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Community series - liver fibrosis and MAFLD: From molecular aspects to novel pharmacological strategies, volume II

Edited by
Juan Armendariz-Borunda, Ana Sandoval-Rodriguez and Aldo Torre

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Community series - liver fibrosis and MAFLD: From molecular aspects to novel pharmacological strategies, volume II

Topic editors

Juan Armendariz-Borunda — University of Guadalajara, Mexico

Ana Sandoval-Rodriguez — University Center of Health Sciences, University of Guadalajara, Mexico

Aldo Torre — National Institute of Medical Sciences and Nutrition Salvador Zubirán, Mexico

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Angel Lanas,
University of Zaragoza, Spain

*CORRESPONDENCE
Juan Armendariz-Borunda
armdbo@gmail.com

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Editorial: Community series - liver fibrosis and MAFLD: from molecular aspects to novel pharmacological strategies, volume II

Ana Sandoval-Rodriguez[1], Aldo Torre-Delgadillo[2] and Juan Armendariz-Borunda [1,3]*

[1]Institute of Molecular Biology in Medicine and Gene Therapy, Centro Universitario de Ciencias de la Salud, University of Guadalajara, Guadalajara, Jalisco, Mexico, [2]Hepatology and Liver Transplantation Unit, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico City, Mexico, [3]Tecnologico de Monterrey, School of Medicine and Health Sciences, Guadalajara, Mexico

KEYWORDS

liver fibrosis, MAFLD, MASLD, steatotic liver disease, non-alcoholic steatohepatitis (NASH)

Editorial on the Research Topic
Community series - liver fibrosis and MAFLD: from molecular aspects to novel pharmacological strategies, volume II

In this consecutive second issue of Frontiers in Gastroenterology editors Armendariz-Borunda, Sandoval-Rodriguez, and Torre-Delgadillo examine new challenges in terminology, from MALFD to Steatotic Liver Disease and the new MASLD argot, retrieved from several manuscripts from 2021 to late 2023, including perspectives, systematic reviews and original research in clinical scenarios to address novel insights in the all-time important fibrosis and the new epidemic-like Metabolic Associated Steatotic Liver Disease (1).

From Thailand, Saokaew et al. present a systematic review of the effect of telemedicine in 285 patients with obesity and NAFLD in ALT, AST, TG, HDL-c levels, and body mass index outcomes. Interestingly, compared with usual care, in mostly urban populations, telemedicine significantly reduced the AST and ALT levels, indicating that some benefits can be obtained using this approach for the follow-up and treatment of patients in geographical areas where usual care is not available. This study also reminds us that even an affordable text message, telephone call, or online class can have potential benefits for the health of participants (Saokaew et al.). However, the weakness of Saokaew's review is that their metanalysis includes only three studies. In another manuscript from South Korea, the authors point out that Helicobacter pylori is a vastly prevalent bacteria that not only causes gastritis, peptic ulcers, and gastric cancer but is also linked to metabolic syndrome and cardiovascular diseases, especially arterial stiffness. Using the logistic regression model, Choi et al. conclude that Hp infection additively increases the risk of arterial stiffness in subjects with NAFLD/MAFLD, which is not surprising since there is a link between Hp infection, NAFLD, and the pathogenesis of insulin resistance and proinflammatory conditions (Choi et al.). Since the term NAFLD was coined more than four decades ago, it has changed twice, in 2020 and 2023, to highlight the importance of metabolic alterations in its pathophysiology (2, 3). In this manuscript, authors use both criteria to

analyze 2,357 subjects included in an NAFLD cohort diagnosed by ultrasound and 3,195 subjects in MAFLD analysis. Regardless of the criteria, male patients had significantly more atherosclerosis risk factors predominant in NAFLD/MAFLD. In both analyses, Hp infection was independently associated with arterial stiffness as well as, a high FIB-4 index and a measurement of fibrosis (Choi et al.).

In an influential review, Falamarzi et al. summarize the role of FGF21 in liver diseases, focusing on NAFLD, AFLD, and HCC. FGF21 and its analogs have been tested to treat these pathologies due to their role in inducing beta-oxidation, improving insulin sensitivity, and decreasing VLDL. FGF21 has also been proposed as a biomarker for NAFLD since its increase correlates with the amount of hepatic fatty acids. Therefore, its use as a delay agent not only for NAFLD but also for AFLD and HCC seems opposite; however, its protective role has been validated in knockout FGF21 mice that showed enhanced inflammation and steatohepatitis. FGF21 administration in experimental alcoholic liver disease demonstrated positive effects in preventing fatty liver progression serum lipid profiles and reduction in oxidative stress. FGF21 analog administration in mice models and human metabolic diseases showed anti-inflammatory, anti-diabetic, and hypolipidemic effects, reversing hepatic steatosis. Especially in HCC, overexpression of FGF21 delays adenoma development, however, it accelerates the progression of tumors to HCC due to FGFR1 interaction. FGF21 decrement has a role in progression to HCC, inducing a microenvironment wherein inflammation, mitotic regenerating factors for hepatocytes, and fibrosis predominate. Furthermore, some genetic variants for FGF21 have been linked to MAFLD, risk behaviors (alcohol, candy, and cigar consumption), and eating habits connecting these variations to obesity and alcohol dependence, diseases in which steatosis pathophysiology is important. In conclusion, FGF21 is a molecule that should be in our minds when thinking about metabolic diseases concerning the liver (Falamarzi et al.).

