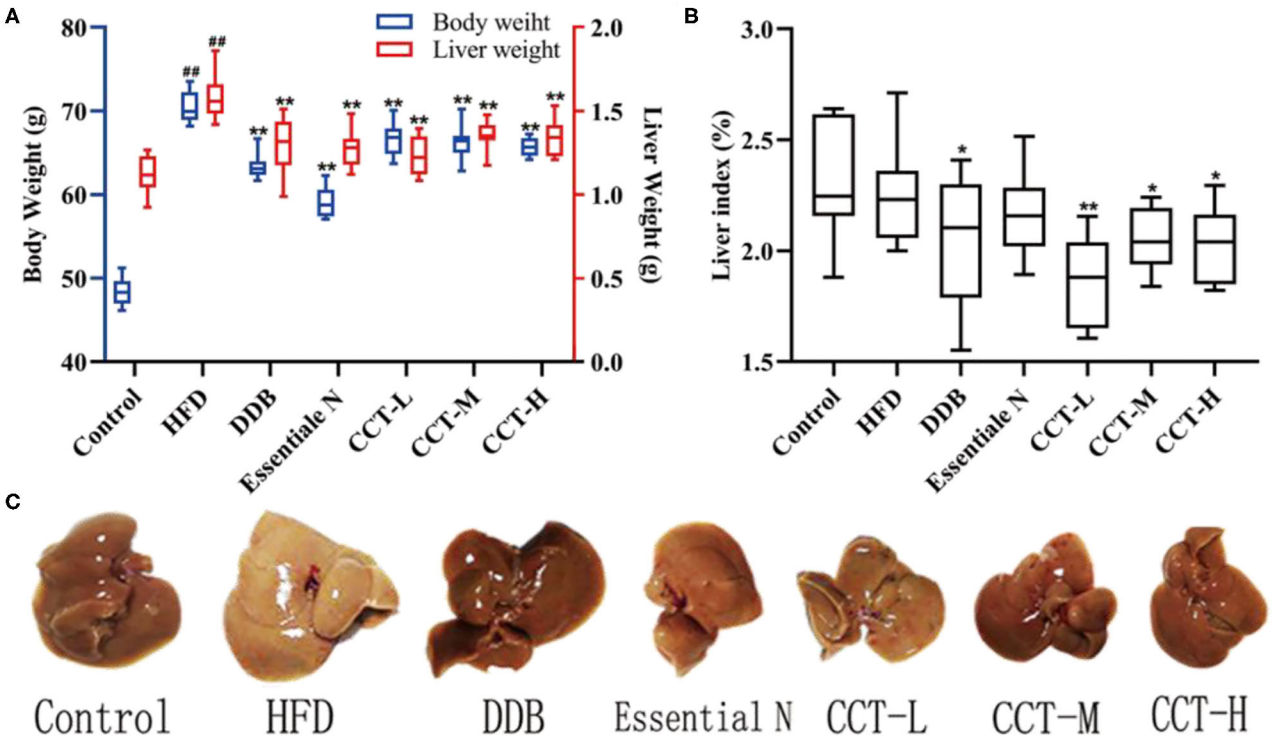

FIGURE 6 | (A) Body weight and liver weight. **(B)** Liver index. **(C)** Liver photographs. $^{\#\#}P < 0.01$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HFD group.

the DBB and Essential N groups (**Figure 7G**). For renal function, the serum levels of BUN, CR, and UA were significantly increased in the HDF group compared with the control group. However, a significant decrease was observed in three dose groups of CCT compared with the HDF group (**Figures 7D–F**).

Effects of CCT on Biomarkers of Oxidative Stress

As shown in **Figure 8**, the serum and liver tissue MDA levels were significantly increased in the HDF group compared with the control group, and there was a significant decrease in the three-dose groups of CCT and DDB group compared with the HDF group (**Figure 8A**). Similar trends were observed in the activities of SOD and CAT indicating that CCT can alleviate oxidative stress induced by NAFLD (**Figures 8B,C**).

Effects of CCT on Biomarkers of Inflammation

To determine whether CCT could inhibit inflammation in an HFD-induced NAFLD-mouse model, the levels of TNF-α, IL-1β, and IL-6 were measured in the liver tissue. As shown in **Figure 9**, TNF-α, IL-1β, and IL-6 levels were significantly increased in the HDF group compared with the control group, but significantly decreased in CCT groups, the DDB group and Essential N group.

Effects of CCT on Liver Histopathology

In the present study, 10 photomicrographs of HE-staining were selected for use in calculating the NAS score at a magnification of ×200. The NAS score in the HFD group was significantly increased compared with the control group and decreased after CCT treatment. This indicated that CCT significantly attenuated inflammation, steatosis, and ballooning (**Figures 10A,B**). Besides, Oil Red O staining (**Figures 10C,D**) showed increased accumulation of lipid droplets in the liver of the HFD group, however, CCT treatment led to a significant decrease in the lipid droplets in the liver.

Effects of CCT on Nrf2 and HO-1

As shown in **Figure 11**, the expression of Nrf2 and HO-1 significantly decreased in the HDF group compared with the control group. After CCT treatment, the expression of Nrf2 and HO-1 significantly increased in all the groups, except for the DDB group compared with the HDF group.

DISCUSSION

Network pharmacology is a newly developing interdisciplinary science based on biology, chemical informatics, and bioinformatics, and is also an effective tool used to explore bioactive ingredients from complex components (38). In recent years, network pharmacology has been widely used in traditional Chinese medicine to investigate the potential therapeutic targets

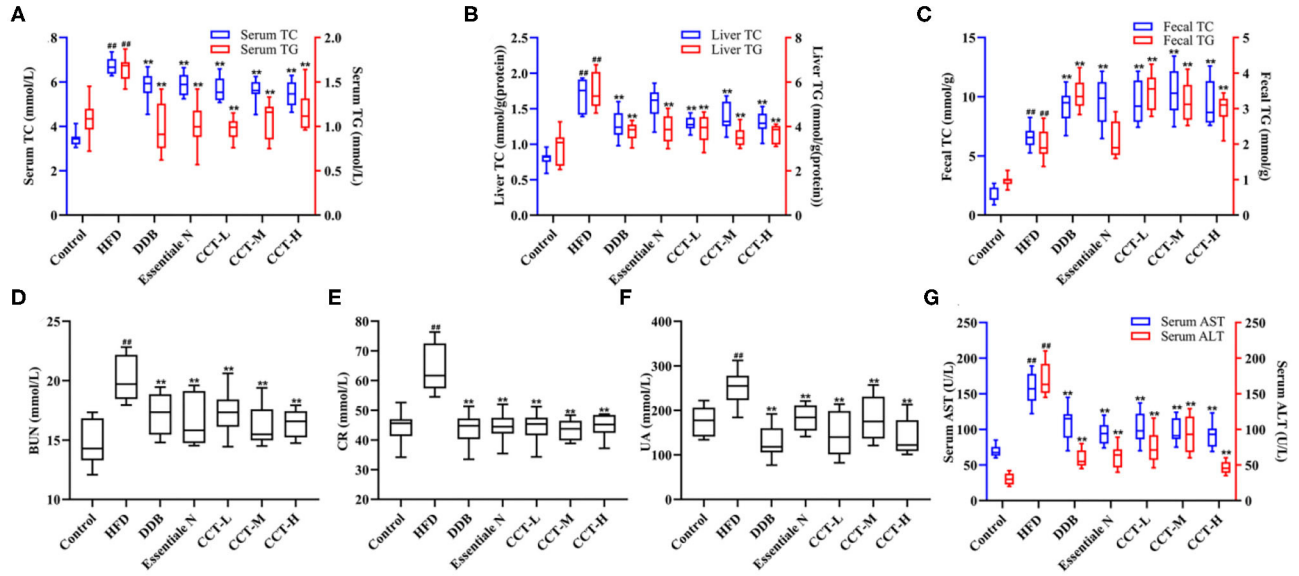

FIGURE 7 | Effects of CCT on lipid, liver and renal function biomarkers. **(A)** Effects of CCT on serum TC and TG. **(B)** Effects of CCT on liver TC and TG. **(C)** Effects of CCT on fecal TC and TG. **(D)** Effects of CCT on BUN. **(E)** Effects of CCT on CR. **(F)** Effects of CCT on UA. **(G)** Effects of CCT on serum AST and ALT. $^{\#\#}P < 0.01$ vs. Control group; $^{**}P < 0.01$ vs. HFD group.

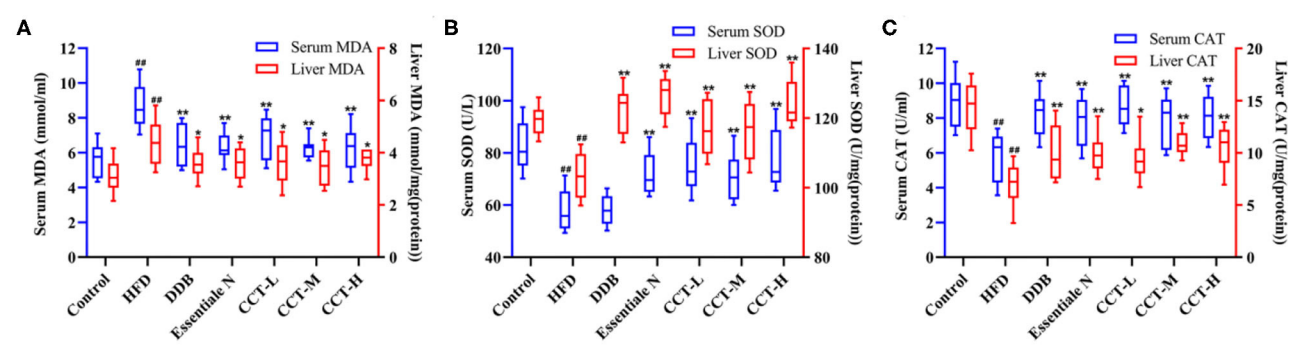

FIGURE 8 | Effects of CCT on oxidative stress biomarkers. **(A)** Effects of CCT on serum MDA and liver TG. **(B)** Effects of CCT on serum SOD and liver SOD. **(C)** Effects of CCT on serum CAT and liver CAT.$^{\#\#}P < 0.01$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HFD group.

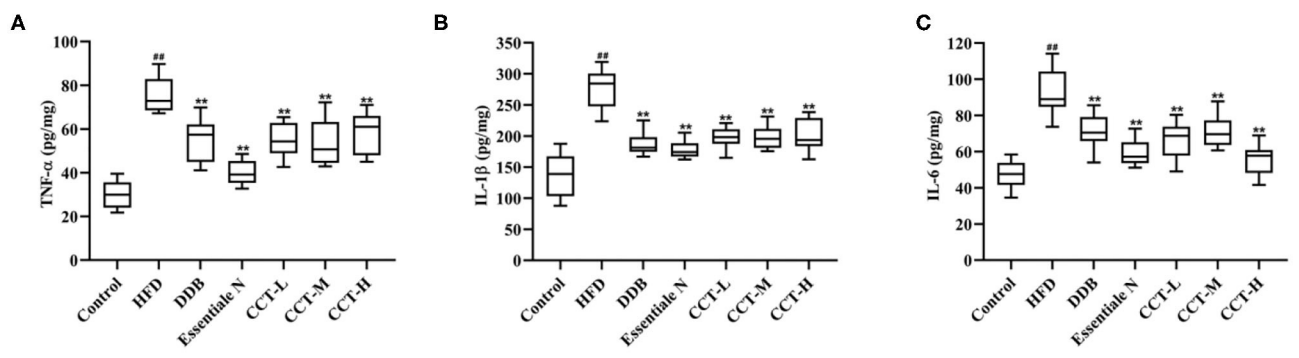

FIGURE 9 | Effects of CCT on inflammation biomarkers. **(A)** Effects of CCT on TNF-α. **(B)** Effects of CCT on IL-1β. **(C)** Effects of CCT on IL-6. $^{\#\#}P < 0.01$ vs. Control group; $^{**}P < 0.01$ vs. HFD group.

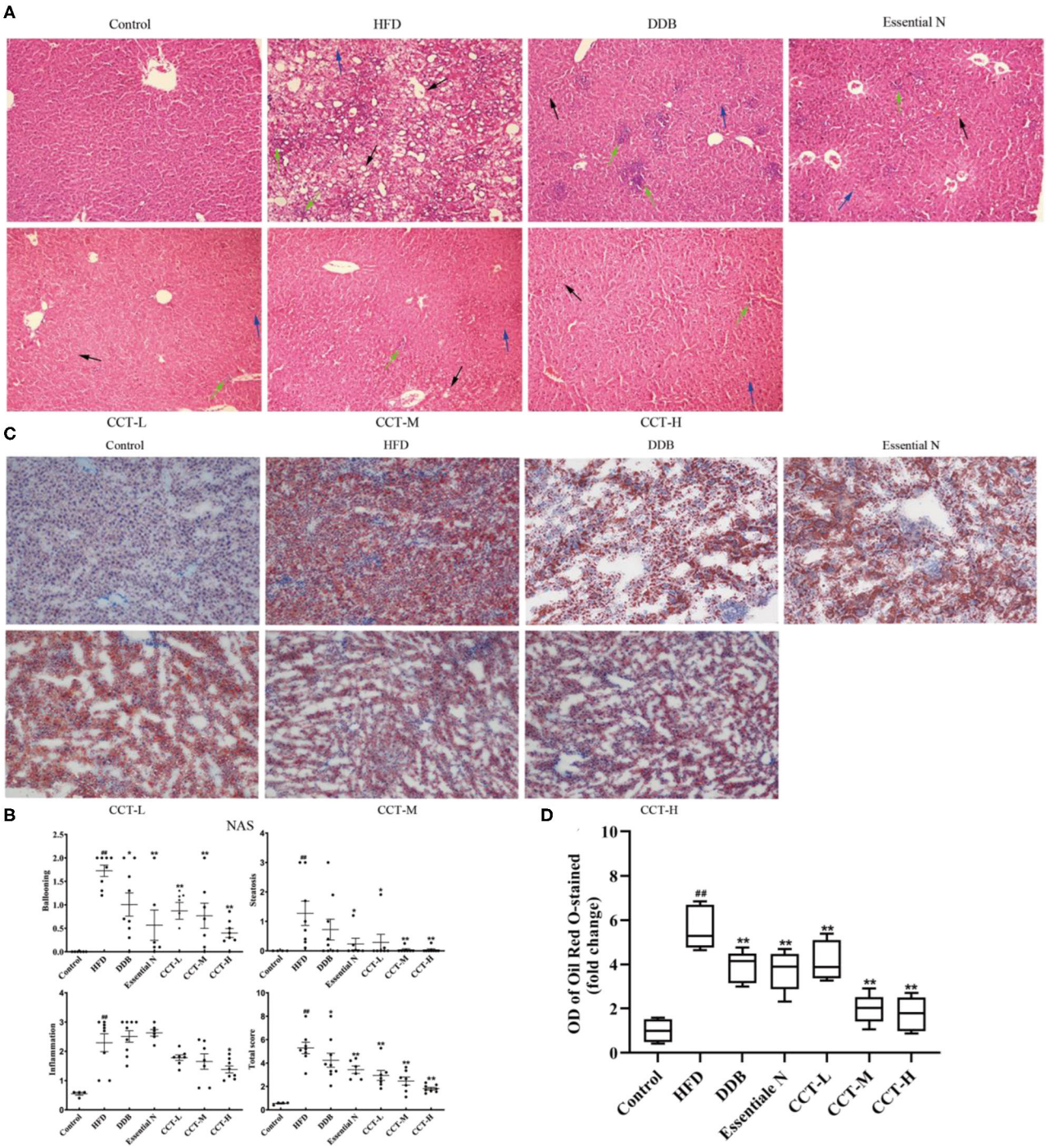

FIGURE 10 | Effects of CCT on liver histopathology. **(A)** Representative photomicrographs of HE staining (×200). The blue arrow represents steatosis, the green arrow represents lobular inflammation, the black arrow represents ballooning degeneration. **(B)** The NAS scores. **(C)** Representative photomicrographs of Oil Red O staining (×200). **(D)** The OD of Oil Red O staining(fold change). $^{\#\#}P < 0.01$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HFD group.

and pharmacological mechanisms in different diseases, however, very few studies have used network pharmacology to study the pharmacological effects of saffron. Consistent with our results, a comparative study of the anti-thrombotic effects of saffron and carthami flos based on network pharmacology indicated that CCT was the key bioactive ingredient of saffron (39). Two other studies on systems pharmacology of saffron emphasized the importance of CCT (40, 41). CCT has various

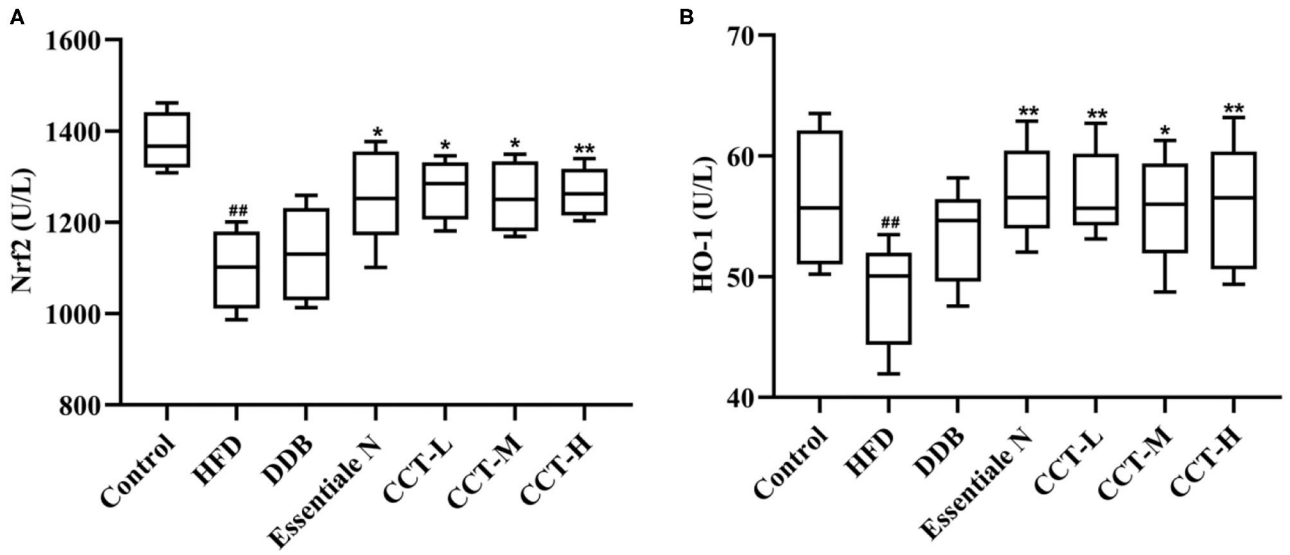

FIGURE 11 | Effects of CCT on Nrf2 and HO-1. **(A)** Effects of CCT on Nrf2. **(B)** Effects of CCT on HO-1. $^{\#\#}P < 0.01$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. HFD group.

biological activities such as anti-tumor, neuroprotective, cardioprotective, hepatoprotective, antidepressant, and improves asthma, Alzheimer's, and diabetes mellitus, etc. (42). In studies of liver diseases, CCT showed comparative beneficial effects on CCL_4-induced and dengue virus-induced liver damage via induction of antioxidant defense (43, 44). In this study, the therapeutic targets of crocin I, crocin II, and CCT were combined, because orally administered crocins are hydrolyzed to CCT before they are incorporated into the blood circulation, and these findings have been confirmed in several pharmacokinetic studies (45–47). As noted above, CCT was reasonable to reckon to be the critical bioactive ingredient of saffron and further used in the verification experiment.

Although several decades of NAFLD research have resulted in major scientific advancements in pathogenesis and therapeutic target, effective drugs are currently under clinical development (48). The main challenge is that the efficacy of a single target is limited because the pathogenesis and progression of NAFLD involve multiple pathogenic pathways, such as insulin resistance, lipotoxicity, oxidative stress, altered immune/cytokine/mitochondrial functioning, and apoptosis (48–50). In the current study, the possible signaling pathways of saffron intervention in NAFLD were analyzed according to the results obtained from GO, KEGG, and PPI analysis. The results of GO enrichment indicated that saffron may interfere with oxidative stress and nuclear receptor activity in the treatment of NAFLD. The results of KEGG enrichment indicated multiple signaling pathways, including AGE-RAGE signaling pathway in diabetic complications, HIF−1, TNF, adipocytokine, NF-kappa B, PPAR, and other diabetes-related signaling pathways involved in the treatment of NAFLD by using saffron. However, the PPI analysis gave very different results, incicating that AKT1, MAPK1, STAT3, PIK3CA, PIK3R1, RELA, TNF, APP, and JUN were the top 10 core targets. In view of the inconsistent results from KEGG enrichment and PPI, it is necessary to systematically analyze the targets and signaling pathways of saffron in the treatment of NAFLD. Taking into consideration that the results of oxidative stress and nuclear receptor activity from GO enrichment, the 206 common targets were reevaluated, of which NFE2L2 (Nrf2) and HMOX1 (HO-1) have attracted considerable attention. Nrf2 is a tran-scription factor that regulates the expression of several anti-oxidant genes. Previous studies show that Nrf2/HO-1-antioxidant response element (ARE)-antioxidant enzyme play a central mechanistic role in the regulation of the complex antioxidant system in the human body and are key nodes in multiple signaling pathways, including PI3K/AKT/MAPK and IL-6/JAK/STAT3 (51–54). Under normal physiological condition, Kelch-like-ECH-associated protein 1 (Keap1), an Nrf2 repressor, binds to Nrf2 to form a complex and resides in the cytoplasm. However, under stimulation with oxidative and electrophilic chemical signals, Nrf2 is released from Keap1 and transferred to the nucleus, where it binds to the ARE. Here, Keap1 plays an important role in cellular responses to chemical and oxidative stress by regulating the stability and nuclear translocation of Nrf2 protein (55, 56). As one of the key target genes of nuclear Nrf2, HO-1 is a downstream antioxidant enzyme and has a broad cytoprotective effect in various diseases (57). It can be inferred from the above results that CCT may play a role in the treatment of NAFLD by co-intervening the expression of Nrf2 and HO-1. The evidences in biomedical literature in support of this hypothesis have been identified. The antioxidant effect of scutellarin on NAFLD is dependent on PI3K/AKT activation with subsequent Nrf2 nuclear translocation, which increases the expression of HO-1 (58). Resolvin D1 mitigates non-alcoholic steatohepatitis by suppressing the TLR4-MyD88-mediated NF-kappa B and MAPK

pathways and activates the Nrf2 pathway in mice (59). In view of the above considerations, Nrf2 and HO-1 were identified as key target genes of the possible signaling pathway and further validated *in vivo* in the current study.

In the validation experiment, HDF was used to induce NAFLD mice to further ascertain whether CCT can ameliorate NAFLD. The results showed that CCT regulates the levels (activities) of TC, TG ALT, AST, and liver index. In the diagnosis of NAFLD, pathological biopsy remains the "golden standard" (60). H&E stained liver sections revealed that CCT can ameliorate hepatocyte ballooning, steatosis, and inflammation to prevent NAFLD. Lipid droplets in the liver were stained using Oil red O, and CCT decreased the lipid droplets. Recent studies have found that NAFLD not only leads to abnormal liver function but also causes extrahepatic disease, such as renal damage (61, 62). Liver steatosis alters the gut barrier function and microbial composition to accumulate toxic metabolites resulting in renal damage (63, 64). In this study, the serum levels of BUN, CR, and UA were analyzed to determine whether CCT can ameliorate renal function caused by NAFLD. The results showed that feeding mice with HFD significantly increased the levels of BUN, CR, and UA, which is consistent with previous clinical trials (65). After CCT treatment, BUN, CR, and UA significantly decreased which was associated with efficient glomerular filtration function and improved NAFLD (66). MDA, a product of lipid oxidation, is used as a marker of oxidative stress. SOD and CAT are enzymatic antioxidant systems, which play important roles in protection against the deleterious effects of hydrogen peroxide and lipid peroxidation in diseases related to oxidative stress (67, 68). In this study, a significant increase in SOD and CAT activities, accompanied by a decrease in MDA level was observed after CCT treatment. These results indicate that CCT can reduce the levels of oxidative stress in the HFD-induced NAFLD model. There is overwhelming evidence that the release of adipocytokines like TNF-α, IL-1β, and IL-6 result in hepatocytes suffering a characteristic variation in their structure developing lobular inflammation and balloon degeneration associated with different degrees of scarring or fibrosis (69). In this study, CCT was found to significantly reduce the levels of TNF-α, IL-1β, and IL-6 which exerts a beneficial effect on NAFLD. After CCT treatment, Nrf2 and HO-1 expression was significantly increased indicating that CCT may activate multiple anti-oxidative signaling pathways to protect against liver lipid peroxidation and hepatitis.

CONCLUSION

In conclusion, this study explored, for the first time, the bioactive ingredients of saffron and their therapeutic targets in NAFLD using network pharmacology and animal experiments. CCT was identified as the bioactive compound that ameliorates HFD-induced NAFLD by decreasing level of antioxidants and proinflammatory factors. This study provides a scientific basis for further analysis of the clinical application of CCT in NAFLD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All experiments were conducted at the Animal experimental research Center of Zhejiang University of Technology. The study was approved by the Animal Protection Research Ethics Committee of Zhejiang University of Technology (20190603064).

AUTHOR CONTRIBUTIONS

PW and YT supervised the project, conceived, and designed the research methods. ZX, SL, QL, and YY performed the experiments. YJ and PF performed network pharmacology analysis. ZX wrote the manuscript. YC revised the manuscript. All authors reviewed and approved the final 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.2021.681391/full#supplementary-material

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Adiponectin, Leptin, and IGF-1 Are Useful Diagnostic and Stratification Biomarkers of NAFLD

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Background: Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease where liver biopsy remains the gold standard for diagnosis. Here we aimed to evaluate the role of circulating adiponectin, leptin, and insulin-like growth factor 1 (IGF-1) levels as non-invasive NAFLD biomarkers and assess their correlation with the metabolome.

Materials and Methods: Leptin, adiponectin, and IGF-1 serum levels were measured by ELISA in two independent cohorts of biopsy-proven obese NAFLD patients and healthy-liver controls (discovery: 38 NAFLD, 13 controls; validation: 194 NAFLD, 31 controls) and correlated with clinical data, histology, genetic parameters, and serum metabolomics.

Results: In both cohorts, leptin increased in NAFLD vs. controls (discovery: AUROC 0.88; validation: AUROC 0.83; $p < 0.0001$). The leptin levels were similar between obese and non-obese healthy controls, suggesting that obesity is not a confounding factor. In the discovery cohort, adiponectin was lower in non-alcoholic steatohepatitis (NASH) vs. non-alcoholic fatty liver (AUROC 0.87; $p < 0.0001$). For the validation cohort, significance was attained for homozygous for PNPLA3 allele c.444C (AUROC 0.63; $p < 0.05$). Combining adiponectin with specific serum lipids improved the assay performance (AUROC 0.80; $p < 0.0001$). For the validation cohort, IGF-1 was lower with

advanced fibrosis (AUROC 0.67, $p < 0.05$), but combination with international normalized ratio (INR) and ferritin increased the assay performance (AUROC 0.81; $p < 0.01$).

Conclusion: Serum leptin discriminates NAFLD, and adiponectin combined with specific lipids stratifies NASH. IGF-1, INR, and ferritin distinguish advanced fibrosis.

Keywords: adiponectin, circulating biomarkers, fibrosis, IGF-1, leptin, lipid metabolism, NAFLD

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic liver conditions and an important risk factor for liver cirrhosis and hepatocellular carcinoma. The NAFLD spectrum varies from simple fat accumulation to non-alcoholic steatohepatitis (NASH), characterized by hepatocellular injury, inflammation, and, eventually, fibrosis (1). In fact, the increasing prevalence of NAFLD parallels that of obesity and is accompanied by rising liver-related morbidity and mortality and, consequently, increased socio-economic burden (2). Clinical practice guidelines recommend NAFLD screening in individuals with metabolic risk factors, such as obesity, type 2 diabetes, and hypertension (metabolic syndrome) (3, 4). Most NAFLD patients are clinically asymptomatic, and when present, the symptoms are unspecific and associated with advanced disease. Furthermore, liver function tests may be within normal range or only slightly increased. Thus, panels composed by anthropometric and blood biochemistry data have been proposed to assess hepatic steatosis (fatty liver index, hepatic steatotic index, NAFLD liver fat score, SteatoTest), although with modest performance compared with imaging biomarkers. In turn, imaging techniques [ultrasonography, magnetic resonance imaging (MRI)] are not fully conclusive at identifying liver inflammation and hepatocyte injury typical of NASH or distinguishing NASH from fatty liver (3, 4). Indeed differential diagnosis between non-alcoholic fatty liver (NAFL) and NASH is an important indicator of increased risk of cirrhosis and other hepatic co-morbidities, although the stage of fibrosis is increasingly recognized as the most relevant NASH prognostic marker (5).

Caspase-3-generated cytokeratin-18 is the most studied biomarker for NASH diagnosis but has limited sensitivity for NASH stage screening (6); hence, other putative biomarkers (inflammatory markers, adipokines, and others) are emerging (7). In parallel, biomarker panels combining several blood parameters are being proposed for fibrosis staging, such as aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio, AST/platelet ratio index (APRI), fibrosis-4 index (Fib-4), or FibroTest, but accuracy has been limited as they mostly rely on liver injury markers (8). To date, liver biopsy is the gold-standard method to accurately discriminate NAFL from NASH, to evaluate the extent of tissue damage and fibrosis, and to rule out other etiologies. However, this is an expensive and invasive procedure with potential for serious complications (9). Moreover, liver biopsy lacks the ability to provide a complete 3D liver overview and is limited by sampling variability. Hence, identification and validation of simple, specific, reproducible, and non-invasive biomarkers that accurately diagnose and monitor NAFLD patients constitute an urgent unmet medical need. NAFLD is generally regarded as a hepatic manifestation of metabolic syndrome (10). Although not yet fully understood, the disease pathogenesis involves disturbances in adipose tissue, insulin resistance, and inflammation (11). Thus, adipokines' and related cytokines' role in NAFLD pathogenesis and severity has been equated (12), and their potential as non-invasive biomarkers of diagnosis and staging deserves further investigation. Moreover, lipid and metabolite signatures in plasma and in liver tissue associated with NAFLD and/or fibrosis (13–15) may further support non-invasive NAFLD diagnosis (16).

We hypothesize that serum adipokines—leptin and adiponectin—and liver-produced insulin-like growth factor 1 (IGF-1) are useful circulating biomarkers for NAFLD diagnosis and stratification. We assessed their potential as non-invasive biomarkers in the discovery and validation cohorts of biopsy-proven NAFLD patients and evaluated their impact on the metabolome.

MATERIALS AND METHODS

Patient Cohorts

Human serum samples were collected from two independent cohorts of morbidly obese adult patients with biopsy-proven diagnosis of NAFLD, who were undergoing bariatric surgery [body mass index (BMI) $\geq$35 kg/m^2], and healthy-liver individuals (17) (BMI <35 kg/m^2) from Santa Maria Hospital (Lisbon, Portugal; discovery cohort) and Donostia University Hospital (San Sebastian, Spain; validation cohort). Up to 20% of the patients were taking statins. The patient inclusion criteria consisted in liver biopsy-proven NAFLD diagnosis and BMI $\geq$35 kg/m^2. The exclusion criteria included alcohol consumption $\geq$20 (females) or $\geq$30 g/day (males), viral hepatitis B and C, and other causes of chronic liver disease. The liver biopsies were obtained during bariatric surgery, under standard procedures.

Abbreviations: ALT, alanine aminotransferase; AHT, arterial hypertension; APRI, AST/platelet ratio index; AST, aspartate aminotransferase; AUROC, area under the ROC curve; DG, diglycerides; DHA, docosahexaenoic; ELF, enhanced liver fibrosis; ELISA, enzyme-linked immunosorbent assay; FA, fatty acids; Fib-4, fibrosis-4 index; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; IGF-1, insulin-like growth factor 1; INR, international normalized ratio; LDL, low-density lipoprotein; LPC, lysophosphatiylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MRI, magnetic resonance imaging; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NFS, NAFLD fibrosis score; NPV, negative predictive value; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PPV, positive predictive value; ROC, uniparameter receiver operating characteristic; SM, sphingomyelin; TG, triglycerides.

Fibrosis was assessed from a distance >0.5 cm from the liver capsule. Blood and tissue samples were collected at a maximum of 1-week interval, while liver imaging was performed within 24 h of surgery. Sample collection was performed after receipt of the patient's informed consent and the Institutional Review Board's approval, in accordance with the Declaration of Helsinki. For histology purposes, formalin-fixed paraffin-embedded tissue sections were routinely stained with hematoxylin–eosin method and with Masson's trichrome for fibrosis evaluation. Histological evaluation was performed by experienced pathologists in a blinded fashion following agreement on the histological features of NAFLD/NASH assessment and NASH diagnosis. NAFLD activity score (NAS 0–8) and fibrosis stage (F0–4) were assigned to each biopsy according to the NASH Clinical Research Network histological scoring system (18). Fibrosis extent was dichotomized as early (F0–2) and advanced (F3–4) fibrosis. Where possible, fibrosis scores APRI (19), Fib-4 (20), and NAFLD fibrosis score (NFS) (21) were calculated. Individuals were classified as healthy non-obese controls (BMI <35 kg/m^2), healthy obese controls [BMI ≥35 kg/m^2, maximum NAS = 1 (if steatosis = 0) and maximum fibrosis score = 1], NAFL (NAS ≤4), or NASH (NAS ≥5). Liver biopsies were obtained from healthy non-obese individuals that were referred for hepatic surgery without an underlying liver disease (17).

The discovery cohort ($n = 51$) included three groups: healthy non-obese controls ($n = 13$), NAFL ($n = 26$), and NASH patients ($n = 12$). Routine biochemical parameters were available; however, detailed histological data was not fully disclosed.

The validation cohort ($n = 225$) encompassed four groups: healthy non-obese controls ($n = 20$), healthy obese controls ($n = 11$), NAFL ($n = 100$), and NASH patients ($n = 94$). Routine biochemical parameters were available, and detailed histological data is described in **Supplementary Table 1**. Information on co-morbidities (arterial hypertension, diabetes, dyslipidemia, and cholelithiasis), genetic polymorphisms [*PNPLA3* rs738409 (c.444C>G, p.I148M), *MBOAT7* rs641738 (c.50G>A, p.G17E), and *TM6SF2* rs58542926 (c.449C>T, p.E167K)], liver hepatic triglyceride (TG) content evaluation by Folch method (17) ($n = 140$), and imaging assessment of liver steatosis (MRI; $n = 122$) were also available.

Serum Hormones and Data Analysis

The serum levels of leptin, adiponectin, and IGF-1 were measured in single determination using specific enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Mediagnost GmbH, Reutlingen, Germany) and correlated with NAFLD activity score, fibrosis stage, biochemical parameters, genotypes, imaging data, and co-morbidities. Data analysis was first performed in the discovery cohort and then expanded to the validation cohort. The normality of values distribution was tested with Kolmogorov–Smirnov ($n \geq 50$) or Shapiro–Wilk ($n < 50$) tests. According to the normality of values distribution, one-way ANOVA or Kruskal–Wallis test followed by Bonferroni or Dunn's multiple-comparison test were used to determine differences between sample groups according to NAS score, steatosis, lobular inflammation and ballooning stages, and genotypes. Differences according to fibrosis dichotomy and presence of co-morbidities were determined with unpaired *t*-test or Mann–Whitney *U*-test. To correlate hormone serum levels and biochemical parameters or imaging data, Pearson or Spearman correlation coefficient and linear regression analysis were applied. Uniparameter receiver operating characteristic (ROC) curve analysis tested the hormones' discriminatory power. Cutoff values were retrieved from Youden index. Variables presenting an area under the ROC curve (AUROC) value superior to 0.60 and $p < 0.05$ were selected for binomial logistic regression analysis. Multi-parameter ROC curves were established for relevant parameter combinations, where an AUROC value >0.80 was considered a very good assay performance. Associated sensitivity, specificity, and positive predictive value (PPV) and negative predictive value (NPV) were estimated. A p-value <0.05 was considered significant. All statistical analysis were performed using GraphPad Prism, version 8.0.2 (La Jolla, California, USA) and IBM SPSS Statistics, version 26 (Armonk, New York, USA).

Metabolomic Analysis

Serum samples from 198 patients in the validation cohort were randomly selected for metabolomic analysis by liquid chromatography–time of flight–mass spectrometry. Two separate liquid chromatography–time of flight–mass spectrometry-based platforms were used: platform 1 to analyze methanol lipid extracts [including fatty acids (FA), acyl carnitines, bile acids, lysoglycerophospholipids, N-acyl ethanolamines, and oxidized FA] and platform 2 for methanol/chloroform lipid extracts (including glycerolipids, cholesterol esters, sphingolipids, and glycerophospholipids) (22). Quality control calibration and validation samples were used to assess data quality. The spectra were mass-corrected by reference to leucine enkephalin. System control and data pre-processing were performed using Masslynx 4.1 software and TargetLynx application manager (Waters Corp., Milford, USA). Peak detection, noise reduction, and data normalization were performed as previously described (23). For univariate analyses, the samples were grouped according to the median serum level of each hormone (leptin = 39.61 ng/ml; adiponectin = 4.84 μg/ml; IGF-1 = 103.65 ng/ml). Log_2 fold change for metabolite levels between higher than median vs. lower than median hormone level groups was determined and compared using unpaired Student's *t*-test ($p < 0.05$) using R v.3.4.1 (R Development Core Team, 2017; http://cran.r-project.org). A p-value <0.05 was considered significant.

RESULTS

Clinical and Biochemical Features of Patient Cohorts

In both cohorts, the NAFL and NASH patients were similar with respecting to age, gender, BMI, gamma-glutamyl transferase (GGT), serum glucose, triglycerides, and cholesterol [total, high-density lipoprotein (HDL) or low-density lipoprotein (LDL)] concentrations (**Tables 1**, **2**). While in the discovery cohort the NAFL and NASH patients had similar circulating AST and ALT, in the validation cohort these liver injury markers

TABLE 1 | Patient demographic and clinical and laboratory data for the discovery cohort.

Parameter	NAFL	NASH
Number	26	12
Age (years)	38.38 ± 1.99	44.83 ± 2.68
Gender (M/F)	10/16	4/8
BMI (kg/m^2)	44.68 ± 1.32	41.56 ± 1.27
Hematology and biochemistry		
AST (U/L)	27.19 ± 2.55	30.92 ± 3.16
ALT (U/L)	39.62 ± 6.96	45.08 ± 5.38
GGT (U/L)	29.36 ± 4.22	37.58 ± 3.42
Glucose (mg/dl)	93.58 ± 2.13	100.58 ± 3.68
Triglycerides (mg/ml)	118.44 ± 5.82	155.91 ± 20.63
Total cholesterol (mg/ml)	191.27 ± 7.41	197.67 ± 10.83
HDL-cholesterol (mg/ml)	46.27 ± 2.69	44.08 ± 2.57
LDL-cholesterol (mmol/dl)	125.00 ± 7.61	125.73 ± 10.11
Total bilirubin (mg/dl)	0.69 ± 0.08	0.74 ± 0.08
Platelets (10^9/L)	280.42 ± 17.32	300.82 ± 11.70
INR	0.97± 0.01	0.94 ± 0.02

Data are expressed as mean ± SEM. Information not available for non-obese controls. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; GGT, γ-glutamyl transferase; HDL, high-density lipoprotein; INR, international normalized ratio; LDL, low-density lipoprotein; M, male; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

and liver fat content evaluated by magnetic resonance imaging and Folch method were increased in NASH vs. NAFL ($p < 0.05$). The prevalence of co-morbidities was similar between NAFL and NASH patients, except for dyslipidemia (higher in NASH vs. NAFL; $p < 0.05$). *PNPLA3* risk allele c.444G frequency vs. wild-type allele was lower in both controls (12.5 vs. 87.5%) and NAFLD (23.7 vs. 76.3%), consistent with previous reports (24). Similarly to previous literature (25), in NAFLD patients, *TM6SF2* risk allele c.499T showed a frequency of 5.7%, while *MBOAT7* risk allele c.50>A presented a frequency of 45.7%.

Leptin Predicts NAFLD and Correlates With Serum Content

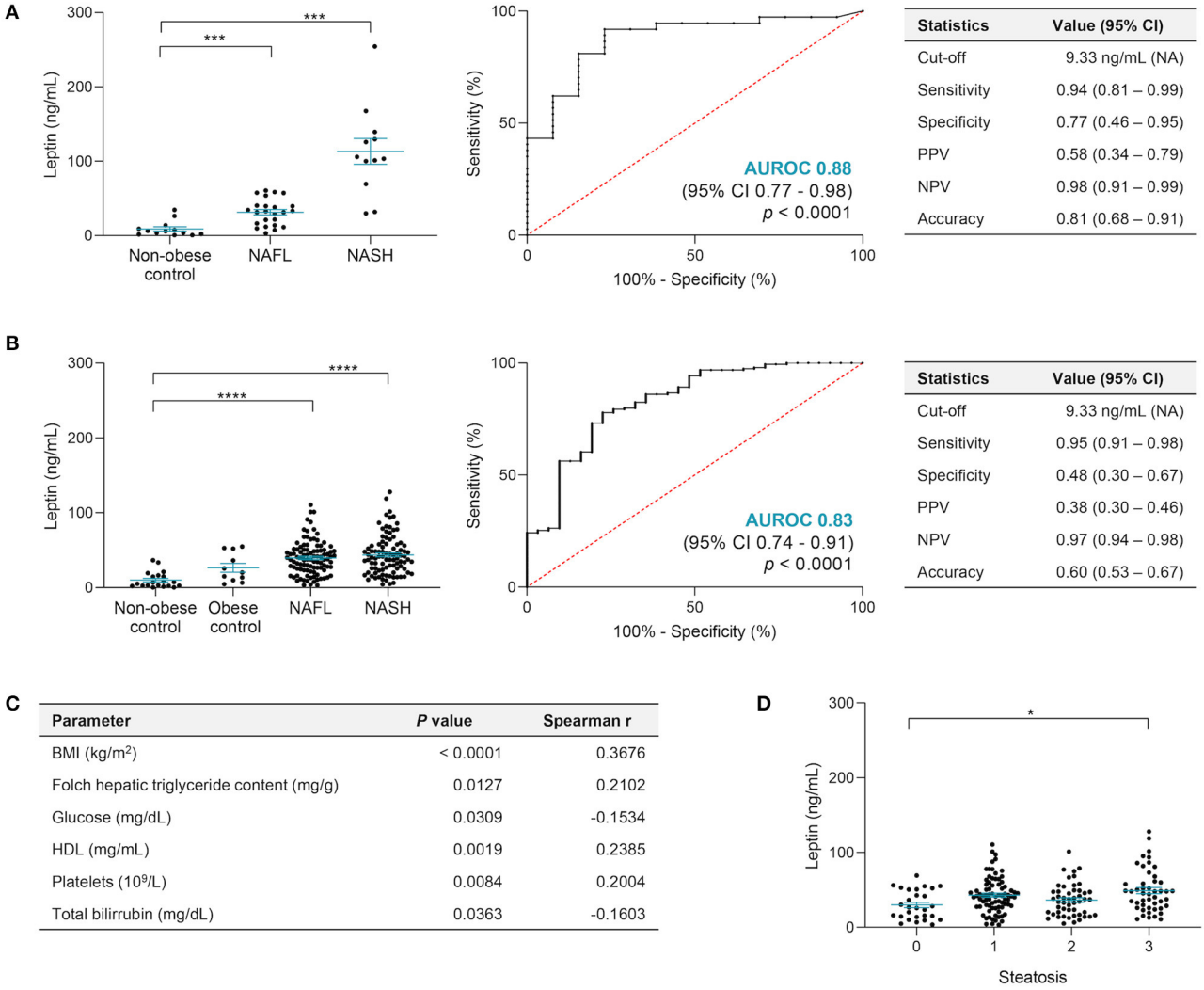
The impact of BMI on leptin and its influence on liver fibrosis remain controversial (26). We observed lower leptin levels in men than in women (discovery cohort: 20.63 ± 3.57 vs. 40.63 ± 4.00 ng/ml, $p < 0.01$; validation cohort: 25.8 ± 2.03 vs. 49.55 ± 1.99 ng/ml, $p < 0.0001$) as reported before (27). In the discovery cohort, serum leptin was higher in NAFL (31.43 ± 3.59 ng/ml, $p < 0.01$) and NASH (37.62 ± 5.12 ng/ml, $p < 0.001$) vs. in non-obese controls (8.92 ± 2.88 ng/ml), showing a good performance when distinguishing non-obese controls from NAFLD patients, with AUROC = 0.88 (95% CI, 0.77–0.98; $p < 0.0001$) and an overall accuracy of 0.81 (95% CI, 0.68–0.91), considering 25% disease prevalence (**Figure 1A**). By applying a leptin cutoff value >9.33 ng/ml for NAFLD inclusion, the assay sensitivity and specificity were 94 and 77%, respectively, and the NPV for ruling out NAFLD diagnosis was 98% (**Figure 1A**). No

TABLE 2 | Patient demographic, clinical and laboratory data for validation cohort.

Parameter	Obese controls	NAFL	NASH
Number	11	100	94
Age (years)	48.10 ± 2.86	45.20 ± 1.27	48.96 ± 1.67
Gender (M/F)	4/6	26/66	34/56
BMI (kg/m^2)	46.03 ± 1.87	44.60 ± 0.67	45.89 ± 0.73
Hematology and biochemistry			
AST (U/L)	23.78 ± 5.93	20.71 ± 0.92	26.24 ± 1.49[a]
ALT (U/L)	22.67 ± 3.43	25.68 ± 1.70	35.18 ± 2.21[a]
GGT (U/L)	21.14 ± 3.52	37.20 ± 4.78	42.27 ± 4.45
Glucose (mg/dl)	108.30 ± 6.77	113.65 ± 3.44	120.22 ± 3.39
Triglycerides (mg/ml)	127.00 ± 32.44	142.22 ± 9.98	158.79 ± 8.43
Total cholesterol (mg/ml)	198.25 ± 4.91	195.75 ± 4.08	206.79 ± 5.41
HDL-cholesterol (mg/ml)	51.50 ± 4.84	48.58 ± 1.47	50.24 ± 1.45
LDL-cholesterol (mmol/dl)	131.30 ± 8.31	118.38± 3.65	125.69 ± 4.12
Total bilirubin (mg/dl)	0.54 ± 0.10	0.55 ± 0.09	0.48 ± 0.03
Platelets (10^9/L)	281.00 ± 33.57	267.78 ± 7.09	251.79 ± 6.22
Hemoglobin (g/dl)	12.40 ± 0.61	13.73 ± 0.13	13.75 ± 0.21
Ferritin (ng/ml)	166.87 ± 31.14	114.15 ± 15.91	134.34 ± 19.79
Albumin (g/dl)	3.96 ± 0.27	4.28 ± 0.05	4.28 ± 0.06
INR	1.01 ± 0.02	0.97 ± 0.01	0.98 ± 0.01
Liver steatosis			
MRI (fat fraction)	n/a	0.15 ± 0.01	0.26 ± 0.01[a]
Hepatic TG content evaluated by Folch method (mg/g)	n/a	57.49 ± 5.47	103.13 ± 6.42[a]
NAFLD-associated polymorphisms			
PNPLA3 (CC/CG/GG)	7/0/1	59/24/6	47/28/9
TM6SF2 (CC/CT/TT)	8/0/0	63/5/3	65/5/0
MBOAT7 (GG/GA/AA)	4/4/0	21/50/18	27/42/15
Co-morbidities			
Arterial hypertension (no/yes)	6/4	46/45	31/59
Diabetes (no/yes)	5/5	66/26	55/34
Dyslipidemia (no/yes)	3/1	55/23	41/41[a]
Cholelithiasis (no/yes)	3/1	52/23	60/19

Data are expressed as mean ± SEM. Information not available for non-obese controls. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; C, cytosine; F, female; G, guanine; GGT, γ-glutamyl transferase; HDL, high-density lipoprotein; INR, international normalized ratio; LDL, low-density lipoprotein; M, male; MRI, magnetic resonance imaging; n/a, not available; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; T, thymine.

[a]*p <0.05 vs. NAFL.*



Statistics	Value (95% CI)
Cut-off	9.33 ng/mL (NA)
Sensitivity	0.94 (0.81 – 0.99)
Specificity	0.77 (0.46 – 0.95)
PPV	0.58 (0.34 – 0.79)
NPV	0.98 (0.91 – 0.99)
Accuracy	0.81 (0.68 – 0.91)

Statistics	Value (95% CI)
Cut-off	9.33 ng/mL (NA)
Sensitivity	0.95 (0.91 – 0.98)
Specificity	0.48 (0.30 – 0.67)
PPV	0.38 (0.30 – 0.46)
NPV	0.97 (0.94 – 0.98)
Accuracy	0.60 (0.53 – 0.67)

C

Parameter	*P* value	Spearman r
BMI (kg/m^2)	< 0.0001	0.3676
Folch hepatic triglyceride content (mg/g)	0.0127	0.2102
Glucose (mg/dL)	0.0309	-0.1534
HDL (mg/mL)	0.0019	0.2385
Platelets (10^9/L)	0.0084	0.2004
Total bilirrubin (mg/dL)	0.0363	-0.1603

FIGURE 1 | Leptin serum levels allow to distinguish NAFLD. **(A)** In the discovery cohort, leptin levels were significantly higher in NAFL and NASH patients compared with non-obese, healthy-liver controls, presenting an AUROC value of 0.88 when distinguishing controls ($n = 13$) from NAFLD patients ($n = 36$), with very high general accuracy and NPV. **(B)** In the validation cohort, the leptin levels did not show differences among healthy controls, non-obese or obese, and confirmed increased levels in NAFL and NASH. Leptin presented good performance assay results when distinguishing healthy controls ($n = 31$) from NAFLD ($n = 194$), with an AUROC value of 0.83 and overall accuracy of 0.60 when applied the cutoff value of >9.33 ng/ml leptin for NAFLD diagnosis established in the discovery cohort. **(C)** Main correlations found between leptin and other parameters in the validation cohort: BMI values ($n = 199$), hepatic TG evaluated by Folch method ($n = 140$; mg TG/g liver tissue), glucose ($n = 198$), HDL ($n = 167$), platelets ($n = 172$), and total bilirubin ($n = 171$). **(D)** Leptin levels increased with steatosis severity in the validation cohort. The *x*-axis represents the histological grade of steatosis, ranging from 0 to 3. There was a significant difference between grade 0 vs. 3 ($n = 28$, grade 0; $n = 80$, grade 1; $n = 49$, grade 2; $n = 48$, grade 3). Leptin levels depicted as mean ± SEM. BMI, body mass index; CI, confidence interval; HDL, high-density lipoprotein; NAFL, non-alcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value. $^*p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

correlations were found between serum leptin and BMI or any biochemical parameter.

In the validation cohort, leptin was higher again in NAFL (39.74 ± 2.25 ng/ml) and NASH (43.72 ± 2.70 ng/ml) vs. non-obese controls (6.21 ± 0.97 ng/ml; $p < 0.0001$) (**Figure 1B**). Although a positive correlation was found between serum leptin levels and BMI ($p < 0.0001$) (**Figure 1C**), no significant differences were evident between non-obese and obese controls (26.52 ± 5.82 ng/ml), suggesting that obesity might not be a confounding factor (**Figure 1B**). Serum leptin showed a good performance again when distinguishing controls (both non-obese and obese) from NAFLD patients (both NAFL and NASH), with AUROC = 0.83 (95%, CI 0.74–0.91; $p < 0.0001$). Considering 25% disease prevalence and by applying the cutoff value >9.33 ng/ml for NAFLD inclusion determined in the discovery cohort, an overall accuracy of 0.60 (95% CI, 0.53–0.67) was achieved, with sensitivity of 95% and specificity of 48% (**Figure 1B**). Serum leptin positively correlated with

hepatic TG content ($p < 0.05$), HDL ($p < 0.01$), platelets ($p < 0.01$), and total bilirubin ($p < 0.01$) (**Figure 1C**). Curiously, diabetic NAFLD patients had significantly lower leptin compared with those who were non-diabetic (34.90 ± 2.87 vs. 45.39 ± 2.17 ng/ml; $p < 0.01$; **Supplementary Figure 1**), supported by the negative correlation between leptin and fasting glucose ($p < 0.05$; **Figure 1C**). Leptin was increased in patients with severe liver steatosis (stage 0 vs. 3, $p < 0.05$) (**Figure 1D**) and tended to increase with lobular inflammation and hepatic ballooning, while no significant differences were found with other co-morbidities, fibrosis stage, or NAFLD-associated polymorphisms (**Supplementary Figure 1**).

Given leptin's association with hepatic fat accumulation, its impact on the serum metabolic signatures of NAFLD patients was further evaluated. Levels of circulating lipids and other metabolites in NAFLD patients were compared between high/low levels of circulating leptin. Higher serum leptin was associated with a general increase in serum lipids, namely, FA ($p < 0.05$), monounsaturated FA ($p < 0.05$), and FA containing palmitoleic acid (16:1; $p < 0.01$) and linolenic acid (18:3; $p < 0.001$), while diglycerides (DG) and TG were decreased (for TG, $p < 0.05$) (**Figure 2**). Other lipid classes such as phosphatidylcholines (PC), lysophosphatiylcholines, phosphatidylinositols ($p < 0.05$), lysophosphatidylinositols, phosphatidylethanolamines (PE), lysophosphatidylethanolamines (LPE), or sphingomyelin (SM; $p < 0.01$) were increased in the serum of NAFLD patients with high circulating leptin levels (**Figure 2**).

Overall, serum leptin predicts NAFLD, correlates with serum lipid changes, and is a potentially valuable tool for NAFLD diagnosis.

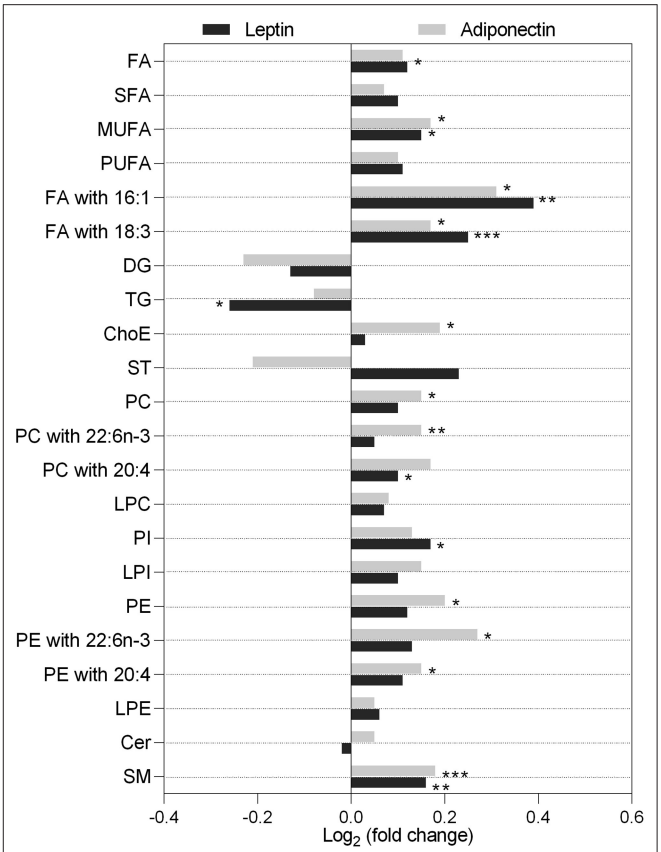
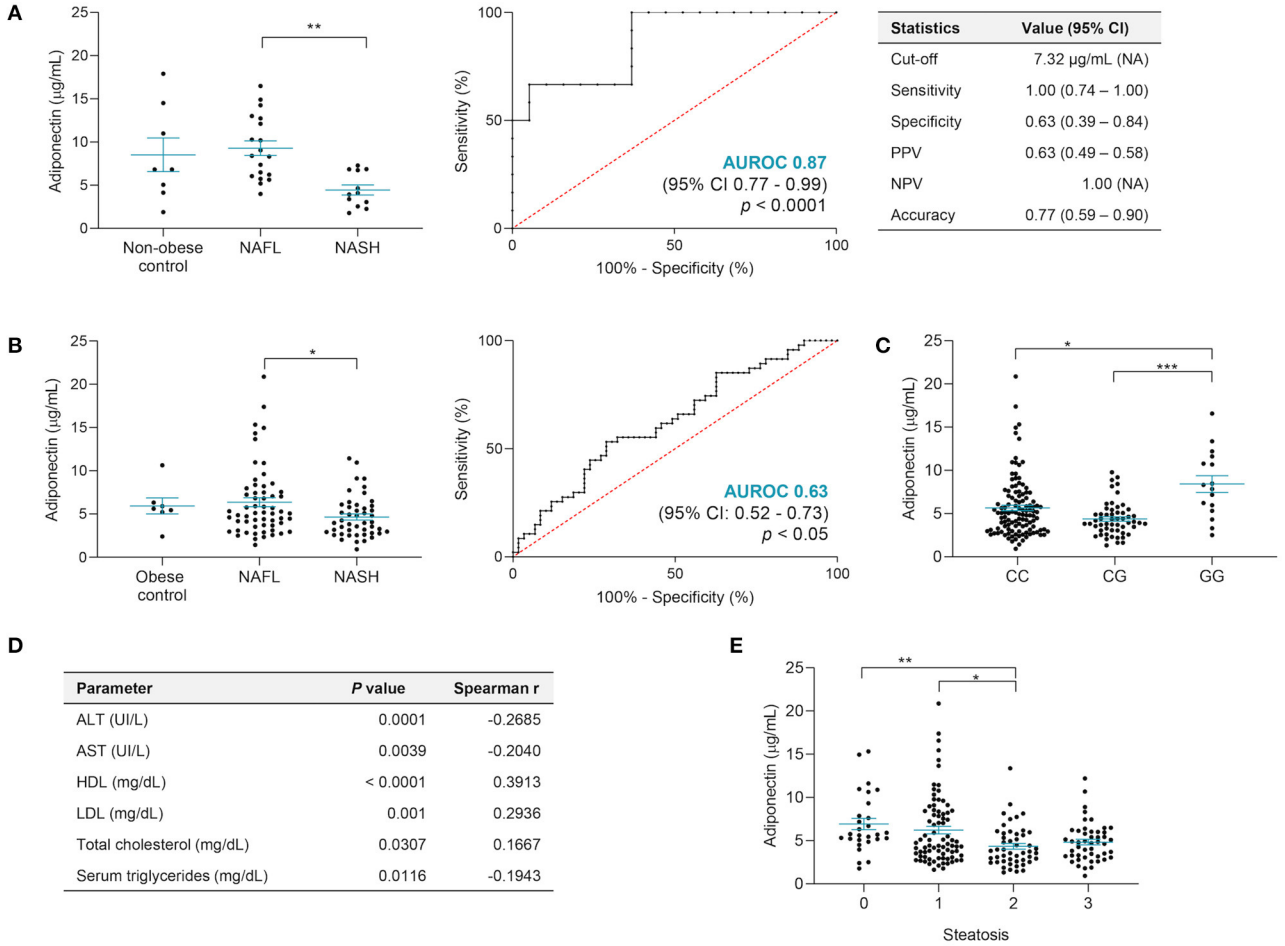


FIGURE 2 | Serum lipidomic signatures associated with higher circulating levels of leptin (dark gray) and adiponectin (light gray). Data presented as $\log_2$ of the lipid level fold change between patients with hormone levels higher than the median *vs.* lower than the median. Leptin median level = 39.61 ng/ml; higher, $n = 99$; lower, $n = 99$. Adiponectin median level = 4.84 μg/ml; higher, $n = 95$; lower, $n = 99$. Cer, ceramide; ChoE, cholesteryl ester; DG, diglyceride; FA, fatty acid; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MUFA, monounsaturated FA; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PUFA, polyunsaturated FA; SFA, saturated FA; SM, sphingomyelins; ST, steroids; TG, triglycerides; 16:0, palmitic acid; 18:3, linolenic acid; 20:4, arachidonic acid; 22:6n-3, docosahexaenoic acid (DHA). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Adiponectin and Specific Lipid Species Distinguish NASH

In the discovery cohort, adiponectin was similar among men and women (6.55 ± 0.98 vs. 7.79 ± 1.04 μg/ml) and significantly lower in NASH vs. NAFL (non-obese controls: 8.51 ± 1.94 μg/ml; NAFL: 9.23 ± 0.85 μg/ml; NASH: 4.45 ± 0.58 μg/ml; $p < 0.01$) (**Figure 3A**). Serum adiponectin showed a good assay performance when distinguishing NAFL vs. NASH (AUROC = 0.87; 95% CI, 0.77–0.99; $p < 0.0001$). This allowed us to establish a cutoff value <7.32 μg/ml for diagnosing NASH, with an overall accuracy of 77%, sensitivity of 100%, and NPV (**Figure 3A**). Additionally, the adiponectin serum levels negatively correlated with the serum markers of liver injury ALT (Spearman $r = -0.46$, $p < 0.01$) and GGT (Spearman $r = -0.42$, $p < 0.05$).

In the validation cohort, adiponectin was lower in men (4.4 ± 0.32 vs. 6.07 ± 0.29 μg/ml, $p < 0.0001$) and, although not significant, were lower in NASH (4.94 ± 0.27 μg/ml) vs. both non-obese (6.21 ± 0.97 μg/ml) and obese (6.56 ± 0.93 μg/ml) controls or NAFL (6.22 ± 0.45 μg/ml). In this cohort, serum adiponectin was only able to distinguish NAFL vs. NASH when NAFLD patients homozygous for *PNPLA3* wild-type allele c.444C were considered, even though with a suboptimal performance (AUROC = 0.63; $p < 0.05$) (**Figure 3B**). By applying the previously established cutoff value of adiponectin <7.32 μg/ml for NASH diagnosis, the PPV and NPV were 49 and 71%, respectively. This AUROC value was paralleled by AST/ALT ratio and only overcome by MRI or Folch method AUROCs, however with lower specificity values (**Table 3**). Curiously, NAFLD patients homozygous for *PNPLA3* risk allele c.444G presented significantly higher adiponectin (GG vs. CC, $p < 0.05$; GG vs. CG, $p < 0.0001$; **Figure 3C**). In the validation cohort, no differences were found between adiponectin and fibrosis stage and ballooning and lobular inflammation, neither for the presence/absence of co-morbidities nor for the other NAFLD risk-associated polymorphisms (**Supplementary Figure 2**). Adiponectin still negatively correlated with AST ($p < 0.01$) and ALT ($p < 0.001$) and positively correlated with total cholesterol ($p < 0.05$), HDL ($p < 0.001$), and LDL ($p < 0.01$), regardless of *PNPLA3* genotype



Statistics	Value (95% CI)
Cut-off	7.32 µg/mL (NA)
Sensitivity	1.00 (0.74 – 1.00)
Specificity	0.63 (0.39 – 0.84)
PPV	0.63 (0.49 – 0.58)
NPV	1.00 (NA)
Accuracy	0.77 (0.59 – 0.90)

Parameter	*P* value	Spearman r
ALT (UI/L)	0.0001	-0.2685
AST (UI/L)	0.0039	-0.2040
HDL (mg/dL)	< 0.0001	0.3913
LDL (mg/dL)	0.001	0.2936
Total cholesterol (mg/dL)	0.0307	0.1667
Serum triglycerides (mg/dL)	0.0116	-0.1943

FIGURE 3 | Adiponectin serum levels differentiate NAFL and NASH, but *PNPLA3* genotype may be a confounding factor. **(A)** In the discovery cohort, the adiponectin serum levels were significantly lower in NASH when compared to NAFL, presenting an AUROC value of 0.87 and very high sensitivity and NPV (non-obese control, $n = 8$; NAFL, $n = 19$; NASH, $n = 12$). **(B)** In the validation cohort, adiponectin distinguishes NAFL from NASH only within *PNPLA3* CC allele carriers, with an AUROC value superior to 0.6 obese controls, $n = 7$; NAFL, $n = 59$, NASH, $n = 47$). **(C)** In the validation cohort, NAFLD patients with *PNPLA3* GG genotype also showed increased levels of adiponectin. The *x*-axis represents the three possible genotypes for rs738409 polymorphism: CC allele, $n = 117$; CG allele, $n = 55$; GG allele, $n = 16$). **(D)** Correlations between adiponectin serum levels and several parameters: AST and ALT serum levels (both $n = 199$), HDL and LDL serum levels (both $n = 167$), total cholesterol levels ($n = 168$), and triglyceride serum levels ($n = 168$). **(E)** The adiponectin levels decreased with higher grades of steatosis. The *x*-axis represents the histological grade of steatosis, ranging from 0 to 3. There was a significant difference between grade 2 vs. grade 1 and vs. grade 0 ($n = 28$, grade 0; $n = 80$, grade 1; $n = 49$, grade 2; $n = 48$, grade 3). Adiponectin levels depicted as mean ± SEM. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(**Figure 3D**). Adiponectin decreased with severity of steatosis and negatively correlated with serum TG ($p < 0.05$; **Figures 3D,E**).

Although not significant, higher serum adiponectin positively correlated with serum lipid classes, except for DG, TG, and steroids (**Figure 2**). Both PE and PC containing arachidonic (20:4) or docosahexaenoic (DHA; 22:6n-3) acids were increased in patients with high circulating adiponectin ($p < 0.05$ and $p < 0.01$). Moreover, the serum levels of 147 metabolites were significantly different between NAFL and NASH, of which 35 were highly correlated with serum adiponectin (**Supplementary Table 2**). Among those, nine TG were already described as capable of distinguishing NAFL from NASH (16). Sixty-five out of the 147 metabolites presented AUROC >0.60 and were considered for binomial regression analysis. This allowed establishing a panel combining adiponectin and nine serum lipids (mainly TG, PC, and SM), reaching an AUROC value of ∼0.80 and an overall accuracy of 74% in distinguishing NAFL from NASH in all patients, regardless of *PNPLA3* genotype. When this panel was applied to homozygous carriers of the *PNPLA3* c.444C allele, the AUROC value increased to 0.83 and the accuracy to 80% (**Table 4**).

Overall, serum adiponectin inversely correlated with liver injury markers and, when combined with nine specific lipids, distinguished NAFL from NASH patients with high accuracy.

TABLE 3 | Performance of serum adiponectin in the differential diagnosis of NAFL vs. NASH in patients homozygous for the wild-type allele CC in the *PNPLA3* rs738409 polymorphism.

	Cutoff	AUROC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)	PPV (95% CI)	NPV (95% CI)	TP	TN	FP	FN
Adiponectin	<7.32 μg/ml	0.63* (0.52–0.73)	0.85 (0.72–0.94)	0.29 (0.18–0.42)	0.54 (0.44–0.64)	0.49 (0.44–0.54)	0.71 (0.52–0.84)	40	17	42	7
AST/ALT	<0.96	0.65** (0.55–0.76)	0.89 (0.77–0.96)	0.49 (0.36–0.63)	0.67 (0.75–0.76)	0.59 (0.52–0.66)	0.85 (0.70–0.93)	42	28	29	5
MRI	>0.15	0.90**** (0.69–0.91)	0.85 (0.68–0.95)	0.68 (0.50–0.83)	0.76 (0.64–0.86)	0.72 (0.61–0.81)	0.82 (0.67–0.91)	28	11	5	23
Hepatic TG content evaluated by Folch method	>42.85 mg/g	0.75*** (0.63–0.87)	0.83 (0.67–0.94)	0.59 (0.42–0.74)	0.70 (0.59–0.80)	0.64 (0.54–0.72)	0.80 (0.65–0.90)	30	24	17	6

Applied in validation cohort. Adiponectin; n = 106; AST/ALT, n = 104; MRI, n = 67; hepatic TG content evaluated by Folch method, n = 77. NASH is considered positive if the levels of each parameter are superior or inferior to the cutoff value as indicated.

AST/ALT, aspartate aminotransferase to alanine aminotransferase ratio; CI, confidence interval; FN, false negative; FP, false positive; MRI, magnetic resonance imaging; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.

**p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

TABLE 4 | Performance of serum lipids and combination panel with serum adiponectin in the differential diagnosis of NAFL *vs.* NASH.

	Cutoff	AUROC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)	PPV (95% CI)	NPV (95% CI)	TP	TN	FP	FN
TG(44:0)	>0.25	0.72* (0.65–0.79)	0.60 (0.49–0.70)	0.75 (0.65–0.83)	0.67 (0.60–0.74)	0.70 (0.61–0.77)	0.65 (0.59–0.71)	54	68	23	36
TG(49:2)	>0.71	0.70* (0.63–0.78)	0.71 (0.61–0.80)	0.58 (0.47–0.69)	0.65 (0.57–0.72)	0.63 (0.56–0.69)	0.67 (0.59–0.75)	64	53	38	26
TG(51:3)	>1.02	0.61* (0.54–0.69)	0.61 (0.50–0.71)	0.56 (0.45–0.66)	0.59 (0.51–0.66)	0.58 (0.51–0.65)	0.59 (0.52–0.67)	55	51	40	35
TG(52:1)	>0.85	0.75** (0.68–0.82)	0.79 (0.69–0.87)	0.59 (0.49–0.70)	0.69 (0.62–0.76)	0.66 (0.59–0.72)	0.74 (0.65–0.81)	71	54	37	19
TG(53:1)	>0.48	0.74* (0.67–0.81)	0.89 (0.81–0.95)	0.49 (0.39–0.60)	0.69 (0.62–0.76)	0.63 (0.58–0.68)	0.82 (0.71–0.89)	80	45	46	10
TG(54:0)	>0.38	0.68* (0.61–0.76)	0.72 (0.62–0.81)	0.57 (0.46–0.67)	0.65 (0.57–0.72)	0.63 (0.56–0.69)	0.68 (0.59–0.75)	65	52	39	25
TG(60:3)	>0.58	0.60 (0.50–0.66)	0.70 (0.59–0.79)	0.47 (0.37–0.58)	0.59 (0.51–0.66)	0.57 (0.51–0.62)	0.61 (0.52–0.70)	63	43	48	27
PC(0:0/14:0)	>0.22	0.70* (0.62–0.77)	0.80 (0.70–0.88)	0.51 (0.40–0.61)	0.65 (0.58–0.72)	0.62 (0.56–0.67)	0.72 (0.62–0.80)	72	46	45	18
SM(38:0)	>1.16	0.71* (0.63–0.78)	0.74 (0.64–0.83)	0.64 (0.53–0.74)	0.69 (0.62–0.76)	0.67 (0.60–0.73)	0.72 (0.63–0.79)	67	58	33	23
Adiponectin + 9 lipids	-	0.796** (0.73–0.86)	0.71 (0.61–0.80)	0.77 (0.67–0.85)	0.74 (0.67–0.80)	0.75 (0.67–0.82)	0.73 (0.66–0.79)	64	70	21	26
Adiponectin + 9 lipids (*PNPLA3* rs738409 CC)	-	0.832** (0.75–0.91)	0.70 (0.55–0.83)	0.88 (0.77–0.95)	0.802 (0.71–0.87)	0.83 (0.70–0.91)	0.79 (0.70–0.85)	33	52	7	14

Applied in the validation cohort, n = 181; PNPLA3 rs738409 CC carriers, n = 106. NASH is considered as positive if the levels of each parameter are superior or inferior to the cutoff value as indicated; for adiponectin, <7.32 μg/ml. p-values corrected with Benjamini–Hochberg procedure, assuming 5% false discovery rate.

CI, confidence interval; FN, false negative; FP, false positive; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NPV, negative predictive value; PC, phosphatidylcholine; PPV, positive predictive value; TG, triglyceride; TN, true negative; TP, true positive; SM, sphingomyelin.

**p < 0.05; **p < 0.01.*

IGF-1 Predicts Advanced Fibrosis in NAFLD

IGF-1 was higher in men in both cohorts (discovery cohort: 152.08 ± 20.78 vs. 81.04 ± 12.24 ng/ml, $p < 0.01$; validation cohort: 126.5 ± 6.78 vs. 103.4 ± 3.87 ng/ml, $p < 0.01$). In the discovery cohort, the IGF-1 levels decreased in NAFL (109.95 ± 16.55 ng/ml) and NASH (113.19 ± 17.39 ng/ml) when compared to non-obese controls (167.42 ± 17.55 ng/ml), although not significantly. In the validation cohort, there were no significant changes in IGF-1 between non-obese controls (115.75 ± 15.69 ng/ml), obese controls (105.83 ± 15.62 ng/ml), NAFL (122.30 ± 6.05 ng/ml), and NASH (103.50 ± 3.98 ng/ml), although an inverse correlation with BMI was observed ($n = 199$; Spearman $r = -0.1647$; 95% CI, 0.30-−0.02; $p < 0.05$) (**Figures 4A,B**). In agreement, in the validation cohort, there were also no significant differences in IGF-1 relating to liver steatosis, lobular inflammation, or hepatocyte ballooning severity (**Supplementary Figure 3**), yet IGF-1 was significantly lower in NAFLD patients with advanced fibrosis (F3–4; $p < 0.05$) (**Figure 4C**). By establishing a cutoff value of IGF-1 inferior to 98.83 ng/ml for the presence of advanced fibrosis, 70% sensitivity, 61% specificity, and 63% overall accuracy were obtained (**Figure 4C** and **Table 5**). Overall, IGF-1, as well as the pre-established biomarker fibrosis panels APRI, Fib-4, and NFS, displayed a suboptimal performance in F0–2 vs. F3–4 discrimination. However, by combining serum IGF-1, ferritin, and INR, the discriminatory power was significantly improved, resulting in AUROC value of 0.81 and overall accuracy of 93%, considering the ∼10% prevalence of advanced fibrosis in this cohort (**Table 5**). Importantly, besides a robust NPV, this panel displayed 71% PPV value, contrasting with the low PPV values from APRI, Fib-4, and NFS. Circulating IGF-1 did not correlate with other parameters or co-morbidities (**Supplementary Figure 3**).

Serum metabolomic analysis showed that higher IGF-1 was associated with an overall increase in main lipid classes, significant for LPE ($p < 0.05$), PC containing arachidonic acid (20:4; $p < 0.05$), diacylglycerophosphocholine ($p < 0.05$), and monoacylglycerophosphoethanolamine ($p < 0.05$) (**Figure 4D**). Moreover, 26 specific serum metabolites were able to distinguish advanced fibrosis, with several bile acids increased in advanced fibrosis, and both cholic acid and PC (17:0/18:2) positively correlated with IGF-1 (**Supplementary Table 3**).

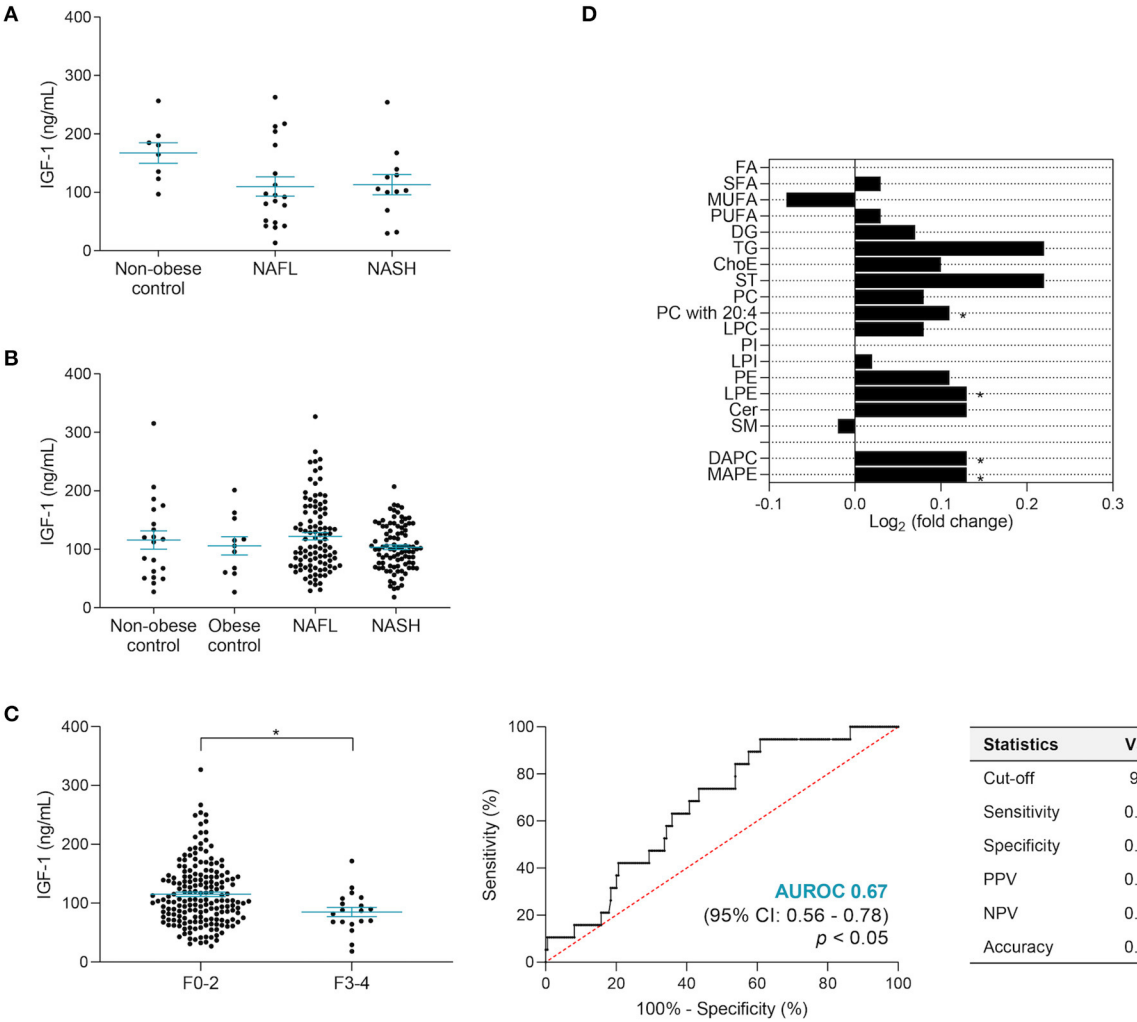
IGF-1 altogether could identify advanced fibrosis, particularly in combination with INR and ferritin, and correlates with serum lipid changes associated with fibrosis.

DISCUSSION

The strong association between obesity and NAFLD, parallel with the continuous rise in the prevalence of obesity, prompts a more detailed study of the role of adipokines and other hormones as potential non-invasive biomarkers for NAFLD (1). Therefore, we measured serum leptin, adiponectin, and IGF-1 in two independent cohorts, comprising both biopsy-proven healthy-liver controls as well as obese NAFL and NASH patients. In the discovery cohort, the serum hormones were correlated with clinical and histological information, including NAS, fibrosis stage, and biochemical parameters. Results were then validated in a second cohort, which further allowed us to explore the role of obesity as a confounding factor by including a control group of obese healthy-liver individuals. Additionally, in this validation cohort, hormone serum levels were correlated with liver imaging, co-morbidities, and NAFLD risk-associated polymorphisms. For a subset of patients, serum metabolomic analysis was also performed. This strategy showed that particularly leptin and adiponectin can be potential diagnosis and stratification biomarkers in NAFLD. The role of these two adipokines in NAFLD pathophysiology, diagnosis, and even treatment has attracted significant attention despite the controversial results.

Leptin is a 16-kDa pro-inflammatory adipokine produced by adipocytes. Serum leptin levels reflect total body mass. Leptin may exert an anti-steatotic action in early NAFLD by promoting FA oxidation and decreased lipogenesis and a pro-inflammatory and pro-fibrotic action at later disease stages by increasing hepatic reactive oxygen species generation, pro-inflammatory cytokine release, and enhanced fibrinogenesis (28, 29). Previous reports have generally found increased serum leptin in NAFLD patients (12, 29, 30). Still the role of obesity as a bias remains poorly explored. Our results in the discovery cohort showed that leptin accurately identified NAFLD patients, where there was no correlation with BMI. Moreover, in the validation cohort, although leptin levels positively correlated with BMI, they were higher in NAFL and NASH compared with both non-obese and obese controls, suggesting that the association between NAFLD and leptin is more robust than the effect of obesity on leptin levels. In this cohort, serum leptin showed again a very good performance as a potential biomarker to distinguish NAFLD patients from healthy individuals. After applying the cutoff value of leptin at >9.33 ng/ml for diagnosing NAFLD in the validation cohort, despite having a lower general accuracy, it still presented sensitivity, specificity, and NPV values far superior than those previously reported (31, 32).

In turn, adiponectin, a 30-kDa protein mainly secreted by adipose tissue, is the most well-studied adipokine in the pathogenesis of NAFLD (30, 33). It is considered to have anti-inflammatory, anti-steatotic, and anti-fibrotic effects. In the liver, adiponectin prevents lipid accumulation by promoting FA oxidation *via* peroxisome proliferator-activated receptor alpha (33). Adiponectin also downregulates pro-inflammatory cytokines produced by Kupffer cells and hepatic stellate cells, thus inhibiting their transformation into myofibroblasts and consequently decreasing liver fibrosis (30, 34–36). Several reports have shown a reverse relation between adiponectin circulating levels and body fat mass and its decrease in obesity, type 2 diabetes, insulin resistance, and dyslipidemia (30, 34). A meta-analysis showed that adiponectin decreases in healthy controls compared to NAFL, and in the latter compared to NASH (37). However, there is significant heterogeneity among studies, and the importance of including biopsy-proven healthy controls is underlined; most differences in adiponectin between individuals with NAFLD and controls were observed when the controls were not subjected to liver



Statistics	Value (95% CI)
Cut-off	98.83 ng/mL (NA)
Sensitivity	0.70 (0.35 to 0.93)
Specificity	0.61 (0.13 to 0.72)
PPV	0.17 (0.11 to 0.25)
NPV	0.95 (0.88 to 0.98)
Accuracy	0.63 (0.53 to 0.72)

FIGURE 4 | IGF-1 serum levels correlated with liver fibrosis score. **(A)** In the discovery cohort, the IGF-1 serum levels were slightly lower in NASH patients compared to NAFL and non-obese controls. **(B)** No significant differences in IGF-1 levels were found among controls and patients with NAFLD in the validation cohort. **(C)** In the validation cohort, NAFLD patients with advanced fibrosis presented significantly lower levels of IGF-1. The *x*-axis represents fibrosis score dichotomy: none to moderate fibrosis (F0-2; $n = 184$) and advanced fibrosis to cirrhosis (F3-4; $n = 19$). IGF-1 alone presents an AUROC value of 0.67 when distinguishing F0-2 *vs.* F3-4. **(D)** Serum lipidomic signature associated with higher IGF-1 circulating levels. Data presented as $\log_2$ of the fold change between higher *vs.* lower than the median IGF-1 level (103.65 ng/ml). Higher, $n = 97$; lower, $n = 97$. IGF-1 levels depicted as mean ± SEM. Cer, ceramide; ChoE, cholesteryl ester; CI, confidence interval; DAPC, diacylglycerophosphocholine; DG, diglyceride; FA, fatty acid; IGF-1, insulin-like growth factor 1; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; LPI, lysophosphatidylinositols; MAPE, monoacylglycerophosphoethanolamine; MUFA, monounsaturated FA; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PUFA, polyunsaturated FA; SFA, saturated FA; SM, sphingomyelins; ST, steroids; TG, triglycerides; 20:4, arachidonic acid. *$p < 0.05$.

biopsy (37). In the discovery cohort, adiponectin presented a very promising performance in distinguishing NAFL vs. NASH. However, when tested in the larger validation cohort, adiponectin only presented a fair discriminatory power in this stratification when homozygous carriers of the *PNPLA3* c.444C allele were considered. Importantly, the discriminatory power of adiponectin regarding NAFL vs. NASH patients was improved when combined with nine specific lipids. Previously, others have shown that combining adiponectin with homeostatic model assessment for insulin resistance and type IV collagen 7S increased the assay sensitivity from 68 to 94% in early-stage NASH prediction (38), while combining adiponectin, resistin, and cleaved cytokeratin-18 predicted NASH with an AUROC of 0.90 (39). Similar approaches combining adiponectin serum levels with a panel of serum lipids, mainly PC species, have been shown to discriminate between healthy individuals, NAFL, and NASH with high accuracy (40). Curiously, previous observations showed that NAFLD patients homozygous for the c.444G allele presented decreased adiponectin (41); however, the opposite was observed here. Nonetheless, these results could explain how this subgroup of NAFLD patients has an improved response to treatment and lifestyle interventions, including bariatric surgery,

TABLE 5 | Performance of IGF-1, alone or in a combination panel, and classical fibrosis scores in distinguishing F0-2 vs. F3-4 grades of fibrosis.

	Cutoff	AUROC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)	PPV (95% CI)	NPV (95% CI)	TP	TN	FP	FN
IGF-1	<98.83 ng/ml	0.67* (0.56–0.78)	0.70 (0.35–0.93)	0.61 (0.51–0.72)	0.63 (0.53–0.72)	0.17 (0.11–0.25)	0.95 (0.88–0.98)	7	60	37	3
APRI	>0.31	0.62 (0.43–0.81)	0.60 (0.26–0.88)	0.71 (0.61–0.80)	0.70 (0.60–0.79)	0.18 (0.11–0.28)	0.95 (0.89–0.97)	6	69	28	4
Fib-4	>0.75	0.66 (0.49–0.82)	0.90 (0.56–0.99)	0.47 (0.37–0.58)	0.51 (0.42–0.61)	0.15 (0.12–0.19)	0.98 (0.88–0.99)	9	46	51	1
NFS	>-0.96	0.74 (0.52–0.89)	0.43 (0.10–0.82)	0.88 (0.77–0.94)	0.83 (0.72–0.91)	0.28 (0.12–0.53)	0.93 (0.88–0.96)	3	56	8	4
Ferritin	>118 ng/ml	0.68* (0.49–0.87)	0.8 (0.44–0.97)	0.65 (0.55–0.74)	0.66 (0.57–0.75)	0.19 (0.13–0.26)	0.97 (0.90–0.99)	8	63	34	2
INR	>1.03	0.78* (0.64–0.93)	0.70 (0.35–0.93)	0.85 (0.76–0.91)	0.83 (0.75–0.90)	0.32 (0.20–0.46)	0.96 (0.91–0.99)	7	82	15	3
IGF-1 + ferritin + INR	-	0.81* (n.d.)	0.50 (0.19–0.81)	0.98 (0.93–0.99)	0.93 (0.87–0.97)	0.71 (0.37–0.91)	0.95 (0.91–0.97)	5	95	2	5

Applied in the validation cohort. NSF, n = 71; others, n = 107. F3-4 is considered as positive if the levels of each parameter are superior or inferior to the cutoff value as indicated. p-values corrected with Benjamini–Hochberg procedure, assuming 5% false discovery rate.

APRI, aspartate aminotransferase-to-platelet ratio; CI, confidence interval; FN, false negative; FP, false positive; IGF-1, insulin-like growth factor 1; INR, international normalized ratio; NFS, NAFLD fibrosis score; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive; n.d., not determined due to the low number of F3-4 patients.

**p < 0.05.*

when compared to CG and CC carriers (42). Additionally, we showed that lower levels of serum adiponectin associated with higher AST and ALT, thus suggesting a role of adiponectin in preventing liver damage. Overall, our observations suggest that adiponectin might be a valuable tool in NAFL vs. NASH stratification; however, its role as a biomarker in NAFLD can be challenged by risk-conferring genetic variants, such as the *PNPLA3* c.444G allele, and can benefit from combination panels, such as the one presented comprising serum lipids.

In fact, NAFLD has been associated with increased lipids in both liver tissue and plasma. Here we observed that higher circulating leptin associated with increased serum lipid profile and increased hepatic steatosis as assessed by histology and imaging. Similarly, higher circulating adiponectin associated with an increased serum lipid profile. The fact that adiponectin is inversely correlated with liver fat contents, as evaluated by MRI, might suggest a protective action for adiponectin by promoting lipid removal from the liver into circulation. For instance, higher adiponectin associated with higher serum PE containing arachidonic acid (C20:4n-6), while lower liver levels of this acid have been observed in NAFLD due to active conversion into pro-inflammatory prostaglandins (13). Furthermore, increased serum PE containing DHA also followed higher adiponectin. DHA, whose lower levels have been associated with both NAFL and NASH, is believed to have anti-inflammatory and metabolic effects and to be able to lower liver TG (13, 14, 43, 44). This might also explain why higher adiponectin inversely correlates with TG as measured by blood biochemistry and serum metabolomics.

In turn, IGF-1, mainly produced in the liver in response to growth hormone stimulation, is known to regulate insulin sensitivity and decrease hepatic TG accumulation in the liver (45). While a few clinical studies have associated low serum IGF-1 with increased severity of steatosis (46, 47) or lobular inflammation (48), our results and those of others (49) failed to show any association. On the other hand, clinical studies are consensual when reporting low serum IGF-1 association with the severity of fibrosis (46, 48–50). In fact, *in vitro* and *in vivo* studies showed that IGF-1 can induce cellular senescence and hepatic stellate cell inactivation through a p53-dependent manner, thus improving experimental fibrosis (51). Accordingly, here we showed that patients with NAFLD and advanced fibrosis presented significantly lower serum IGF-1. More importantly, we found that the 63% accuracy of serum IGF-1 alone in distinguishing low/mild fibrosis from advanced fibrosis is further improved to 93% if IGF-1 is combined with ferritin and INR. These results are very similar to those determined for the Enhanced Liver Fibrosis (ELF) test, recommended by the NICE guidelines for the evaluation of fibrosis in NAFLD (9). A poor PPV value has still been reported for the generality of fibrosis tests, including ELF (52). The panel here proposed displayed high PPV value, being matched by the recent Hepamet fibrosis scoring system where a PPV of 76% was achieved (53). Recent reports suggest that IGF-1 biodisponibility might be decreased during disease progression toward NASH and liver fibrosis and advance IGF-1/intact IGFBP3 ratio as a fibrosis predictor (54). Moreover, IGF-1 might associate with a fibrosis-specific serum metabolite profile, where many bile acids were found increased in advanced

fibrosis, namely, cholic acid, which also correlated with serum IGF-1. Increased levels of many bile acids, including cholic acid, have already been associated with liver fibrosis (55).

Inclusion of a patient group comprising lean-NAFLD would allow further inquiries on adipokines as NAFLD biomarkers, particularly for leptin. It is also recognized that the number of healthy non-obese controls is relatively small, mostly due to the difficulty in obtaining biopsies from healthy-liver individuals. Lastly, the performance of the proposed biomarker panels comprising serum adiponectin and lipids for NAFL vs. NASH stratification and serum IGF-1 with INR and ferritin for identification of advanced fibrosis should be prospectively tested in a different cohort.

CONCLUSION

In conclusion, we suggest that serum leptin can identify NAFLD without obesity as a confounding factor, whereas adiponectin combined with specific serum lipids might be advantageous for NAFL vs. NASH stratification. In turn, IGF-1, together with ferritin and INR, could embody a valuable biomarker panel to identify advanced fibrosis, which could be easily implemented in clinics and presents a better PPV than the regularly used algorithms. Serum metabolomics correlate with hormone levels and offer new opportunities for improved non-invasive biomarker panels.

DATA AVAILABILITY STATEMENT

The data that support the current study are available within the article/**Supplementary Materials**, and from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by sample collection was performed after patient informed consent and Institutional Review Board approval, in accordance with the Declaration of Helsinki, by the ethics committees of Hospital de Santa Maria (Lisbon, Portugal) and Donostia University Hospital (San Sebastian, Spain). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VM contributed to the methodology, validation, formal analysis, investigation, writing—original draft, writing—review and editing, and visualization. MA contributed to the supervision, methodology, writing–review and editing, and visualization. NB, MP, RL, AC, EA, and JA conducted the investigation. FD-R contributed to the formal analysis, writing—review, and editing. AS-L contributed to data curation, writing—review, and editing. RJ-A and EE contributed to data curation. LB, DT, and BF contributed to writing—review and editing. MM contributed to data curation. CA, MK, and FL contributed to the investigation and writing—review and editing. HC-P contributed to the resources, data curation, and writing—review and editing. JB contributed to the resources, data curation, supervision, funding acquisition, writing—review, and editing. RC contributed to writing—review and editing. AN contributed to the conceptualization, resources, writing—review, and editing. CR contributed to the supervision, conceptualization, funding acquisition, resources, writing—review, and editing. All the authors read and approved the final 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.2021.683250/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Is Fatty Liver Associated With Depression? A Meta-Analysis and Systematic Review on the Prevalence, Risk Factors, and Outcomes of Depression and Non-alcoholic Fatty Liver Disease

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Background and Aims: Both non-alcoholic fatty liver disease (NAFLD) and depression have a high global prevalence which is projected to increase further. While studies exploring the association have been done, there are conflicting data. This study aims to assess the prevalence and association between depression and NAFLD.

Methods: Medline and Embase were searched from inception to March 3, 2020. Meta-analysis of proportions using the generalized linear mix model was conducted to analyze the pooled prevalence of depression in NAFLD patients. Risk factors for depression in NAFLD patients were evaluated in conventional pairwise meta-analysis.

Results: Ten studies involving 2,041,752 NAFLD patients were included. Pooled prevalence of depression was 18.21% (CI: 11.12–28.38%) in patients with NAFLD and 40.68% (CI: 25.11–58.37%) in patients with non-alcoholic steatohepatitis (NASH). NAFLD resulted in significantly higher risk of development of depression (OR: 1.29, CI: 1.02–1.64, $p = 0.03$). NASH patients had a significantly higher risk of depression compared with NAFLD patients (RR: 2.83, CI: 2.41–3.32, $p < 0.001$). Diabetes, body mass index (BMI), female sex, smoking, and history of pulmonary disease were significant risk factors for depression in NAFLD patients.

Conclusion: This study demonstrated a high prevalence of depression in NAFLD patients and a significant association between both conditions. Furthermore, patients with NASH had a significantly higher risk of depression compared with those with NAFLD. Diabetes, BMI, history of lung disease or smoking, and female gender were significant risk factors. Further studies investigating the pathophysiological mechanism underlying depression and NAFLD are needed.

Keywords: mood disorder, metabolic disease, fatty liver, evidence-based practice, mental health

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease, with an estimated global prevalence of 25%. This is further projected to increase in conjunction with the rising rates of metabolic syndrome and obesity (1–4). However, the influence of depression on NAFLD has yet to be well-described. The Global Burden of Disease study from 1990 to 2017 reported a 49.86% increase in the incidence of depression (5), with at least one in five people experiencing depression in their lifetime (6–8). Depression is often comorbid of many chronic diseases and incrementally worsens health outcomes (9, 10). Multiple community- and population-based studies have reported that patients with depression have a 2-fold increased risk of developing metabolic syndrome (11–13). Depression is also highly prevalent in the diabetic population, affecting more than one-quarter of both type 1 and type 2 diabetics (14). A meta-analysis of longitudinal studies also confirmed the reciprocal relationship between obesity and depression (15) and increased cardiac mortality in patients with this comorbidity (16).

While studies have been conducted to describe the relationship between depression and NAFLD, previous literature has reported conflicting results, varying from a strong association (17, 18) to no association (19, 20). A study involving a database of 567 patients with biopsy-proven NAFLD estimated that 67.5% of patients had depressive symptoms and showed that they were associated with histological severity of NAFLD (21). It is unclear if depression affects NAFLD due to underlying risk factors or if depression is independently associated with NAFLD (22). Thus, this meta-analysis and systematic review aims to assess the prevalence, associations, risk factors, and outcomes between depression and NAFLD.

METHODS

Search Strategy

This review was synthesized with reference to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRSIMA) guidelines (23). A search was conducted on Medline and Embase databases to identify relevant articles from inception up to March 3, 2020. Keywords and MeSH terms synonymous to "NAFLD" and "depression" were applied in the search strategy to identify relevant articles. The full search used was as follows: *(depress*.tw. or exp Depression/or MDD.tw.) AND (NAFLD or NASH or ((liver or hepatic) AND (fatty or steatosis or steatoses))).tw. or (exp Fatty Liver/or steatohepat*tw.).* In addition, a sieve was conducted on the references of included articles. Abstracts were imported into EndNote X9 for removal of duplicates and for the initial sieve.

Abbreviations: BMI, body mass index; CES-D, Korean Center for Epidemiological Studies-Depression Scale; COPD, chronic obstructive pulmonary disease; DSM-IV-TR, Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision; HADS, Hospital Anxiety and Depression Scale; ICD-9 and ICD-10, International Classification of Disease, Ninth Revision and Tenth Revision; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; MDD, major depressive disorder; PHQ-9, Patient Health Questionnaire-9.

Study Selection and Data Extraction

Two authors (XJL and LLKE) were involved in the screening of abstracts to check the eligibility for inclusion, with disputes being resolved through consensus from a third independent author. Retrospective and prospective cohort studies, case–control, and cross-sectional studies were considered for inclusion, while editorials, systematic reviews, meta-analyses, and commentaries were excluded. Additionally, only English language articles were considered for inclusion. Studies were included according to the following criteria: i) studies regarding the prevalence, risk factors, and outcomes of depression and ii) studies where patients had a diagnosis of NAFLD made *via* liver biopsy, imaging techniques (radiologic testing and abdominal ultrasound), or International Classification of Diseases. Studies relating to the diagnosis of non-alcoholic steatohepatitis (NASH) by self-reported physician diagnosis were also included. Since NASH is a subgroup of NAFLD, a definitive diagnosis of NASH would equate to the presence of NAFLD. As with a previous article, the diagnosis of depression was subclassified into self-reported, self-rated, and clinician-rated (24). Self-reported diagnosis includes identification of depression through self-reporting of medical history, while self-rated diagnosis of depression comprises of patient responses from the Patient Health Questionnaire-9 (PHQ-9), Hospital Anxiety and Depression Scale (HADS), Korean Center for Epidemiological Studies-Depression Scale (CES-D), and Beck's Depression Inventory scale. Clinician-rated diagnosis comprises of depression diagnosed by a psychiatrist according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR) and International Classification of Disease, Ninth Revision (ICD-9) and Tenth Revision (ICD-10) codes. Pediatrics studies were excluded from the analysis.

Relevant data from each article were extracted by a pair of independent authors onto a structured proforma. Baseline demographics, including but not limited to author, year, sample size, country, mean age, percentage of diabetes, and prevalence of depression, were extracted. The main outcomes of interest were the prevalence of depression in NAFLD and NASH patients and risk factors including gender, diabetes mellitus, body mass index (BMI), hypertension, hyperlipidemia, history of smoking, history of lung disease, history of cancer, history of heart diseases, history of neurologic diseases, and race.

Statistical Analysis and Quality Assessment

All analysis was done in R studio (Version 1.3.1093) and RevMan 5.4. Statistical significance was considered for outcomes with a p-value < 0.05. A single-arm analysis of binary outcomes was pooled in the form of proportions using the generalized linear mixed model (GLMM) with Clopper–Pearson intervals to stabilize the variance (25, 26). Simulation studies have found that the GLLM model provides the most accurate estimate in single-arm meta-analysis (25). A sensitivity analysis was done to include only studies from biopsy-proven NAFLD. Next, a subgroup analysis was conducted to account for the differences in the rate of depression between clinician-diagnosed, self-reported,

and self-rated diagnosis. Based on the pooled proportions of single-arm studies, the respective relative risks (RR) of depression in NASH (p_1) vs. NAFLD (p_2) patients was calculated as the ratio of the pooled proportions $\frac{p_1}{p_2}$ of patients with depression in each subgroup (27). The lower (*LCL*) and upper (*UCL*) bounds for the 95% confidence intervals were estimated using the Katz-logarithmic method, wherein n_1 and n_2 represent the respective number of patients receiving transplanted allografts fixated using each of the three methods. Additionally, the *p*-value was calculated after a natural log transformation of the relative risk *z*-score (28).

$$LCL = RRe^{\left(-1.96 \times \sqrt{\frac{1-p_1}{n_1 p_1} + \frac{1-p_2}{n_2 p_2}}\right)}$$

$$UCL = RRe^{\left(1.96 \times \sqrt{\frac{1-p_1}{n_1 p_1} + \frac{1-p_2}{n_2 p_2}}\right)}$$

$$p - value = e^{\left(-0.717 \times \left|\frac{\ln RR}{\frac{\ln UCL - \ln LCL}{2 \times 1.96}}\right| - 0.416 \times \left(\frac{\ln RR}{\frac{\ln UCL - \ln LCL}{2 \times 1.96}}\right)^2\right)}$$

In the analysis of risk factors, a conventional pairwise analysis was done using odds ratios (OR) and mean difference (MD) with the Mantel–Haenszel and inverse variance, respectively (29, 30). For outcomes with insufficient articles for a meta-analysis, a systematic synthesis of literature was used to represent available data. Statistical heterogeneity was assessed *via* I^2 and Cochran *Q* test values, where an I^2 value of 0 to 40% indicates low heterogeneity, while values of 30–60, 50–90, and 75–100% were classified as moderate, substantial, and considerable heterogeneity, respectively (29, 31). A Cochran *Q* test with *p*-value of ≤ 0.10 was considered significant for heterogeneity. A random effects model was used in all analysis regardless of heterogeneity as recent evidence suggests that it provides more robust outcome measures compared with the alternative fixed effects models. Publication bias was not assessed with the lack of a suitable tool in single-arm meta-analysis to assess publication bias and small quantity of included studies (32, 33). Quality assessment of the included articles was done with the Joanna Briggs Institute (JBI) Critical Appraisal Tool (34). The JBI assessment rates the risk of bias of cohort studies on the premises of appropriateness of sample frame, sampling method, adequacy of sample size, data analysis, methods for identification and measurement of relevant conditions, statistical analysis, and response rate adequacy and is the most widely used tool in prevalence meta-analysis.

RESULTS

Summary of Included Articles

In the initial search strategy, 1,766 references were identified, of which 1,315 references were screened after the removal of duplicates. After initial screening, a full-text review was done for 50 articles, and finally, 10 articles involving 2,041,752 NAFLD patients were included in the analysis (**Figure 1**). Of the included studies, one is a prospective study (35), while the remainder are retrospective studies (17, 21, 22, 36–41). Depression was identified through clinician-rated scales [National Institute of Mental Health Diagnostic Interview Schedule (DIS), Version III (36); DSM-IV-TR (35) and International Classification of Disease, Ninth Revision (ICD-9) and Tenth Revision (ICD-10) (41, 42)], self-rated scales [HADS (21), CES-D (38), Beck Depression Inventory scale (22)], and self-reported physician diagnosis (17, 37, 40). NAFLD was diagnosed through liver biopsy in four studies (17, 21, 35, 40) and imaging techniques in four studies (17, 22, 38, 40) and identified through ICD-9 and ICD-10 codes in two studies (41, 42). NASH was diagnosed through liver biopsy in one study (36) and identified through self-reported physician diagnosis in another study (37). A summary of the included articles and quality assessment can be found in **Supplementary Tables 1**, **2**, respectively.

Development of Depression in NAFLD

Five studies assessed the association of depression with NAFLD (21, 22, 36, 38, 42). In the pooled analysis of the four retrospective studies involving 38,407 patients, NAFLD resulted in a significant increase in the risk of depression (OR: 1.29, 95% CI: 1.02–1.64, $p = 0.03$, **Figure 2**). A retrospective follow-up study by Labenz et al. assessed the incidence of depression in 19,871 NAFLD patients over 10 years. The 10-year incidence of depression was 21.2% in patients with NAFLD compared with 18.2% of individuals without NAFLD. The hazard ratio was 1.21 (95% CI: 1.14–1.26, $p < 0.001$) risk increase in development depression in NALFD patients after adjusting for confounders including diabetes mellitus, cardiovascular diseases, asthma, chronic obstructive pulmonary disease (COPD), and cancer (42).

Prevalence of Depression in NAFLD

The overall pooled prevalence of depression in patients with NAFLD was 18.21% (CI: 11.12–28.38%, **Figure 3**) in 2,041,752 individuals. The pooled prevalence of depression in biopsy-proven NAFLD was 22.68% (CI: 8.93–46.75%).

A subgroup analysis was conducted to compare between NASH and NAFLD. The prevalence of depression in NASH patients is 40.68% (CI: 25.11–58.37%), significantly higher than the pooled prevalence of depression in NAFLD patients at 14.39% (CI: 8.89–22.45%). Compared with patients with NAFLD, patients with NASH had a significantly higher risk of depression (RR: 2.83, CI: 2.41–3.32, $p < 0.001$).

To account for differences in the diagnosis of depression as a potential source for heterogeneity, estimates were stratified by diagnostic criteria of depression. Prevalence of depression in NAFLD patients in four studies where depression was identified through clinician-rated scales was 20.26% (CI: 9.38–38.42%) (35, 36, 41, 42), which was lower than self-reported diagnosis used in three studies where prevalence was 32.43% (CI: 27.03–38.33%) (17, 37, 40) and higher than self-rated depression reported in three studies where prevalence was 8.19% (CI: 4.66–14.01%) (21, 22, 38).

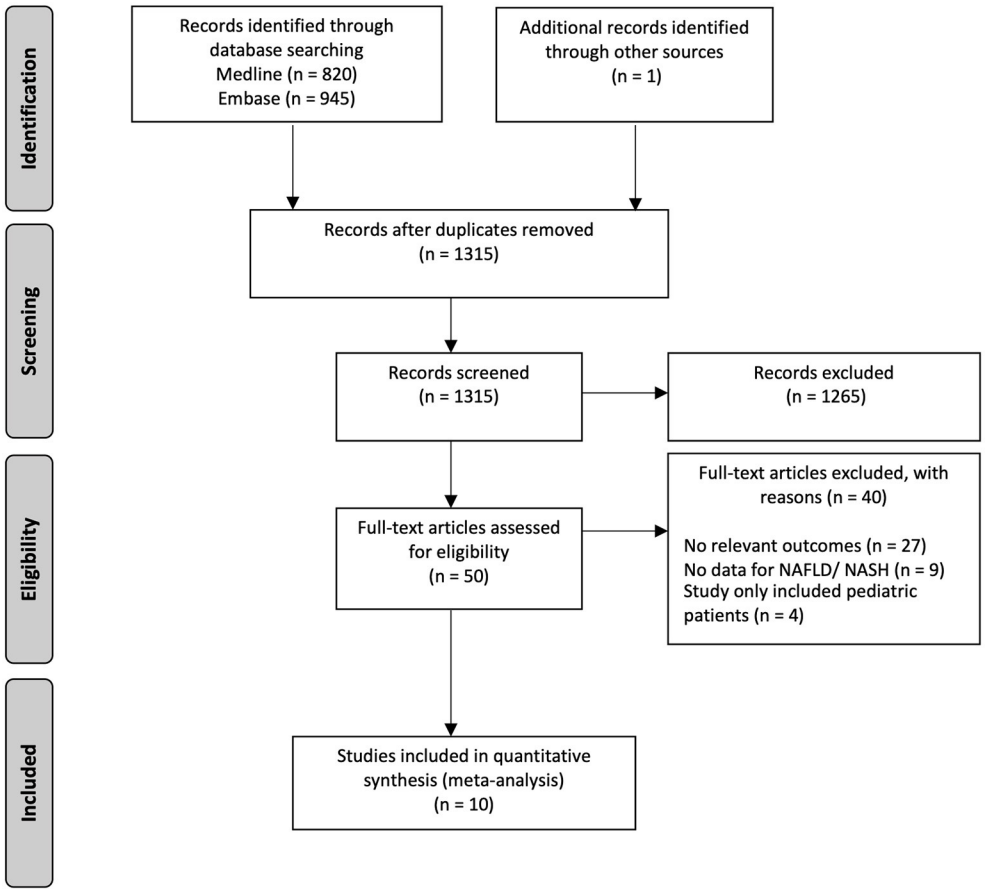

FIGURE 1 | PRISMA flowchart of the included studies.

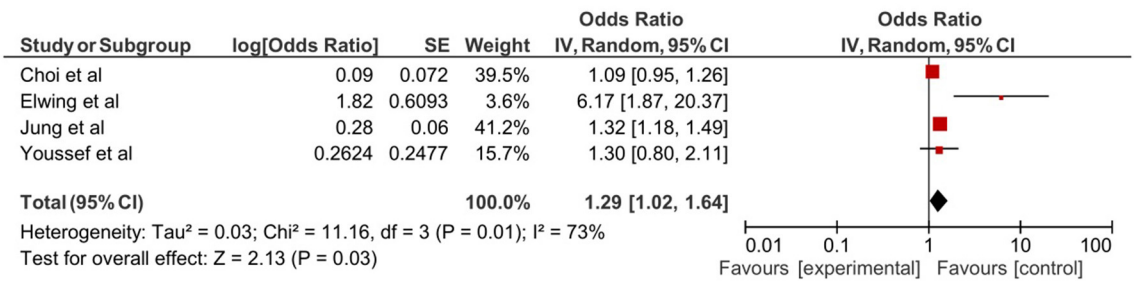

FIGURE 2 | Forest plot of depression in non-alcoholic fatty liver disease (NAFLD).

Factors Associated With Depression in NAFLD

When comparisons were made between NAFLD patients with depression and NAFLD patients without depression, diabetes (OR: 1.71, CI: 1.15–2.55, $p = 0.007$), BMI (MD: 1.89, CI: 0.97–2.80, $p < 0.0001$), and female sex (OR: 0.57, CI: 0.35–0.93, $p = 0.02$) were significant risk factors associated with depression in NAFLD patients. However, hypertension (OR: 1.54, CI: 1.00–2.37, $p = 0.05$) and hyperlipidemia (OR: 1.18, CI: 0.61–2.28, $p = 0.623$) were not significant risk factors for depression in NAFLD patients.

In addition, Weinstein et al. (17) reported history of smoking and history of lung disease as statistically significant risk factors independently associated with depressions in NAFLD patients (OR: 4.132, CI: 1.224–13.95, $p = 0.0096$ and OR: 4.621, CI: 1.346–15.92, $p = 0.0087$, respectively). A previous history of cancer (OR: 1.370, CI: 0.394–4.765, $p = 0.621$), heart diseases (OR: 2.750, CI: 0.377–20.07, $p = 0.319$), or neurologic disease (OR:

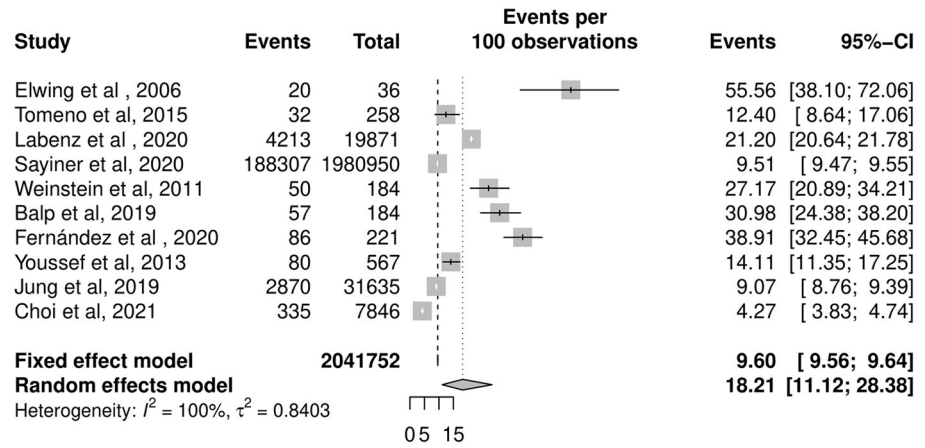

FIGURE 3 | Pooled prevalence of depression in NAFLD patients.

2.787, CI: 0.544–14.29, $p = 0.219$) had no significant association with depression. African American descent was not associated with the development of depression (OR: 0.198, CI: 0.024–1.629, $p = 0.147$).

Outcomes of NAFLD With Depression

Two studies, Tomeno et al. and Sayiner et al. reported on the outcomes of NAFLD patients comorbid with depression. Tomeno et al. compared the clinical response of 32 NAFLD patients with major depressive disorder (MDD) and 226 NAFLD patients without MDD after 48 weeks of standard care involving basic education, lifestyle change counseling, and medication control. Parameters used in the calculation of hepatic steatosis index and fatty liver index such as body weight, serum AST, ALT, and GGT levels were significantly improved in NAFLD patients without MDD after 48 weeks of standard care. In contrast, there were no statistically significant improvements in these parameters for NAFLD patients with MDD (35). Sayiner et al. analyzed depression as an independent predictor of overall 1-year mortality in NAFLD patients in both inpatient and outpatient settings. The odds for overall 1-year mortality of NAFLD patients with depression was statistically significant in both inpatient (OR: 1.07, CI: 1.05–1.09) and outpatient settings (OR: 1.21, CI: 1.18–1.25) (41).

DISCUSSION

The projected rise in the incidence of NAFLD mirrors the obesity epidemic, which at present affects at least one-third of the world (43). This systematic review and meta-analysis reports 15.76% prevalence of depression in NAFLD patients. The risk of development of depression was significantly increased in patients with NAFLD (OR: 1.29, 95% CI: 1.02–1.64, $p = 0.03$). A 10-year follow-up by Labenz et al. also showed a significant association between depression and NAFLD (HR: 1.21, 95% CI: 1.14–1.26, $p < 0.001$). Female sex, diabetes, BMI, history of smoking, and history of lung disease were associated with the development of depression. The relative risk of depression between NASH and NAFLD patients was RR: 2.83 (CI: 2.41–3.32, $p < 0.001$, **Figure 4**). In truth, the actual prevalence of clinically diagnosed depression is likely to be higher than that reported, especially among Asians where traditional screening methods for depression have been reported to have a poorer detection rate (44).

The incidence of depression is likely to be higher in patients with NAFLD as compared with those without, as shown by Labenz et al. in their large population-based study (42). Importantly, the authors controlled for variables including diabetes and obesity, demonstrating increased incidence of depression in patients with NAFLD independent of these comorbidities. While the studies included in our manuscript are unable to show causality, there is emerging evidence, which may shed light on the associations between depression and NAFLD. Systemic inflammation plays a putative role in the pathogenesis of NAFLD and depression, and the progression of both diseases is seen in states of increased oxidative stress (45). Increased levels of proinflammatory cytokines such as interleukin-6, interleukin-1 beta, and tumor necrosis factor alpha may contribute to systemic inflammation leading to depression in NAFLD patients (42, 46, 47). Another plausible association between NAFLD and depression would be the role of serotonin in the pathogenesis of depression as there is increased expression of serotonin catalyzing enzymes in patients with NASH (22).

In our study, diabetes was a significant risk factor for the development of depression (OR: 1.71, CI: 1.15–2.55, $p = 0.007$). Egede et al. reported diabetic patients to be almost twice as likely to be diagnosed with depression compared with the general population (48). BMI was identified as a significant risk factor for the development of depression (MD: 1.89, CI: 0.97–2.80, $p < 0.0001$). There are also several studies reporting an increased prevalence of depression with higher BMI (49, 50). Luppino et al. in their meta-analysis of obesity and depression showed that obesity at baseline had increased the risk of onset of depression at follow-up, with OR 1.55 (95% CI 1.22–1.98, $p < 0.001$) (15).

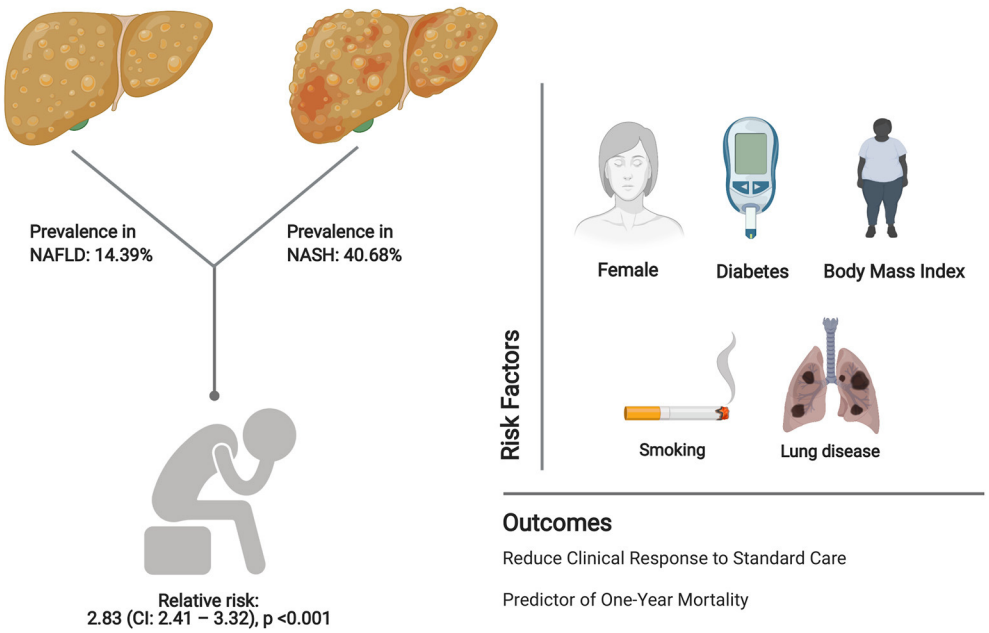

FIGURE 4 | Summary of prevalence, risk factors, and clinical outcomes of depression in NAFLD patients. Image created with Biorender.com.

The risk factors of depression in NAFLD patients were associated with increased systemic inflammation as observed in smokers and patients with chronic lung disease. Female gender was also associated with increased odds of depression in NAFLD patients. It is known that females are at higher risk of depression than males, and a study in 2015 by Moieni suggested that during an induced state of inflammation, women encountered greater increases in depression (51). Smoking has also been identified as a risk factor for depression, possibly due to the role of nicotine receptors as neuromodulators of various neurotransmitter pathways to the brain, including those involved in depression (52). This in turn may have contributed to history of lung disease being a risk factor for depression as corroborated by Goodwin et al. (53).

Interestingly, the prevalence of depression is markedly higher in patients with NASH as compared with patients with simple hepatic steatosis. NASH with or without fibrosis is a more severe disease compared with simple steatosis. It occurs at a rate of about 25% in 3 years in patients in the simple steatosis stage (54). This would not come as a surprise given that an increase in circulating inflammatory cytokines is seen in steatohepatitis compared with simple steatosis, and patients with depression similarly see an increase in peripheral and central inflammation. In fact, the bidirectional relationship between depression and obesity, which is also closely related to NAFLD, has already been elucidated in animal studies (55). Therefore, it is likely that dietary initiatives protective toward NAFLD progression may also improve mental health in patients with depression (56). Evidently, depression and NAFLD share multiple upstream hits in the pathophysiology of the disease; thus, it is no wonder that they are gradiently associated.

In a study by Tomeno et al. the presence of depression was associated with a decreased effect of lifestyle intervention on weight loss, resulting in an increased challenge for clinical management (35). Although there are multiple pharmacotherapies with promising results in phase 3 trials for NASH, at present, weight loss and lifestyle change are the cornerstones of NAFLD treatment. Furthermore, overall weight loss has been shown to be beneficial for associated comorbidities such as diabetes and hypertension (57, 58). Yet, many antidepressant classes result in weight gain, and thus, the possibility of successful treatment outcome may be grim in the coexistence of depression and NAFLD (59).

It may be tempting to consider bariatric surgery for this subgroup of patients with both NAFLD and depression. After all, bariatric surgery has shown promising results in improvement in NASH as seen in several studies (60–62). Furthermore, weight loss following bariatric surgery may be associated with improvements in mood, at least initially. However, it is not without adverse outcomes. For example, a 10-year cohort study in Sweden reported an increase in suicides among obese patients following surgery compared with the general population (OR: 2.85, 95% CI: 2.40–3.39) in addition to alcohol and substance abuse (63). Several reasons for increased suicide rates post-bariatric surgery have been suggested, such as genetic susceptibility and changes in gut peptide release. It has also been theorized that addiction transfer could explain this phenomenon. In this theory, binge eating is hypothesized to

serve as a coping mechanism to ameliorate negative emotional states (64). Consequently, in the post-bariatric group, this behavior is substituted by alcohol consumption due to the patient's inability to regulate emotions through overeating. However, this model has neither been adequately developed nor empirically validated and should be explored in future research (65).

Depression in NAFLD is associated with poorer outcomes such as decreased response to medical treatment and is even an independent predictor of all-cause mortality at 1 year. It is unclear if treatment of depression (be it pharmacological or behavioral) would positively influence NAFLD progression. Nevertheless, physicians should be cognizant of the fact that the prevalence and incidence of depression in NAFLD patients is higher and may consider adopting a low threshold in the use of screening scores for mood disorders in these patients as clinically required.

Strengths and Limitations

To our best knowledge, this is the first meta-analysis of the prevalence, associative risk factors, and outcomes of depression in NAFLD patients. The limitation of this study is the heterogeneity in the included studies. However, we circumvented the issue by reclassification of depression diagnosis into clinician-rated, self-reported, and self-rated scales (66). Of note, the prevalence of depression was higher in self-reported and self-rated studies. Moreover, larger sample sizes are often associated with an increased I^2 in simulation studies (67, 68). Thus, large I^2 value (>90%) in prevalence meta-analysis may be attributed to the larger sample sizes involved (69, 70). Secondly, while the prevalence of NAFLD in Asia is similar to the Western population, only three out of the 10 studies were conducted in Asia. Thus, this meta-analysis may not adequately represent Asian NAFLD patients. Lastly, while our study reports factors associated with depression in NAFLD patients, more confirmatory studies are needed to show causation through further understanding of the pathogenesis and bidirectional relationship between the two conditions.

CONCLUSION

In summary, this meta-analysis and systematic review assesses the association between depression and NAFLD. The analysis demonstrates the high prevalence of depression in NAFLD in spite of heterogeneity due to differences in diagnostic criteria of depression. Furthermore, patients with NASH were found to have a significantly higher risk of depression compared with those with NAFLD. Diabetes, BMI, history of smoking, history of lung disease, and being female were also identified as significant risk factors. NAFLD comorbid with depression has serious complications as evident in reduced clinical response after standard care and increased all-cause 1-year mortality. This paper, thus, highlights the significant clinical implications of NAFLD and depression as public health concerns. Further studies to validate the findings and explore potential pathophysiological mechanism underlying the association between depression and NAFLD are needed.

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.

AUTHOR CONTRIBUTIONS

JX, LL, and CN contributed to the acquisition of data, analysis and interpretation of data, and drafting of the article. DT, WL, CH, ET, AS, and MM aided in revising the article critically for important intellectual content. All authors read and gave final approval of the version to be submitted.

SUPPLEMENTARY MATERIAL

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REVIEW
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Complement Component C3: A Novel Biomarker Participating in the Pathogenesis of Non-alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disorder worldwide. The pathological spectrum of NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) that induces progressive liver cirrhosis and eventually hepatocellular carcinoma (HCC). However, the molecular mechanisms driving the transformation of NASH are obscure. There is a compelling need for understanding the pathogenic mechanisms of NASH, and thereby providing new insight into mechanism-based therapy. Currently, several studies reported that complement system, an innate immune system, played an important role in the pathogenesis of NAFLD, which was also proved by our recent study. Complement component 3 (C3), a protein of the innate immune system, plays a hub role in the complement system. Herein, we present a review on the role and molecular mechanism of C3 in NASH as well as its implication in NASH diagnosis and treatment.

Keywords: non-alcoholic fatty liver disease, complement C3, complement C3 receptor, pathogenic mechanism, therapy

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become a very common liver disease worldwide. The disease spectrum includes non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis(NASH), nutritional fibrosis and hepatocellular carcinoma Cancer (HCC). Epidemiological investigations show that the prevalence of NAFLD is 25–30% in western countries (1). With the change of diet structure and lifestyle, the prevalence of NAFLD is 17–46% in China (2). Notably, the prevalence of NAFLD in young children is currently increasing year by year, and the high prevalence rate is alarming (3). Among NAFLD patients, the prevalence of NASH is about 20% (4). However, the diagnosis of NASH relies on liver biopsy and the non-invasive diagnostic methods are limited. To date, there's no Food and Drug Administration (FDA)-approved drug for NAFLD and NASH treatment. Therefore, there is an unmet clinical need for the diagnosis, prevention and treatment of NAFLD and NASH. In recent years, innate immunity is thought to play an important role in the development of NAFLD. Therefore, we summarized previous studies and provided a holistic framework concerning the relationship between complement and NAFLD.

THE OVERVIEW OF COMPLEMENTS SYSTEM

In the early stage of NAFLD, bacterial endotoxin, free fatty acid (FFA), cholesterol and many other substances in the body can activate the complement system through danger associated molecular patterns (DAMPs) or pathogen associated molecular pattern (PAMPs) (5, 6). Complement system, which is considered as an important innate immunity (7–10), has been confirmed to be cascade-activated through the following three pathways: classical, lectin and alternative, all of which converge in the formation of fraction C3. Briefly, in the activated complement signaling pathway, complement component C3 is cleaved into C3a and C3b through C3 converting enzyme, in which C3b binds with C3 converting enzyme complex to form C4bC2aC3b complex in classical pathway and lectin pathway and C3bBbC3b complex in alternative pathway. Both complexes are converting enzymes of complement molecule C5, and then further activate the downstream molecules of Complement system to form membrane attack complex (MAC/C5b-9). Furthermore, the classical activation pathway mainly involves the binding of antigen with immunoglobulin (IgM or IgG) or C-reactive protein. In the lectin pathway, Complement activation is triggered by the interaction complex carbohydrate residues with the surface of pathogens to circulating mannose binding lectins (MBL) or ficolins. The alternative pathway is activated by the direct combination of hydrolyzed C3b and bacterial membrane surface (**Figure 1**).

COMPLEMENT C3 AND NAFLD

Currently, the role of Complement system in NAFLD disorders has been extensively investigated in clinical epidemiological studies (11). Serum complement C3 levels are positively associated with the severity of NAFLD. A Turkish case-control study involving 46 NAFLD patients demonstrated that the level of Complement component C3 was significantly higher in peripheral blood of NAFLD patients than that of healthy control group and chronic hepatitis B control group (12). Moreover, a Dutch cross-sectional study involving 523 middle-aged and elderly patients with NAFLD found that the level of C3a, the active product of complement C3, was closely related to liver fat content (13). Consistently, two large sample epidemiological studies recruited thousands of cases in China showed that the level of serum complement C3 was an independent risk factor for the diagnosis of NAFLD and related to the prevalence and the severity of NAFLD (14, 15). Apart from the serum, the deposition of C3 is also identified in the liver tissue of NAFLD patients accompanied with the deposition of MAC-C9 (16). The deposition of C3 was proved to be mainly located around the hepatocytes with macrovesicular steatosis. Subsequently, clinicopathological examination confirmed that a large number of hepatic parenchymal cells were apoptotic in the liver tissue with complement C3 activation. Complement C3 activation could lead to a large number of neutrophils infiltration and abnormal increase of IL-8 and IL-6 expression in liver tissue, while C9 deposition could lead to increased IL-1β expression in liver cells. Additionally, in other patients with NAFLD, there was a close correlation between serum C3 level and NAFLD. For example, Pan et al. demonstrated that C3 was the only highly predictive factor in diagnosing NAFLD from 648 recruited patients with chronic kidney disease by Logistic regression analysis (17). Ursini et al. recruited 164 patients with rheumatoid arthritis, of which 25% (41/164) were complicated with NAFLD. Further logistic regression analysis also confirmed the high correlation between C3 and NAFLD analysis (18). Most importantly, Himoto et al. found that the increased serum C3 levels are closely related to the abnormal metabolism of the body including obesity, insulin resistance, and/or hepatic steatosis in those patients with chronic hepatitis C, which had nothing to do with chronic HCV infection (19). Collectively, the complement system is closely related to NAFLD. It is speculated that the complement system is largely activated to regulate the immune inflammatory response in the pathogenesis of NAFLD, which directly participates in the occurrence, development and prognosis of NAFLD.

THE PATHOGENIC C3 ACTIVATION IN NAFLD PROGRESSION

NAFLD severity was closely associated with accumulation of activation products of C3 around steatotic hepatocytes. However, the underlying mechanism by which complement C3 in NAFLD remained elusive. Hepatocytes are confirmed to be the predominant origin of complement components including C3 protein. Because C3 is the key molecule in the pathway of complement system activation (7), several hypotheses are proposed that C3 plays an important role in lipid metabolism in the pathogenesis of NAFLD (**Figure 2**): Firstly, C3 is mainly synthesized by hepatocytes and identified to appear in lipoprotein particles such as high density lipoprotein and chylomicron (20, 21). When complement C3 gene was knocked out in mice, serum triglyceride levels increased 58% higher compared with wild-type mice, and the increased lipoprotein profile is mainly low-density lipoprotein and very low-density lipoprotein (21), indicating a potential role of C3 in lipid metabolism regulation (22). Secondly, Complement system directly regulates the oxidative stress in hepatocytes with excessive fat accumulation. It is well-known that multiple-hit hypothesis has been widely accepted in the pathogenesis of NAFLD. The first hit is closely linked with insulin resistance under fat accumulation. With the response of hepatocytes to oxidative stress, a large number of inflammatory cytokines are secreted in the liver, which further cause the second hit in hepatocytes (23). Complement component C3, as a major player in innate immune response, might be activated by the first hit and forming the second hit in NAFLD

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; C3, Complement component 3; NAFL, non-alcoholic fatty liver; FDA, Food and Drug Administration; FFA, free fatty acid; DAMPs, danger associated molecular patterns; PAMPs, pathogen associated molecular patterns; MAC, membrane attack complex; MBL, mannose-binding lectin; IL, interleukin; KCs, Kupffer cells; NLR, neutrophil to lymphocyte ratio; NAS, NAFLD activity score.

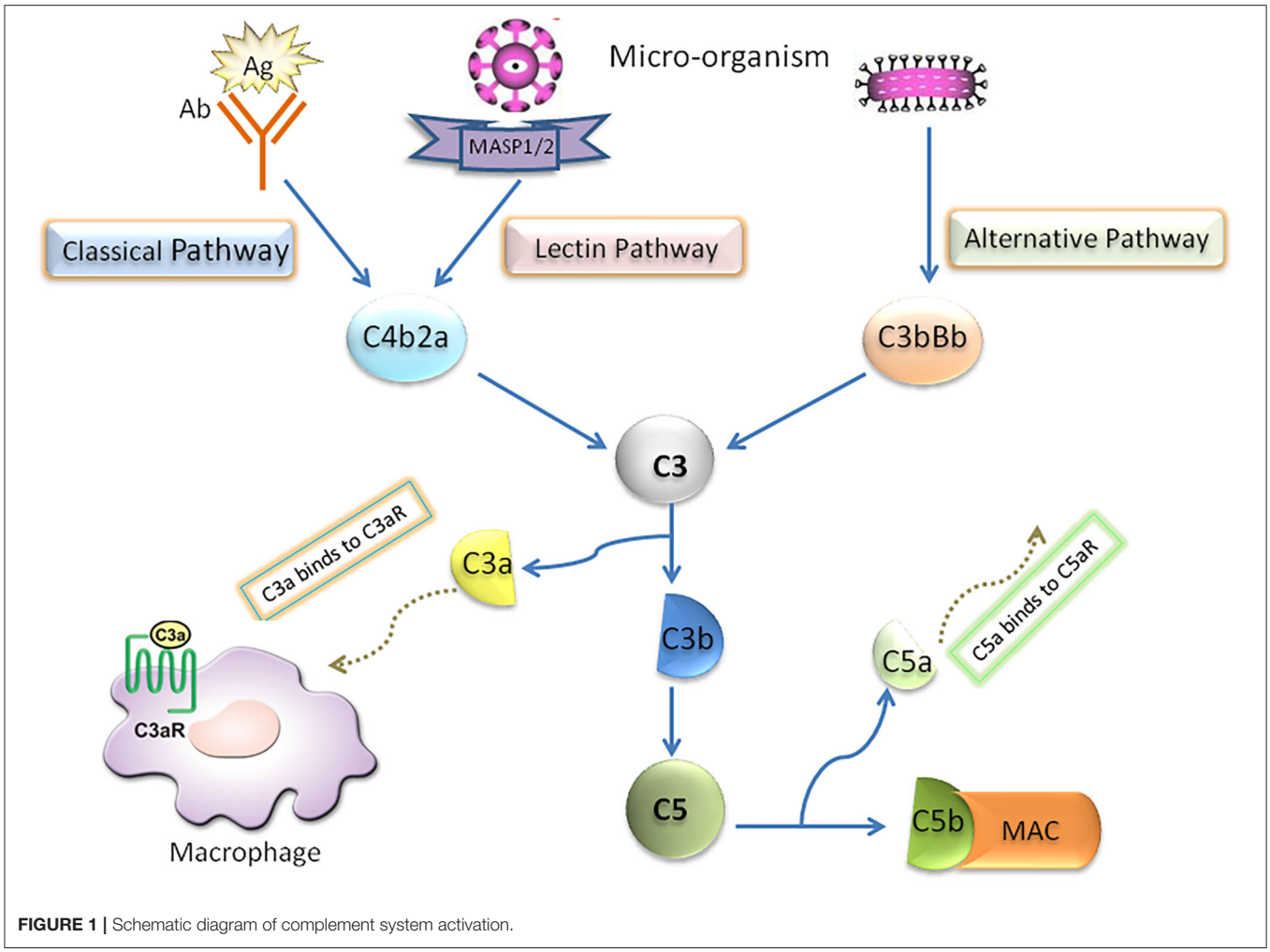

FIGURE 1 | Schematic diagram of complement system activation.

pathogenesis (24). Thirdly, the phenomenon of apoptosis is a typical pathological feature in the liver with NAFL and NASH. Due to the accumulated fat droplets in hepatocytes, a variety of apoptotic cascade pathways are activated including caspases 3 and 7 or cleavage of cytokeratin 18, resulting in a large number of hepatocytes apoptosis. Complement system was found to be quickly activated by the apoptotic hepatocyte debris. Furthermore, the activated Complement system immediately recognized and cleaned the apoptotic pathological liver cells, thus maintaining the homostasis in liver (25). Thus, elevation of serum complement C3 might act as a protective response in NAFLD mediated by apoptosis. Finally, Complement activation is indicated to be involved of novel molecular mechanism in the pathogenesis of NAFLD. Acylation-stimulating protein (ASP), a C3 derivative involved in adipocyte lipid metabolism by stimulating triglyceride synthesis, was reported to be increased in NAFLD patients (26). A vicious cycle has been further confirmed in the pathogenesis of NAFLD. ASP can promote the fat accumulation in liver cells to exacerbates hepatic steatosis. On the other hand, the fatty liver promotes the activation of complement system to increase ASP synthesis (12). In brief, there is a potential balance in the pathogenesis of NAFLD between complement system activation and hepatocyte lipid metabolism signaling, which maintains the stability of liver internal environment. Once the balance is broken, the process of hepatocyte lipid metabolism will enter a vicious circle, which exacerbates irreversibly hepatic steatosis as a consequence.

C3a/C3aR ACTIVATION AND METABOLIC FUNCTION

Recently, increased evidence showed that C3a can change the storage, transportation and utilization of glycolipids in adipocytes and directly affect adipogenesis, glucose uptake and lipolysis in the pathogenesis of NAFLD. Oral administration of selective C3aR antagonist for 8 weeks can significantly improve the typical symptoms of metabolic syndrome in diet-induced obese rats, including weight loss, visceral fat reduction, glucose and insulin intolerance improvement, adipose tissue inflammation relief, blood lipid concentration dropping, etc. The above research results innovatively provide two novel mechanisms of C3a involved in energy metabolism on theoretical and experimental basis, that is, C3a can not only promote

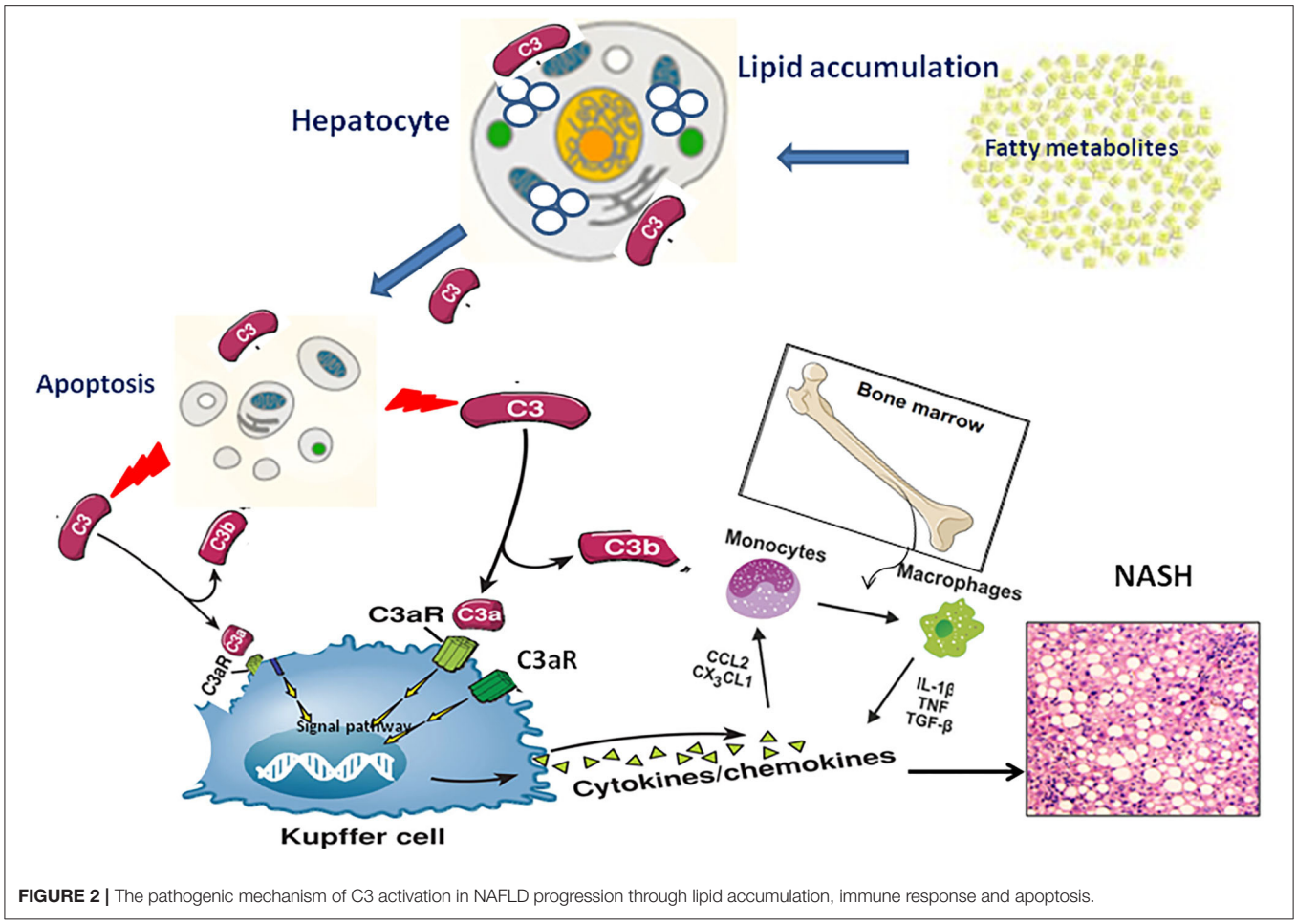

FIGURE 2 | The pathogenic mechanism of C3 activation in NAFLD progression through lipid accumulation, immune response and apoptosis.

the uptake of fatty acids and glucose by adipocytes, but also inhibit fat burn-off by inhibiting cAMP synthesis and lipolysis in adipocytes (27). These studies fully elucidate the important correlation between complement C3a/C3aR signaling pathway and energetic metabolism, and further reveal the molecular mechanism of abnormal immune response aggravating obesity and metabolic dysfunction. More importantly, antagonists targeting C3a/C3aR signaling pathway is considered as a novel strategy for the treatment of metabolic dysfunction, including NAFLD. Consistently, our recent result also found that C3a/C3aR signal pathway was closely related to the development of NASH-fibrosis. In C3aR$^{-/-}$ mice model, we also demonstrated that C3aR depletion significantly reduced the progression of NASH related liver fibrosis (28).Studies have confirmed that C3a is an important derivative produced by C3 cleavage when complement system is activated. C3a has significant biological characteristics of anaphylactic toxin and is an important pro-inflammatory molecule in the body. It can directly trigger mast cells degranulation, inflammatory reaction, chemotaxis effect, granulocyte activation as well increasing vascular permeability, promoting smooth muscle contraction and clearing away the immune complexes, etc (29). In other words, C3a plays an important role in the pathogenesis of a variety of clinical diseases, including organ ischemia-reperfusion injury, sepsis and metabolic inflammation (27, 30–37). Structurally, C3a is composed of 77-aa polypeptide containing three to four helical regions. A series of irregular amino acid residues is also proved in the C-terminal of C3a protein, which is flexible in spatial conformation. There is evidence that these flexible C-terminal residues are necessary to stabilize the conformation on binding C3aR by the upstream a helix (38–40). It has been demonstrated that C3a exerts its biological effect mainly by binding to its receptor C3aR, which belongs to G protein-coupled receptor containing seven trans-membrane regions. C3a/C3aR interaction is generally regulated by G protein-coupled receptor kinase-mediated receptor phosphorylation (41). In the past, it has been considered that C3aR is the only specific receptor of C3a. It shares close homology with C5a specific receptors C5aR1 and C5aR2. However, recent studies have showed that the interaction of C3a/C3aR seems to be more complex than expected. For example, C3aR has been found to be able to couple with heterotrimeric G proteins depending on different cell types (42–44). In human granulocytes, C5a can inhibit the activity of C3aR. Ruan et al. confirmed that C3a can form a complex with CpG oligonucleotides to improve the release of IFN-a in monocytes (45). Neuropeptide TLQP-21 (a cleaved fragment

of VGL propeptide) was found to specifically binds on C3aR and fully activate the biological function of C3aR in mice, which completely comply with the conformational change of ligand/receptor interaction (46, 47). Additionally, recent studies have demonstrated that C3a can bind to the receptor of advanced glycation end products (RAGE) with very high affinity, but this high affinity interaction cannot be explained by a simple ligand upon receptor binding (45, 48).

C3a/C3aR AXIS AND IMMUNE RESPONSE IN NAFLD

Kupffer Cells/Macrophages and C3a/C3aR Axis in NAFLD

Liver is the largest reservoir of macrophages in the body, and macrophages in liver play a key role in the pathogenesis of NAFLD (23, 49, 50). According to different origin, macrophages in liver can be divided into two types, one is Kupffer cells fixed in liver, the other is monocytes/macrophages derived from bone marrow. They mainly play the role of innate immunity such as phagocytosis and secretion of inflammatory cytokines. At the early stage of NAFLD, Kupffer/macrophage cells are the first defense lines against the accumulation of excessive lipid metabolites in the liver. Firstly, steatotic hepatocytes disturb hepatic sinusoidal perfusion because of the "gap occupying" effect and Kupffer cells are subsequently attracted by neutrophils to the sinusoidal gap to participate inflammation. Secondly, free fatty acids (FFA) excessively interact with the FFA specific receptors on Kupffer/macrophage cells surface to regulate inflammatory response. Thirdly, Kupffer/macrophage cells mistakenly attribute the abnormal hepatocytes full of excessive lipid accumulation to the harmful substances and immediately phagocytize and destroy them, which further aggravate hepatocyte damage (51). Under the above conditions, these overactivated Kupffer/macrophage cells secrete a large number of inflammatory cytokines (such as TNFα, IL-6, IL-1β) as well as inflammatory chemokines (such as CCL2, CCL3, CCL5, CXCL16, CX3CL1,). In addition, Kupffer cells also recruit a large number of bone marrow-derived monocytes/macrophages into the liver to expand the inflammatory response and accelerate the liver from simple steatosis to NASH (52). CCL2/CCR2 interaction is proved to be the first signal pathway in Kupffer/macrophage recruiting monocytes from bone marrow (53). Afterwards, some other chemokines signal pathways are also confirmed through Kuppfer cells mechanisms such as CXCL10/CXCR3, CCL5/CCR1, and CCL1/CCR8 (11). In recent years, a number of research groups have got the highly consistent results by various ways to delete macrophages in NAFLD mice model (54–56). Namely, knocking-out monocytes/macrophages can reduce significantly the severity of liver steatosis and inflammation and further delay the process of NAFLD. However, so far, there is no systematic and in-depth study on the specific molecular mechanism of regulating macrophage activation in the whole pathogenesis of NAFLD.

As mentioned above, hepatocytes are the main origin to synthesize C3a molecules, and C3aR is predominantly located in the cell membrane of monocytes/macrophages. In the chronic phase of liver inflammation, C3a/C3aR signaling activity is showed more significant in monocytes/macrophages than that of neutrophils (57, 58). Under this condition, C3a can activate the signal pathway of peripheral blood monocytes with the co-stimulation of TLR-4, and further induce the secretion of various proinflammatory factors such as IL-1 β, TNF-α, IL-6 and PGE2 (59–63). This view is supported by evidence that liver steatosis have modest pathology reductions in C3aR1$^{-/-}$ mice models (57). Therefore, we speculate that the C3a/C3aR interaction may be a novel signaling pathway by activating macrophages to regulate the occurrence and development of NAFLD. In the early stage of NAFLD, a large amount of fat accumulates in hepatocytes, resulting in the broken balance between complement system activation and hepatocyte lipid metabolism. Subsequently, Complement cascades enter into an overactivated state and excessive amount of C3 molecular is cleaved to release C3a. Through the specific interaction of C3a/C3aR, Kupffer/macrophages cells in the liver are directly activated to secrete inflammatory chemokines to recruit monocytes from peripheral blood into the liver, which further expand the inflammatory response in the steatotic liver (24) (**Figure 2**).

Hepatic Stellate Cell and C3a/C3aR Axis in NAFLD

Under normal physiological conditions, component C3 is mainly expressed in hepatic parenchymal cells. However, recent evidence has demonstrated that C3 is also slightly expressed in other type cells such as bone marrow cells, lymphocytes, fibroblasts and endothelial like cells (64). Activated hepatic stellate cells, as the initiating factor of hepatic fibrogenesis, have obvious characteristics of fibroblasts. Up to now, there are relatively few reports about complement C3 directly involved in the activation of hepatic stellate cells (HSCs) about NAFLD. A recent study showed that activated hepatic stellate cells could promote the hepatocarcinogenesis through C3 signaling pathway by inhibiting the proliferation of CD4 + and CD8 + cells, promoting the exhausting anti-tumor T lymphocytes as well as improving the differentiation of bone marrow-derived suppressor cells (MDSC) (65). Consistently, our recent results demonstrated that C3a/C3aR signaling pathway was activated in the mouse model of nutritional fibrosis. The related mechanism is being further explored in C3aR$^{-/-}$ mice (28). However, Component cascade activated C5 molecular is found to be a key factor that contributes to hepatic fibrosisgenesis by enhancing the migration ability of hepatic stellate cells. Furthermore, the C5a/C5aR axis was shown to directly mediate inflammatory, chemotactic and anaphylatoxic properties in innate and adaptive immunity as well as to modulate activation and migration of HSCs (66).

T Cell and C3a/C3aR Axis in NAFLD

It has been confirmed that T cells play an important regulatory role in the pathogenesis of NAFLD. The balance has a directly effect in the pathogenesis of NAFLD between Th1-secreted pro-inflammatory cytokines and Th2-secreted anti-inflammatory

cytokines (67). The number of $CD4^+$ helper T cell 17 (Th17) subsets was significantly higher than that of regulatory T cells (Treg) in the pathogenesis of NAFLD (68–72). With the development from simple steatosis to NASH, the number of Th17 cells in liver and peripheral blood increased continuously in a clinical epidemiological study of 104 human subjects (including 30 patients with NASH, 31 patients with simple steatosis and 43 healthy controls), resulting in a significant increase in Th17/resting T regulatory cell ratio (70). There is no doubt that C3a molecule directly participates in T cells proliferation and differentiation as well as regulates the biological function, but the mechanism is complex (73, 74). It is still controversial whether C3aR is widely expressed in T cells (75). When C3aR gene was knocked-out as a target, the number of T cells was significantly reduced in $C3aR^{-/-}$ mice model (73, 76). Further studies confirmed that increasing the intracellular expression of C3a in T cells can significantly prolong their survival (77, 78). TCR stimulation can significantly up-regulate the expression of C3aR mRNA in T cells (79). Therefore, it is speculated that C3a further regulates T cells proliferation, differentiation and biological functions through a potential autocrine way. Recent studies have shown that C3a can promote T cell proliferation, enhance T cell reaction and prolong inflammatory response by inhibiting Treg production (73, 76). After adoptive transfer of $C3aR^{-/-}$ T cells into wild-type animals, the immunological function of Treg was seriously changed (76). In addition, there is evidence that C3aR activation in antigen-presenting cells (APCs) can inhibit Th2 polarization and further block IL-4 secretion (80). Under the synergistic effect of C5aR1 signaling pathway, C3aR can suppress the production of TGF -β1 by dendritic cells, reduce the stimulation of Treg differentiation, and then eliminate the inhibitory response to Th1 (76).

Neutrophil and C3a/C3aR Axis in NAFLD

Neutrophil infiltration is usually observed in the liver of NAFLD patients, and the severity of infiltration is closely related to disease progression (81, 82). The excessive fat accumulation overloads the normal metabolic capacity of hepatocytes. Subsequently, the abnormal metabolic injury results in neutrophils to be overactivated and recruited to the steatosic liver. After administration the neutrophil-specific antibody 1A8 into mice, hepatic lipid accumulation and inflammation were significantly attenuated in HFD diet induced NAFLD models, which further slowed down the progress of NASH (83). Currently, neutrophil to lymphocyte ratio (NLR) has been clinically used as an effective biomarker to predict the severity of NAFLD (84–86). Accumulating evidence shows that NLR is positively correlated with NAFLD activity score (NAS) and an independent predictor of NAFLD prognosis. The higher the NLR value, the higher the severity of the disease, and the worse the prognosis of NAFLD. In the study of 101 NASH patients, the NLR value was significantly higher than that of controls without NAFLD (mean 2.5 vs. 1.6, $P < 0.001$) (87). In the stage of NASH related fibrosis, the NLR value in advanced fibrosis stage (f3-4) was significantly higher than that of patients with early fibrosis stage (f1-2) (median: 2.9 and 1.8, $P < 0.001$) (87). So far, it has been controversial for C3a/C3aR interaction in neutrophils because the pure neutrophils was isolated difficultly over a long period in the past, which resulted in the doubt that C3a induced neutrophil activation may be contaminated by non-neutrophils (88). At present, C3aR is definitely identified to express with high level on the surface of neutrophils. Curiously, although C3a/C3aR interaction can activate downstream ERK1/2/Akt signaling pathway, C3a alone does not play chemotactic function as well as stimulate neutrophil degranulation. Recent study suggested that the signaling produced by C3a stimulation of neutrophils was found to be dependent under the synergistic effect of C5aR2 (89). Another study demonstrated that C3a directly prevents neutrophils migration from bone marrow to peripheral circulation by antagonizing neutrophils migration factors (such as G-CSF) (72). Altogether, the specific mechanism of C3a/C3aR in neutrophils in the pathogenesis of NAFLD needs to further explore in the coming future.

C3a AS A BIOMARKER FOR NON-INVASIVE NASH DIAGNOSIS

Although NASH is increasingly prevalent, it's hard to be diagnosed. Yet liver biopsy is recognized as the gold standard, but it is limited by its sampling bias, poor acceptability, and severe complications. Therefore, non-invasive methods are urgently needed to avoid biopsy for diagnosing NAFLD. Currently, some serum biomarkers have been widely accepted for the diagnosis of NASH such as the circulating serum levels of CK-18, the single nucleotide polymorphisms located in PNPLA3 as well as the non-coding RNAs, etc. (90). Especially, the most recent efforts concentrating on "omics" approaches (lipidomics, proteomics, and metabolomics) using high-throughput technologies have shown promising results to identify novel biomarkers of NAFLD, NASH, and advanced fibrosis (91). However, those diagnostic accuracy need to be further improved by combining other different approaches.

Complement system activation has been demonstrated in liver biopsies from patients with NAFLD compared to healthy controls. It has been confirmed that complement C3 levels increased in patients with NASH, but not in those with viral liver disease. More recently, circulating C3 levels have been demonstrated to predict the presence of NAFLD in a large cohort from general population independently of the most plausible confounders such as the presence of metabolic syndrome and obesity. In addition, some similar evidence was also confirmed in the other disease combined with NAFLD. Ursini et al. provide an important evidence for the potential role of complement C3 as a surrogate biomarker of NAFLD in a large cohort of Rheumatoid arthritis (RA) patients at the best cut-off value of 1.23 g/l for complement C3 with a sensitivity of 76% and a specificity of 64% (18). Pan et al. demonstrated the predictive role of complement C3 as a candidate biomarker for diagnosing NAFLD in chronic kidney disease (CKD) patients at the best cut-off value of 993.5 mg/L for complement C3 with a sensitivity of 63.9% and a specificity of 70.1% (17). Therefore, serum C3 may be fully used as a non-invasive diagnostic marker in the coming clinical diagnosis of NASH.

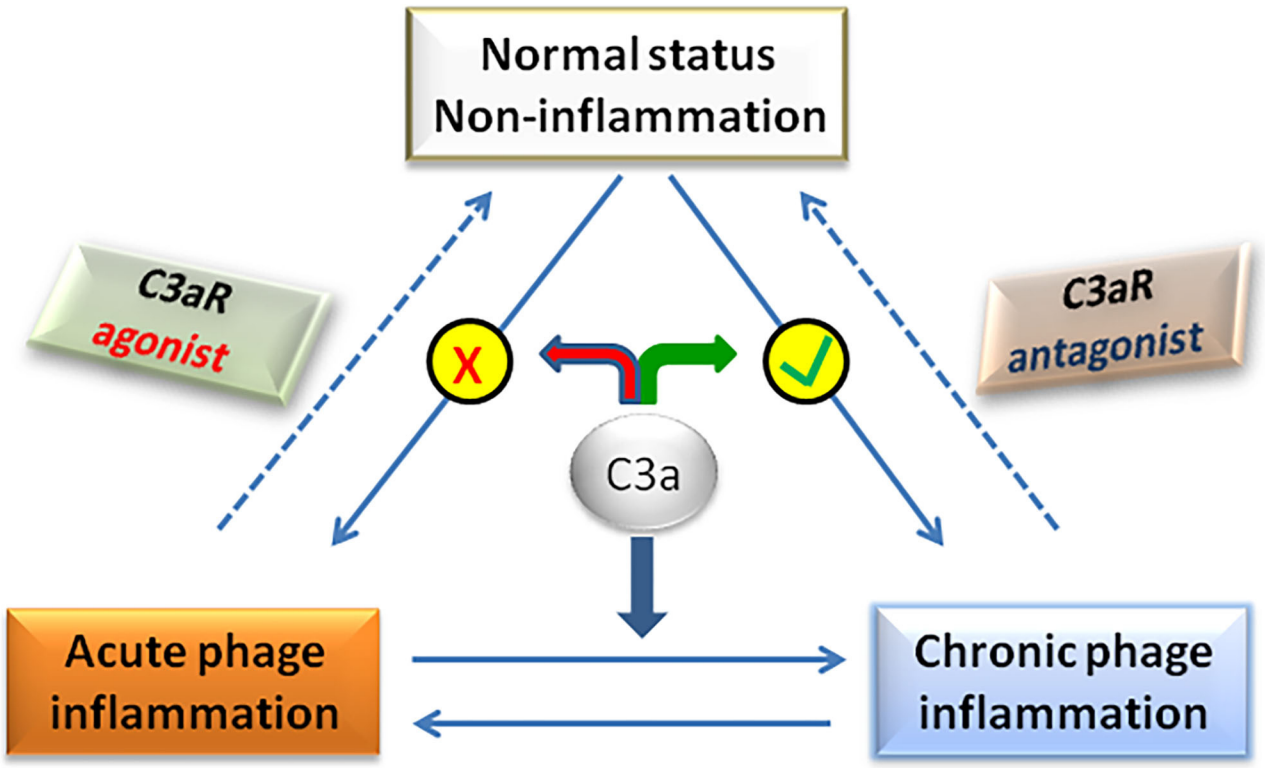

FIGURE 3 | The balance of C3a actions determines the disease phenotype.

THE POTENTIAL BENEFIT FROM ANTAGONIZING C3aR IN NASH THERAPY

With the increasing incidence of global NAFLD, more and more strategies are explored to prevent effectively NASH in medical treatment nowadays. It is of great significance to find the specific signal pathway leading to the occurrence of NAFLD. Intriguingly, C3aR was indicated to be a remarkable gene closely related to obesity and potential insulin resistance in the mice model intercrossed among different strains by integrated genomic analysis (92). On this base, it is speculated to be a very promising treatment through targeting C3a/C3aR in the pathogenesis of NAFLD. So far, researchers have designed a variety of small molecule antagonists for C3a/C3aR signaling pathway and verified their pharmacological effects. For example, a series of diaminoisoindoline compounds can play a significant role as C3a antagonist at the micromolar level (93). A new oral selective antagonist of C3a receptor, discovered by heterocyclic hinge control conformation, can significantly block the recruitment and activation of macrophages and neutrophils and then play the role of inhibiting the expression of inflammatory mediators (94). Also known is FLTChaAR (IC50 240 nM, Ca2+, macrophages), a significant hexapeptide C3a antagonist, which provide an important reference for the coming antagonist design (95). As C3aR antagonist obtained through high-throughput screening, Sb290157 is thought as the most promising drug for the treatment of metabolic syndrome including NAFLD in the clinical future (88, 96). In the obese rat models fed by high carbohydrate and saturated fat diet, SB 290157 can attenuate the inflammatory response by mainly controlling macrophages into adipose or liver tissue (97), which significantly reduce the obesity and body weight by effectively improving liver metabolism (27). Subsequently, it was further confirmed that the IC50s of sb290157 were 27.7 nm in RBL-C3aR cells and 28 nm human neutrophils, respectively. Most importantly, sb290157 is found to acts selectively on C3aR not C5aR or other six chemotactic G protein coupled receptors, which indicates very potential effect in the clinical application (96).

Recent evidence has elucidated C3 as a potent lipogenic hormone in the pathogenesis of NAFLD according to preclinical and translational evidence. Theoretically, initial discussions primarily relied on considerations of C3 deficiency which often leads to a broader range of susceptibilities to infections. As a matter of factor, it can be effectively avoided by developing therapies targeting C3a/C3aR, which participates in inflammatory responses such as anti COVID-19 and anti-HCC treatment (97–102). Complement C3 activation may interfere with NAFLD with at least two distinct mechanisms by enhancing adipose tissue inflammation via the local engagement of C3a and C5a receptors as well as by providing the substrate for the conversion of C3a into ASP that may exerts its effects systemically (103). Regretfully, no clinical registered trial is found to target C3a or C3aR in NASH therapy so far. Our previous studies have shown that C3a/C3aR participates in the pathogenesis of

NASH even fibrosis by regulating various signaling pathways and mentioned the protective function in the C3aR$^{-/-}$ mice model (28). Therefore, in-depth investigations and awareness of the roles of C3a/C3aR in NASH are urgently needed that will lead to a further expansion of potential indications for complement treatments in the future.

Traditionally, C3a/C3aR signaling axis plays a pro-inflammatory role in the pathogenesis of NAFLD. However, recent studies have found that in the early stage of NAFLD, C3a plays an anti-inflammatory role by preventing neutrophils from accumulating in liver tissue (58). In fact, whether C3a is pro-inflammatory or anti-inflammatory is not mutually exclusive in the pathogenesis of NAFLD, but depends on the balance between pro-inflammatory and anti-inflammatory effects of C3a, which determines the final outcome of the disease (**Figure 3**). For example, C3a plays a pro-inflammatory role in the NASH stage of NAFLD by activating Kupffer cells under the effect of neutrophil elastase. If neutrophils were deleted at that time, the activation of Kupffer cells was significantly delayed (104). Therefore, the dual role of C3a/C3aR signaling axis should be considered for the rational designing therapeutic strategies targeting C3a/C3aR in the effective treatment of NAFLD.

CONCLUSION

Complement system is one of the most important innate immune barriers in the body, among which complement C3 is the critical component in complement cascade activation. Clinically, increased epidemiological evidence has shown that C3 is closely related to the pathogenesis of NAFLD. Serum fatty acids, adipose tissue-derived cytokines and gut derived endotoxin can take part in complement activation. After complement activation, C3 interacts with different types of liver innate immune cells, and ultimately participates in the pathogenesis of NAFLD. C3a is an important derivative from C3 when complement cascades are activated. Accumulating studies indicate that C3a plays an important role in the pathogenesis of NAFLD by interacting with its receptor C3aR. Targeted inhibition of C3aR activation is a potential strategy for the prevention and treatment of NAFLD. Although SB 290157 is an effective and selective C3aR antagonist in some experimental animal models, the preclinical and clinical evidence of SB 290157 needs to be explored to prevent dietary obesity, metabolic dysfunction and NAFLD in the coming future.

AUTHOR CONTRIBUTIONS

JH and XZ were involved in study design and drafted the paper. XZ supervised and reviewed the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of Carnitine in Non-alcoholic Fatty Liver Disease and Other Related Diseases: An Update

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Carnitine is an amino acid-derived substance that coordinates a wide range of biological processes. Such functions include transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix, regulation of acetyl-CoA/CoA, control of inter-organellar acyl traffic, and protection against oxidative stress. Recent studies have found that carnitine plays an important role in several diseases, including non-alcoholic fatty liver disease (NAFLD). However, its effect is still controversial, and its mechanism is not clear. Herein, this review provides current knowledge on the biological functions of carnitine, the "multiple hit" impact of carnitine on the NAFLD progression, and the downstream mechanisms. Based on the "multiple hit" hypothesis, carnitine inhibits β-oxidation, improves mitochondrial dysfunction, and reduces insulin resistance to ameliorate NAFLD. L-carnitine may have therapeutic role in liver diseases including non-alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma, alcoholic fatty liver disease, and viral hepatitis. We also discuss the prospects of L-carnitine supplementation as a therapeutic strategy in NAFLD and related diseases, and the factors limiting its widespread use.

Keywords: carnitine, non-alcoholic fatty liver disease, L-carnitine supplementation, targeted therapy, therapeutic diet

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INTRODUCTION

Carnitine (3-hydroxy-4-N-trimethylammoniobutanoate) is an essential water-soluble molecule with multiple functions in the human body (1). Examples of such functions include reducing oxidative stress, increasing expression of pro-inflammatory cytokines (2–4), and improving mitochondrial dysfunction (5) and insulin resistance (IR) (6, 7). Moreover, it plays an important role in the development of many metabolic diseases, such as hypertension, diabetes, polycystic ovary syndrome (8), and osteoarthritis (9). Besides, carnitine has been reported to be closely associated with the development of non-alcoholic fatty liver diseases (NAFLD). Numerous studies have shown that NAFLD has become a major healthcare concern and economic burden worldwide (10); therefore, its prevention and treatment have gained increased attention among researchers. In this review, we summarize and discuss the relationship between carnitine and NAFLD, effects of L-carnitine (the biologically active form of carnitine) supplementation in NAFLD, and related diseases, including non-alcoholic steatohepatitis (NASH), cirrhosis, hepatic cellular cancer (HCC), alcoholic fatty liver disease, and viral hepatitis.

BIOLOGICAL CHARACTERISTICS OF CARNITINE

Classification and Distribution in Human Body

Carnitine is an amino acid belonging to a quaternary ammonium cationic complex. It has two stereoisomers: bioactive L-carnitine and abiotic enantiomeric isomer D-carnitine. L-carnitine is the predominant carnitine used in biological and medical fields and is commonly referred to as "carnitine." The human body contains about 300 mg/kg of L-carnitine, 98% of which is intracellular, with 80% present in the muscles, 5–10% in the gastrointestinal tract, and 3% in the liver (11). Although D-carnitine has no biological activity in humans, it adversely impacts biochemical processes by inhibiting the carnitine acetyltransferase (12). D-carnitine supplementation has been found to induce liver inflammation, oxidative stress, and apoptosis in animal studies (13). In addition, D-carnitine can cause secondary carnitine deficiency (SCD), so researchers often use D-carnitine supplements to feed mice to establish carnitine lack animal models (14, 15). For these reasons, D-carnitine has been rarely studied in humans.

Endogenous Synthesis and Exogenous Sources

In the average adult diet, it is estimated that about 75% of the daily carnitine requirement mainly comes from meat, fish, and dairy products (16), while the remaining 25% is derived from endogenous synthesis (1). In strict vegetarians, more than 90% of the daily carnitine requirement is obtained by endogenous synthesis (17–19), whereby humans can synthesize ∼1–2 μmol carnitine/kg/day (20). The synthesized carnitine is formed when 6-N-trimethyl-lysine (TML) is released during protein degradation (**Figure 1**) (21). After release, TML is hydroxylated into 3-hydroxyl-6-N-trimethyl-lysine (HTML) by trimethyl dioxygenase (TMLD), which is then broken down into 4-N-trimethyl-butylaldehyde (TMABA) and glycine by HTML aldolase (HTMLA) (1). TMABA produces 4-N-trimethylaminobutyrate (γ-butyrobetaine) under the action of dehydrogenase (11). Finally, γ-butyrobetaine dioxygenase (BBD) is used to hydroxylate γ-butyrobetaine to produce endogenous carnitine (**Figure 1**) (22, 23). BBD is confined to the human liver, kidneys, testis and brain, thus the biosynthesis of carnitine occurs only at these locations (24). Other tissues, such as skeletal muscle, obtain carnitine from the blood (25).

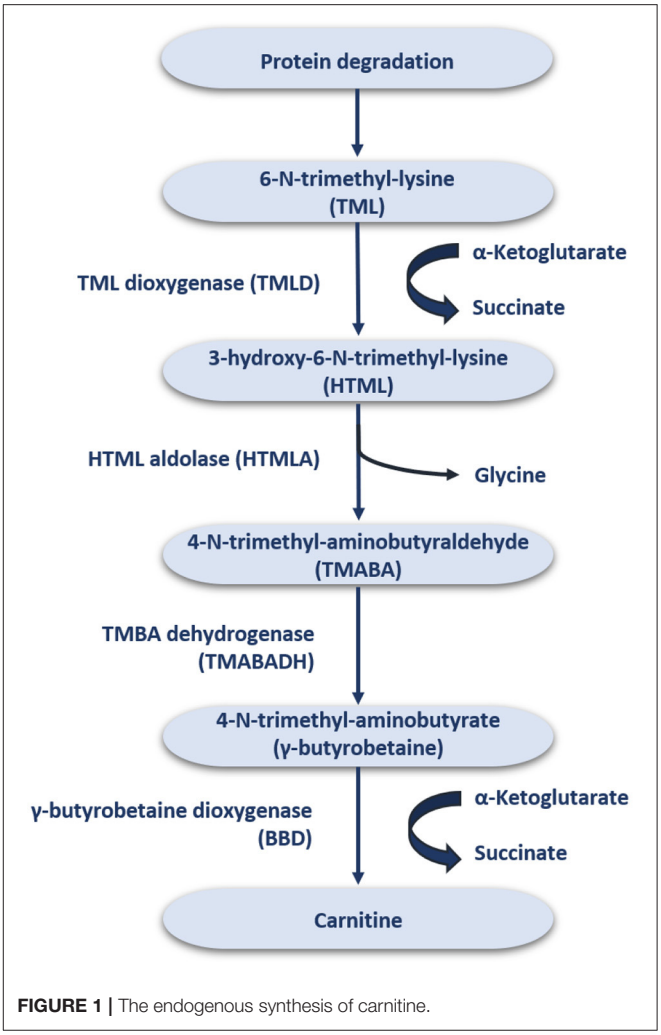

FIGURE 1 | The endogenous synthesis of carnitine.

The distribution and homeostasis of carnitine within the body is controlled by organic cationic transporters (OCTN) (26). OCTN act on intestinal absorption and renal reabsorption of carnitine, and plays an important role in tissue distribution by catalyzing carnitine to enter cells *in vivo* (27). Among these, OCTN2 is the most important physiologically transporter of carnitine due to its high affinity and wide expression (27). OCTN2 plays a crucial role in carnitine homeostasis. Notably, BB, a direct precursor of carnitine, is also a good substrate for OCTN2. The liver and kidneys have a strong ability to convert BB to carnitine. Loss or mutation of OCTN2 function results in primary systemic carnitine deficiency (PCD) with severe clinical

Abbreviations: ACS, acyl-CoA synthetase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; AST, aspartate aminotransferase; BMI, body mass index; CACT, carnitine-acylcarnitine translocase; CAT, catalase; CHB, chronic hepatitis B; CHC, chronic hepatitis C; COT, carnitine octyltransferase; CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; CRP, C-reactive protein; ER, endoplasmic reticulum; FBG, fasting blood glucose; FFAs, free fatty acids; GPx, glutathione peroxidase; GR, glutathione reductase; HbA1c, glycosylated hemoglobin; HC, hip circumference; HCC, hepatocellular carcinoma; HDL-C, high dense lipoprotein cholesterol; HE, hepatic encephalopathy; HOMA, homeostatic model assessment; IGF-1, insulin-like growth factor-1; IMM, inner mitochondrial membrane; IR, insulin resistance; LCFA, long-chain fatty acids;LDL-C, low density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NCT-A, number connection test-ANf-κB, nuclear factor kappa B; NOX, nicotinamide adenine dinucleotide phosphate oxidase; OMM, outer mitochondrial membrane; PCD, primary carnitine deficiency; PDHC, pyruvate dehydrogenase complex; PPARγ, peroxisome-activated receptor-γ; PT, prothrombin time; ROS, reactive oxygen species; SOD, superoxide dismutase; SOD2, superoxide dismutase 2; TACE, transarterial chemoembolization; TB, total bilirubin; TC, cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor-α; UPR, unfolded protein response; WC, waistline circumference; XO, xanthine oxidase; αSMA, α-smooth muscle actin; γ-GT, γ-glutamyl transpeptidase.

consequences such as cardiac and skeletal myopathy, cardiac hypertrophy and NAFLD (28).

Biological Functions

Transport of Long-Chain Fatty Acids Into the Mitochondrial Matrix

The essential function of carnitine is to transport LCFAs from the cytoplasm to the mitochondrial matrix for subsequent degradation by β-oxidation, known as "carnitine shuttle" (5). LCFA activation occurs in the cytosol, but the enzymes required to catalyze LCFA oxidation exist in the mitochondrial matrix (29). In this process, LCFA must be first activated into lipoyl-CoA via acyl-CoA synthetase (ACS) (30). Then, lipoyl-CoA is transported into the mitochondria. Since the inner mitochondrial membrane is impermeable to lipoyl-CoA (29), the entry of lipoyl-CoA relies on a shuttle system, which requires carnitine.

The carnitine shuttle has three main steps. First, CoA must be transferred from lipoyl-CoA to the hydroxyl group of carnitine to form lipoyl-carnitine. This transesterification is catalyzed by carnitine palmitoyl transferase I (CPT I) in the outer membrane (1). Second, the lipoyl-carnitine ester enters the matrix by facilitated diffusion through carnitine-acylcarnitine translocase (CACT) located in the inner mitochondrial membrane (31). In the final step, lipoyl-CoA is enzymatically transferred from carnitine to intramitochondrial CoA by carnitine palmitoyl transferase II (CPT II) (32). This isozyme, located on the inner face of the inner mitochondrial membrane, regenerates lipoyl-CoA and releases free carnitine into the matrix. Carnitine enters the intermembrane space again via CACT (**Figure 2**).

Regulation of Acetyl-CoA/CoA Ratio

Under physiological conditions, carnitine can buffer excess acetyl-CoA in the mitochondria via the formation of acetyl-carnitine (33), which requires the presence of carnitine acyltransferase and carnitine acylcarnitine translocase. Acetyl-CoA is either metabolized through the tricarboxylic acid cycle (TCA cycle) or exported as acetyl-carnitine by carnitine. When there is persistent excess or underutilization of certain fatty acids, non-metabolizable acyl-CoAs accumulate. In such situations, carnitine acts as a receiver for these acyl groups by removing them from the tissues and excreting them in the urine (20), or they get separated from carnitine and reused (34). Carnitine regulation of acetyl-CoA/CoA reduces the inhibition of many intramitochondrial enzymes involved in glucose and amino acid catabolism (35).

Inter-organellar Acyl Transfer

Long-chain fatty acids (LCFA) and branched chain fatty acids are oxidized in peroxisomes. In contrast to mitochondrial β-oxidation, incomplete peroxisomal oxidation of fatty acids yields acetyl-CoA and shortened medium-chain acyl-CoAs. In order to completely oxidize these substances into CO_2, the products of peroxisome fatty acid oxidation must be transported to the mitochondria (20). Since CoA and CoA esters cannot penetrate the cell membrane, they must be converted into their respective carnitine esters by catalase and carnitine octyltransferase (COT) in peroxisomes. Therefore, the carnitine esters are transported from peroxisomes to mitochondria through peroxisome and mitochondrial carnitine-acylcarnitine translocase (CACT), then reconverted into CoA esters by mitochondrial CPT II in the mitochondrial matrix (20). These are then oxidized into CO_2 and H_2O through mitochondrial β-oxidation, TCA cycle, and electron transfer.

Reduction of Oxidative Stress

Carnitine has several protective effects on oxidative stress. These include direct scavenging of free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide dismutase and hydrogen peroxide, and metal chelation to catalyze free radical formation, such as Fe^{2+}; inhibition of reactive oxygen species-producing enzymes such as xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate oxidase (NOX); upregulation of antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) glutathione peroxidase (GPx), heme oxygenase, endothelial nitric oxide synthase, and other protective proteins (5).

In addition to the above biological functions, carnitine also plays an important role in anti-apoptosis and protection of mitochondrial biogenesis and integrity, which are beyond the scope of this study (5).

Application in Diseases

Carnitine, a natural compound closely related to the above-mentioned functions, has recently been found to alter the underlying disease pathology with fewer side effects (5, 36). It has been reported that L-carnitine as a supplement can be useful in the treatment of hypertension (37), diabetes mellitus (6, 7), NAFLD (34), heart failure (38), coronary artery disease (39), liver cirrhosis (40), muscle injury (41), dyslipidemia (42), migraine (43), Alzheimer's disease (44), and other chronic diseases.

ROLE OF CARNITINE IN THE PATHOGENESIS OF NAFLD AND ASSOCIATED DISEASES

Carnitine and "Multiple Hit" Hypothesis in the Pathogenesis of NAFLD

The prevalence of NAFLD is increasing at a tremendous rate, currently affecting about 24% of the world's population. It has been pinpointed as the most common cause of liver disease globally (45). In the last decade, the clinical burden of NAFLD has been associated to liver-related morbidity and mortality and also to the extra-hepatic manifestations involving other organs and regulatory pathways. The pathogenesis of NAFLD is multifactorial and only partially understood (46). At present, the highly recognized pathogenic mechanism is a "multiple hit" hypothesis, which considers the combined effects of multiple insults on genetically susceptible subjects to induce NAFLD and provides a reasonable explanation for the development of NAFLD (47). The first hit is the accumulation of fat in the liver, followed by the development of necrotic inflammation and fibrosis. In addition, nutritional factors, intestinal microflora, and genetic and epigenetic factors exhibit significant influence (48).

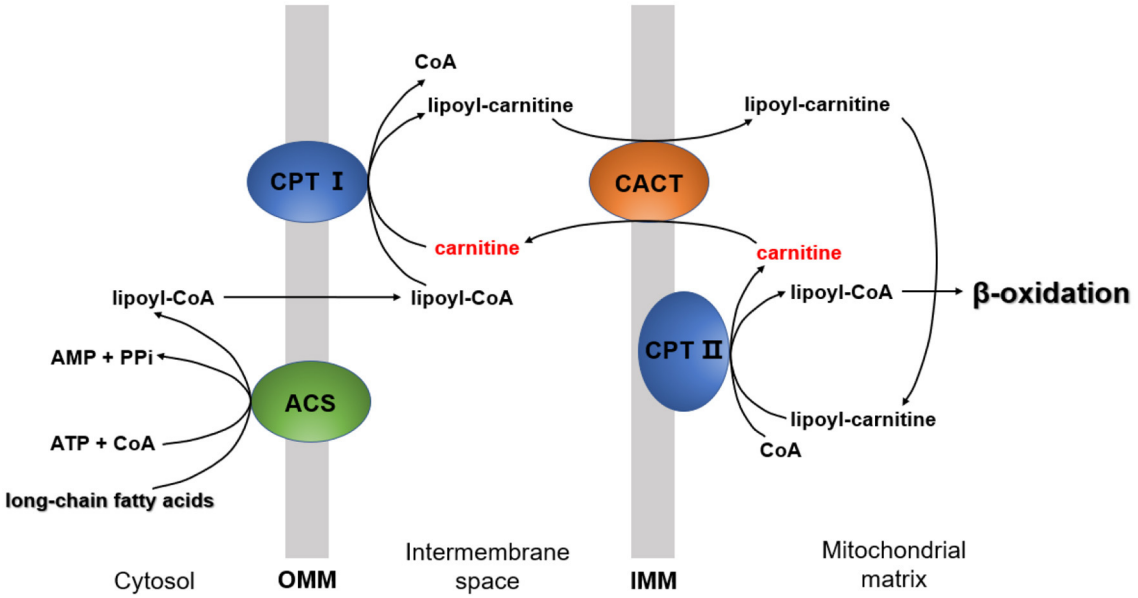

FIGURE 2 | The mechanism by which long-chain fatty acids enter the mitochondria. CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; ACS, acyl-CoA synthetase; CACT, carnitine-acylcarnitine translocase; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

Inhibition of β-oxidation, mitochondrial dysfunction, and insulin resistance (IR) are the three most important links in these "hits" as well as targets for carnitine to ameliorate NAFLD. Herein, we attempt to explain the role of carnitine in the pathogenesis of NAFLD, as shown in **Figure 3**.

Supplementation with carnitine may have a positive effect on the β-oxidation of fatty acids in mitochondria, thereby promoting lipid metabolism by boosting the uptake of fatty acids and eventually reducing fat accumulation in hepatocytes. It is known that NAFLD is associated with an imbalance in a variety of metabolic networks, among which abnormal lipid metabolism is the core pathological metabolic process (49). The immediate cause of NAFLD is high levels of triglycerides (TG) and serum free fatty acids (FFAs). Excess TG comes from hepatic fat production and dietary fat supply, while FFAs accumulate due to lipolysis of visceral adipose tissues (50). The most important strategy to reduce NAFLD is to decrease dietary fat consumption and promote the catabolism of FFAs. β-oxidation is the core process of FFA decomposition, and carnitine acts as an FFA transporter. Carnitine plays an extremely important role in β-oxidation through the "carnitine shuttle," as described above (51). The carnitine-mediated entry process is a rate-limiting step for mitochondrial LCFA oxidation and, thus, a major regulating point. Increased levels of carnitine may improve the originally inhibited β-oxidation, which is a great way to reduce fat accumulation in the liver (34). Recent studies have emphasized the key role of mitochondrial dysfunction in the occurrence and development of NAFLD (52). The effect of carnitine on mitochondrial dysfunction has been confirmed by various experiments (5). Increased FFAs can lead to hepatic lipotoxicity, generation of reactive oxygen, and damage to the mitochondrial membrane (52). Carnitine treatment could increase the mRNA expression of carnitine palmitoyltransferase 1A and peroxisome proliferator-activated receptor-γ (PPAR-γ), while preventing lipid membrane peroxidation and ROS. This further leads to the reversal of mitochondrial dysfunction, thereby increasing mitochondrial β-oxidation and reducing intracellular oxidative stress to prevent hepatic lipotoxicity (53, 54).

An important mechanism by which carnitine improves IR is enhancing the oxidation of mitochondrial long-chain acyl-CoAs (**Figure 2**) (55). Accumulation of long-chain acyl-CoAs and other FA metabolites impairs insulin signaling and leads to the development of IR, a crucial pathophysiological factor for carbohydrate metabolism disorders consistent with the development of NAFLD (49). Since abnormal mitochondrial function is associated with IR (56), carnitine therapy can also reduce IR by improving mitochondrial function (56).

Other possible mechanisms to improve insulin sensitivity in NAFLD reported in the literature include the regulation of the acetyl-CoA/CoA ratio in mitochondria, modulation of the pyruvate dehydrogenase complex (PDHC) activity, altering the expression of glycolytic and gluconeogenic enzymes as well as the expression of genes associated with the insulin signaling cascade, and stimulation of the insulin-like growth factor-1 (IGF-1) axis and IGF-1 signaling cascade (34, 55). As mentioned above, carnitine improves the glucose metabolism by reducing acetyl-CoA/CoA and reduces IR. Both insulin and IGF-1 act through their homologous receptor tyrosine kinases to coordinate metabolism and cellular responses to nutrient supply (57). When IGF-1 axis is activated, multiple signaling pathways

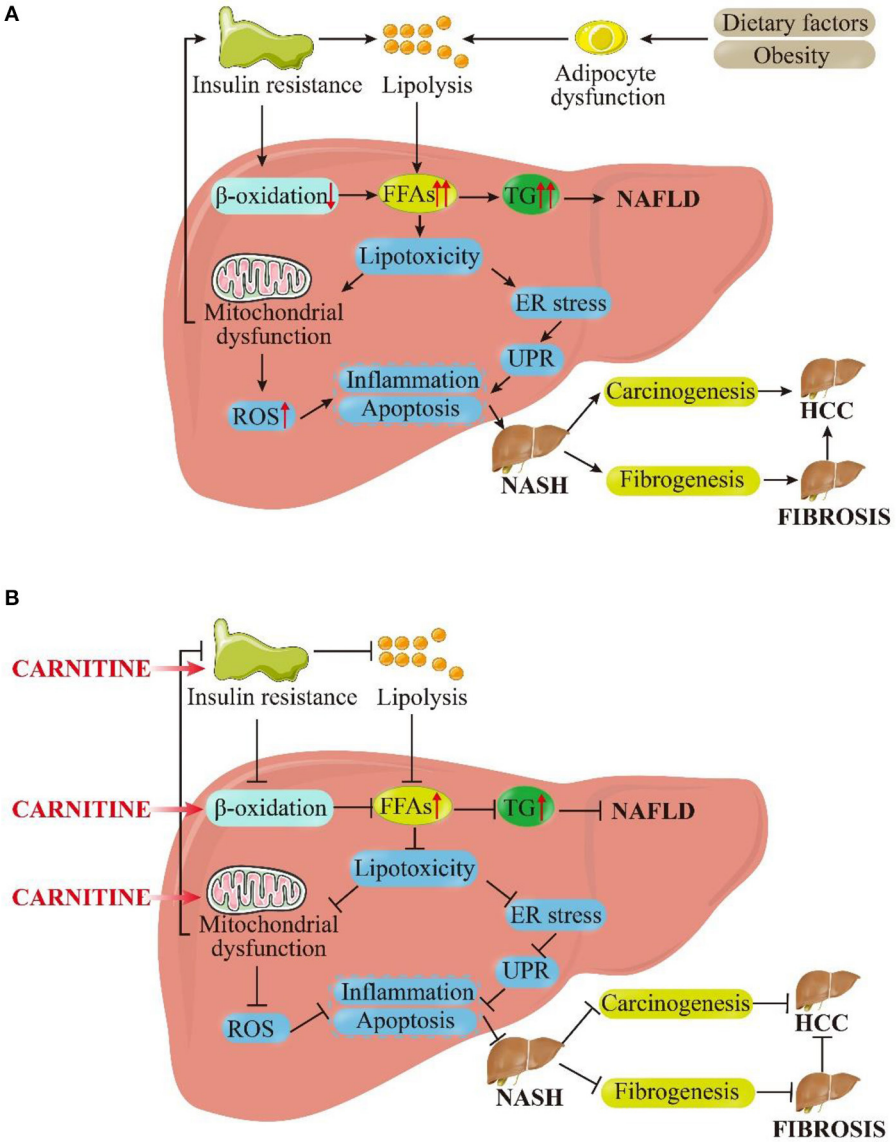

FIGURE 3 | Pictorial representation of the **(A)** pathophysiological mechanism of NAFLD based on the "multiple hit" hypothesis and **(B)** inhibitory effects of carnitine on NAFLD and its progression. As shown in **(A)**, dietary and environmental factors, together with obesity, lead to the proliferation and dysfunction of adipocytes. Insulin resistance acts on adipose tissue and worsens adipocyte dysfunction, which in turn worsens lipolysis. In the liver, insulin resistance inhibits β-oxidation. Under the dual attack of increased lipolysis and weakened β-oxidation, the influx FFAs to hepatocytes is increased greatly, leading to the synthesis and accumulation of TG and enhanced liver lipotoxicity. Excessive TG eventually induces NAFLD. Increased lipotoxicity leads to mitochondrial dysfunction and oxidative stress on endoplasmic reticulum (ER) by the activation of ROS, which leads to liver inflammation and fibrosis. At the same time, mitochondrial dysfunction promotes insulin resistance, exacerbating above process in a vicious circle. These pathological processes can cause the liver to persist in the stable stage of disease (NAFLD) or develop in to NASH. In the late stage of disease progression, NASH can progress to fibrosis or even HCC under the stimulation of certain factors. As shown in **(B)**, carnitine supplementation reduces insulin resistance, promotes β-oxidation and improves mitochondrial function. Subsequently, the cellular concentration of FFAs and TG in hepatocytes get reduced and lipotoxicity is alleviated. The level of ROS is restrained to a certain extent, and inflammation and apoptosis were also improved. These above interlocking effects could alleviate NAFLD and NASH, and they may even have positive therapeutic effects on liver fibrosis and HCC. FFAs, free fatty acids; TG, triglycerides; ER, endoplasmic reticulum; UPR, unfolded protein response; ROS, reactive oxygen species; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma.

corresponding to its kinase domain are also activated, including PI3K/Akt pathway and Raf/MEK/ERK level linkage pathway, which can ultimately reduce IR and prevent cell apoptosis (58, 59), thereby improving NAFLD.

Role of Carnitine in NAFLD Progression and Development of Associated Diseases

The liver is the main organ responsible for detoxification and metabolism of various compounds that produce reactive oxygen

species (ROS). As such, liver disease may lead to increased ROS production and, subsequently, increased peroxidation of lipid membranes and inflammatory factors production, leading to hepatocyte injury and cell death (60–63). Injury or death of hepatocytes results in increased release of liver enzymes (64). Patients with chronic liver disease, especially liver cirrhosis, usually suffer from secondary carnitine deficiency (65, 66). Because the liver is one of the main sites for carnitine synthesis, liver disease can impair carnitine synthesis, which further aggravates the above-mentioned pathological processes and promotes disease progression. A recent meta-analysis showed that L-carnitine supplementation improves liver function by reducing histological steatosis and NAS scores in patients with NASH (67). In addition, Okabayashi et al. demonstrated that L-carnitine could improve postoperative liver function in hepatectomized patients (68). L-carnitine reduces inflammatory response via the transfer of β-oxidized long-chain fatty acids in mitochondria and the excretion of toxic substances in fatty acid metabolism (69). It also plays the role of free radical scavenger by reducing the production of ROS (70). Hence, L-carnitine may contribute by reducing oxidative stress, decreasing inflammatory response, and activating enzymes involved in the defense against oxidative damage, thereby reducing elevated serum liver enzymes in patients with liver disease.

Primary carnitine deficiency (PCD) is an autosomal recessive FA oxidation disease (34). Both serum and intracellular carnitine levels are very low in PCD patients. Therefore, FAs cannot be accumulated as an energy source (71), leading to increased ROS production (72). Different from PCD, secondary carnitine deficiency (SCD) is mostly caused by drugs (such as valproic acid, anticancer drugs, omeprazole, amphoteric drugs, etc.) or diseases (such as fatty acid oxidation disorder, organic acidemia, etc.) (1). These drugs or diseases increase the excretion of carnitine in the form of acylcarnitine in urine, reduce renal tubule reabsorption and endogenous biosynthesis, and inhibit carnitine transporters, resulting in increased carnitine consumption (73, 74). Carnitine deficiency impairs the mitochondrial β-oxidation of FAs, leading to acute metabolic decompensation, elevated aminotransferase, and hepatic encephalopathy similar to NAFLD (75). It has been reported that long-term oral carnitine treatment for 6 months in a patient with carnitine deficiency resulted in increased muscle strength, significant reduction in heart size, relief from cardiomyopathy, and partial repletion of carnitine levels in plasma and muscle with complete repletion of liver functions (71). L-carnitine has been approved by the US Food and Drug Administration for the treatment of PCD and SCD (76).

Alcoholic fatty liver disease is another major cause of fatty liver disease, for which carnitine supplementation may also be beneficial for improvement (77). The endogenous biosynthesis of FAs is the main cause of hepatic steatosis in chronic alcoholism (78). Hepatic steatosis is further aggravated by impaired fatty acid oxidation in an ethanol-impaired liver (79). Sachan et al. evaluated the lipid-lowering effects of carnitine and its precursors (lysine and methionine) in rats with chronic alcoholism and concluded that carnitine effectively prevented alcohol-induced hyperlipidemia and liver fat accumulation. Carnitine was also found to be more effective than its precursors (77).

The efficacy of L-carnitine supplements in the treatment of chronic hepatitis B (CHB) remains controversial. A Korean study compared the combination of entecavir and carnitine with entecavir alone to determine their therapeutic effects in CHB. Results indicated that the ALT normalization rate in the combination group was higher than that in the entecavir group, but the HBV-DNA normalization rate and change in serum HBV-DNA levels were similar (80). However, a recent trial showed that besifovir dipivoxil maleate combined with L-carnitine did not lead to any improvement in hepatic steatosis in CHB patients (81). On the other hand, carnitine has a higher recognition for the therapeutic effect in chronic hepatitis C (CHC). A recent meta-analysis showed that long-term use of low-dose (≤2,000 mg/day) L-carnitine could reduce TC and TG in overweight patients with liver disease, especially those with chronic hepatitis C (82). Another study also suggested that L-carnitine may be an effective adjuvant for anti-HCV therapy, because it reduces the oxidative stress induced by JFH-1 infection, inhibits HCV assembly, and exhibits anti-HCV activity through its anti-adipogenic activity in HCV-infected cells (83).

DOWNSTREAM MECHANISM OF CARNITINE IN NAFLD AND RELATED DISEASES

The molecular regulation mechanism of the role of carnitine in fatty acid metabolism has been preliminarily clarified. Typical genes, such as OCTN2 (encoded by SLC22A5) and /or BBD (encoded by BBOX1), regulated by PPARα play regulatory roles in the cellular fatty acid uptake, fatty acid activation, intracellular fatty acid transport, and β-oxidation of mitochondrial and peroxisomal fatty acids (84). With the increase of FFA oxidation, PPARα is activated, and the expression of BBD and OCTN2 in oxidative tissue is simulated to facilitate disposal (85). At the same time, increased expression of BBD and OCTN2 also plays a role in regulating carnitine homeostasis by stimulating the intake and biosynthesis of the carnitine, thus increasing the concentration of carnitine (86). The transcriptional regulation of PPARα on carnitine homeostasis related genes is consistent with their basic role in fatty acid catabolism (85).

Many experimental studies have been conducted to understand the impact of carnitine supplementation in NAFLD. For instance, one study employed a mouse model to demonstrate that L-carnitine supplementation could oppose the NAFLD progression. Results revealed a reduction in hepatic lipid accumulation and oxidative stress, hepatic fibrosis via modulation of α-smooth muscle actin (αSMA), peroxisome-activated receptor gamma (PPARγ), and nuclear factor kappa B (Nf-κB) expression (87). Among them, PPARγ may regulate cell apoptosis through the p21, p53 and p27 pathways, and exert an inhibitory effect on the progression of HCC (88). Another research using a mouse model showed that L-carnitine supplementation can inhibit the NAFLD development by decreasing TNF (receptor superfamily member 9 or CD137), CCL23 (C-C motif chemokine 23), MMP1 (matrix metalloproteinase 1), and FGF21 (fibroblast growth factor 21)

TABLE 1 | Studies reporting the outcomes of L-carnitine supplementation on NAFLD.

References	Type of study	Subject	Sample size	Duration	Intervention group	Control group	Results*
Somi et al. (97)	Randomized control trial	Humans	80 (40 vs. 40)	24 weeks	L-Carnitine (250 mg bid)	Without treatment	↓: AST, ALT, BMI, weight, NAFLD sonographic grade
Lim et al. (98)	Randomized control trial	Humans	45 (29 vs. 16)	12 weeks	Carnitine (600 mg/d)	Without treatment	↓: AST, ALT, TB ↑: peripheral mitochondrial DNA copy number
Mollica et al. (87)	Randomized control trial	Mice	30 (10 vs. 10 vs. 10)	6 weeks	Group 1: methionine-choline-deficient diet (6 weeks) + L-carnitine (200 mg/kg each day) (last 3 weeks); Group 2: methionine-choline-deficient diet (6 weeks)	Regular diet (6 weeks)	LC treatment ameliorated hepatic fat accumulation, limited ROS production, improved the fibrosis progression of NAFLD, significantly reduced the NF-kB protein content
Fujisawa et al. (99)	Randomized control trial	Oryzias latipes	80 (20 vs. 20 vs. 20 vs. 20)	6 weeks	Group 1: regular diet (4 weeks) + L-carnitine (1 mM) (2 weeks) Group 3: high-fat diet (4 weeks) + L-carnitine (1 mM) (2 weeks)	Group 2: regular diet (4 weeks) Group 4: high-fat diet (4 weeks)	↓: lipid accumulation ↑: the expression of SOD2, acetyl-CoA, ATP
Kathirvel et al. (100)	Randomized control trial	Mouse	40 (10 vs. 10 vs. 10 vs. 10)	24 weeks	Group 1: high-fat diet Group 2: high-fat diet + ALC + LA	Group 3: standard diet Group 4: standard diet + ALC + LA	↓: size of the mitochondria in liver; ALT; AST ↑: carbamoyl phosphate synthase 1

**Only show indicators that have statistically significant changes after L-carnitine intervention.*
ALT, alanine aminotransferase; AST, aspartate aminotransferase; TB, total bilirubin; BMI, body mass index; ROS, reactive oxygen species; SOD2, superoxide dismutase 2.

in the plasma (89). Another study on hepatoma HepG2 cells revealed the distinct benefits of L-carnitine in fructose-mediated lipid accumulation through adenosine monophosphate-activated protein kinase (AMPK) activation (90). L-carnitine was found to increase PGC1α expression and ameliorate mitochondrial damage (90). Numerous works have revealed that the combination of L-carnitine and nicotinamide nucleoside can enhance the transfer of fatty acids across mitochondrial inner membrane and increase the content of nicotinamide adenine nucleotide (NAD +), which is necessary for β-oxidation and the TCA cycle. It can also reverse the harmful effects of a high-fat diet on liver metabolic pathways and related regulators, such as ACOX, SCAP, SREBF, PPARGC1B, and INSR (91). These mechanisms support the administration of L-carnitine as a novel drug to reduce NAFLD.

TREATMENT OF NAFLD WITH L-CARNITINE SUPPLEMENTATION

Since the effects of NAFLD are reversible, it is important to control the disease in its early stages (92). Unfortunately, there are no drugs specifically approved for the treatment of NAFLD at present (93). Available treatments for NAFLD mainly focus on changing lifestyle habits and encouraging weight loss. In addition, several molecules have been studied as an adjuvant therapy, including L-carnitine, CoQ_{10}, vitamin E, vine tea polyphenol, cytoprotective agents (ursodeoxycholic acid), and insulin sensitizers (pioglitazone and metformin) (94).

L-carnitine, the only biologically active form of carnitine, has been one of the most widely studied molecules for the treatment of NAFLD. Several meta-analyses showed that L-carnitine supplementation improved steatosis and NASH (67) and carnitine supplementation in NAFLD patients could reduce AST, ALT, TG, and HOMA-IR (95). In yet another meta-analysis, L-carnitine supplementation was found to significantly improve the circulating levels of ALT, AST, and gamma-glutamyl transpeptidase (γ-GT), which may have a positive impact on liver function (96).

The research trials conducted to study the effects of L-carnitine supplements in NAFLD, its progression, and the associated diseases are summarized in **Tables 1**, **2**. Most of these studies reported that L-carnitine supplementation can normalize or reduce the serum levels of liver enzymes (94, 97, 98, 101–104). Some have confirmed that L-carnitine supplementation could reduce the incidence and severity of NAFLD (103) and, thus, improve liver attenuation index on CT (101), NAFLD sonographic grade (97), and histological scores (104). Other studies propose that L-carnitine supplementation could improve blood glucose (including FBG and HbA_1c) and IR (including HOMA-IR) (103), blood lipid profile (104), and mitochondrial function (98, 102) in NAFLD patients with diabetes. Two studies demonstrated that L-carnitine can improve liver function and prognosis in patients with liver cancer after surgery (68, 106). Several randomized controlled

TABLE 2 | Studies reporting the effects of L-carnitine supplementation on NAFLD progression and related diseases.

References	Type of study	Subject	Disease	Sample size	Duration	Intervention group	Control group	Results*
Alavinejad et al. (94)	Randomized double blind pilot study	Humans	NAFLD + Diabetes	54 (28 vs. 26)	12 weeks	L-Carnitine (750 mg tid)	Placebo (750 mg tid)	↓: AST, ALT
Bae et al. (101)	Randomized double blind pilot study	Humans	NAFLD + Diabetes	78 (39 vs. 39)	12 weeks	Carnitine-orotate complex (824 mg tid)	Placebo (824 mg tid)	↓: ALT, liver attenuation index on CT, HbA_1c
Hong et al. (102)	Randomized double blind pilot study	Humans	NAFLD +\ Diabetes	52 (26 vs. 26)	12 weeks	Metformin (250 mg tid) + carnitine-orotate complex (300 mg tid)	Metformin (250 mg tid)	↓: ALT, urinary 8-hydroxy-2'-deoxyguanosine ↑: mtDNA copy number
Hamza et al. (103)	Prospective case-control interventional study	Humans	Obesity (suspected NAFLD)	50	24 weeks	Comparison before and after L-Carnitine (50 mg/kg/d) therapy		↓: AST, ALT, Liver span, WC, HC, waist/hip ratio, FBG, Chemerin, HOMA index, incidence and severity of NAFLD (after LC therapy)
Malaguarnera et al. (104)	Randomized double blind pilot study	Humans	NASH	74 (36 vs. 38)	24 weeks	L-carnitine (1 g bid) + 1,600-calorie diet	Placebo (1 g bid) + 1,600-calorie diet	↓: AST, ALT, γ-GT, TC, LDL-C, TG, FBG, HOMA-IR, CRP, TNF-α, histological scores ↑: HDL-C
Ishikawa et al. (105)	Randomized control trial	Mice	NASH	unclear	4 weeks	High-fat diet + L-carnitine	high-fat diet	↓: TNF-α mRNA, NAFLD activity score ↑: CPT II
Okabayashi et al. (68)	Randomized control trial	Humans	liver cancer (after hepatectomy)	208 (102 vs. 106)	2 weeks	L-carnitine (30 mg/kg) (oral before liver resection)	Usual intake	↓: hospital stay, ammonia levels, neutrophil/lymphocyte ratio, post-hepatic liver failure ↑: PT
Hassan et al. (106)	Randomized control trial	Humans	liver cancer (after TACE)	50 (24 vs. 26)	12 weeks	L-Carnitine (300 mg bid)	Usual intake	↓: Child-Pugh score, TB ↑: PT, serum albumin
Malaguarnera et al. (107)	Randomized control trial	Humans	Cirrhosis + HE	120 (60 vs. 60)	60 days	L-Carnitine (2 g bid)	Placebo (2 g bid)	↓: NH_4^+; NCT-A
Cecere et al. (108)	Randomized control trial	Humans	hepatic cirrhosis	27 (16 vs. 11)	4 weeks	L-Carnitine (3 g bid)	Placebo (3g bid)	↓: NH_4^+

**Only show indicators that have statistically significant changes after L-carnitine intervention.*

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transpeptidase; HDL-C, high dense lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; FBG, fasting blood glucose; TB, total bilirubin; TG, triglyceride; TC, cholesterol; WC, waistline circumference; HC, hip circumference; CRP, C-reactive protein; TNF-α, tumor necrosis factor-α; HOMA, homeostatic model assessment; HbA_1c, glycosylated hemoglobin; TACE, transarterial chemoembolization; PT, prothrombin time; HE, hepatic encephalopathy; NCT-A, number connection test-A.

clinical trials conducted by Malaguarnera et al. in patients with cirrhosis and hepatic encephalopathy (ranging from mild hepatic encephalopathy to coma) confirmed that L-carnitine supplementation significantly improved hepatic encephalopathy parameters (107–111). The results of these trials suggest that L-carnitine supplementation is advantageous for the treatment of cirrhosis with hepatic encephalopathy. Experimental trials in animals further conclude that L-carnitine supplementation can reduce lipid deposition and increase the metabolites related to β-oxidation, which confirm the importance of L-carnitine in controlling oxidative stress, steatosis, and fibrosis in the liver (87, 99).

At least 11 studies reported no obvious adverse reactions in humans (112–122) and support the use of L-carnitine as an ingredient in dietary supplements. As recognized by several experiments, L-carnitine has also attracted widespread attention since it can be easily obtained from meat at a low-cost.

The recommended dose of L-carnitine is 15 g/day for healthy individuals (123) and 100–400 mg/kg/day for patients with carnitine deficiency (96). Some side effects have been observed after high-dose supplementation of L-carnitine, such as diarrhea, intestinal problems, and trimethylamine production that causes a fishy odor. However, these can be effectively addressed by appropriately reducing the supplemental dosage (124). Hence, it is important to measure the plasma L-carnitine levels to determine the optimal dose of L-carnitine for each patient.

LIMITATIONS AND PROSPECTS OF CARNITINE FOR THE TREATMENT OF NAFLD

Despite promising results, the studies on the role of L-carnitine in NAFLD, listed in **Tables 1**, **2**, face many limitations. First, most trials used the compound preparation of L-carnitine and other substances in the intervention group. The presence of other substances can influence the outcomes of L-carnitine, making it difficult to assess its role in isolation. Second, the bioavailability of L-carnitine was not assessed in the reported studies, so there is no clear reference value for the amount of L-carnitine present in the blood after ingestion. Third, the sample sizes of the reported trials were small, which affects the credibility of the results.

In fact, there are still many unclear aspects of L-carnitine supplementation. For instance, the suggested improvement of metabolism by L-carnitine supplements is based primarily on short-term supplementation (125). A meta-analysis reported that the body weight of obese patients decreased significantly after supplementation with L-carnitine (126). However, in the subgroup analysis, it was found that L-carnitine had no effect on the body weight of subjects whose BMI was <25 kg/m^2 or when L-carnitine supplementation was administered for more than 24 weeks (126). Thus, it is not explicit whether the effects of L-carnitine supplementation on NAFLD will result in similar time-dependent effects. Although carnitine supplementation was given orally in most of the studies (**Tables 1**, **2**), some trials administered carnitine intravenously (127, 128). Therefore, the optimal route to administer L-carnitine supplements for NAFLD remains unclear. While many substances can be used as dietary supplements to improve NAFLD, no studies have directly compared them to determine which is most effective. Furthermore, the studies on the effects of L-carnitine supplements in NAFLD have yielded conflicting results. Bruls et al. showed that L-carnitine infusion could neither alleviate lipid-induced insulin resistance and metabolic inflexibility nor change the availability of skeletal muscle carnitine (129). Fujiwara et al. found that high-fat diet feeding and L-carnitine supplementation increased STAT3 phosphorylation in HCC tissues and could synergistically promote the development of liver cancer (130).

To improve the accuracy of future studies on the effects of L-carnitine supplementation in NAFLD treatment, the following aspects could be considered: use of L-carnitine in isolation in the intervention group; assessment of the bioavailability of L-carnitine; multicenter trials with larger sample size; longer follow-up to evaluate time-dependent effects; and use of intravenous L-carnitine supplementation. In addition, experimental studies are recommended to compare the effects of other substances that have been proven effective in enhancing NAFLD via L-carnitine and to understand the downstream mechanism.

CONCLUSION

Carnitine plays an important role in transporting FAs to the mitochondrial matrix for β-oxidation, regulating acetyl-CoA/CoA, exporting acetyl- and chain-shortened acyl products from peroxisomes, and reducing oxidative stress. L-carnitine supplementation can normalize or reduce serum levels of liver enzymes, decrease the incidence and severity of NAFLD, and improve both the lipid profile and mitochondrial function. L-carnitine may have therapeutic effects on liver diseases, including NASH, cirrhosis, HCC, alcoholic fatty liver disease, and viral hepatitis. In addition, L-carnitine supplementation is safe, low cost, and easy to administer. Due to the limited and inadequate studies on the effects of L-carnitine supplementation on NAFLD, future research should aim to determine the exact role of L-carnitine for the treatment of NAFLD.

AUTHOR CONTRIBUTIONS

NL: study design and drafting. HZ: study design and critical review/revision. All authors contributed to the article and approved the submitted version.

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