Griffett and Burris elegantly review how LXR inverse agonist molecules enabled the recruitment of corepressors that silence *de novo* lipogenesis (DNL)-related genes act in dyslipidemia and MAFLD. As nuclear receptors, LXRs are master regulators of lipid and cholesterol metabolism, intricately involved in the regulation of DNL in the liver, meaning they are very interesting potential drug targets. The use of LXR agonists displays anti-atherogenic properties by increasing cholesterol efflux from peripheral tissues; however, this can also lead to outcomes in hepatic steatosis. Since LXRα and LXRβ can recruit either coactivators or corepressors, this research group has developed LXR ligands (SR9238 and SR9243) that selectively enhanced the ability of LXRs to recruit corepressors like NCoR ID1 and NCoR ID2 peptides and in consequence decreases the expression of *Fasn*, *Srebf1c*, and *Scd1*, specifically in the liver not affecting reverse cholesterol transport (RCT) in peripheral tissues. SR9238 administration decreased the expression of genes encoding DNL enzymes and in consequence diminished hepatic steatosis in DIO mice, ob^{-}/ob^{-} mice fed with a diet high in cholesterol, fructose, and trans-fat, mice under Lieber-DiCarli diet, ASH rodent model using chronic ethanol consumption plus "binge" ethanol doses, and even in a dual model of mice fed with high cholesterol/trans-fat/fructose and ethanol. Interestingly, these compounds decrease plasma LDL-C, with potential efficacy for hypercholesterolemia. Due to their beneficial effects, some other LXR inverse agonists with similar pharmacological profiles have been developed by other researchers and pharma companies, reaching phase I clinical trials for the treatment of severe dyslipidemia—TLC-2716- (Griffett and Burris).

In another contribution to this issue, researchers from Singapore use data from the National Health and Nutrition Examination Survey (1999–2018) of the general non-institutionalized population. Their results show that 30% of NAFLD patients had concomitant depression. Using AASLD-NAFLD criteria and defining depression as the use of antidepressants or $\geq$10 scores on the Patient Health Questionnaire-9 (PHQ-9); in multivariate analysis older age, female gender, diabetes, higher BMI, hypertension, being Hispanic or Caucasian; resulted in an increased risk of depression amongst individuals with NAFLD. The impact of suffering depression in NAFLD patients—adjusting for age, gender, race, BMI, and diabetes- is linked to complications like CVD and stroke, and a 50% increased risk of mortality (no significance for CVD mortality but weighted for cancer-related mortality). These complications are reduced in depression-treated NAFLD patients, compared to untreated depression. The link between depression and NAFLD comes from insulin resistance onset, which can alter insulin signaling in the brain, overexpression of TNFa, IL-6, and monoamine oxidase-A that may also potentiate mood disorders and the presence of concomitant diabetes and obesity, resulting in oxidative stress and inflammation (Ng et al.). Other studies have also found an association between depression and NAFLD (4) reminding us of the importance of including periodic screening for depression in clinical practice guidelines and holistic care for patients with NAFLD. An innovative study by Meyer et al. approaches an ancient issue in NAFLD dynamics that is involved with fibrosis development. Using data from seven published clinical studies in which patients had biopsy-proven NAFLD or NASH, they developed a computational continuous-time Markov chain model that claims to capture the well-known clinical heterogeneity of fibrosis progression to provide alertness for clinical trial design. As a proof of principle, they applied the model to quantify pioglitazone effects on fibrosis progression. The advantages of continuous-time Markov chain models are the fact that they are probabilistic and time-independent. In this particular model, five potential states represent each stage of fibrosis (according to Kleiner or Brunt scoring), and progressors move through the stages of fibrosis with a probability of progression or regression that is independent of how long the subject was in that fibrosis score. After data fitting and sensitivity analysis, the authors assessed how an intervention, such as pioglitazone, impacts the forward and reverse model parameters. Using the model, the results indicate that pioglitazone slows disease progression and reverses fibrosis, based on the faster transition to lower fibrosis scores, showing good fitting of observed and predicted data. Since fibrosis progression in NAFLD and NASH is not well characterized, this model could predict, given an initial distribution, a percentage of progressors from non-progressors, also this model estimates that intervention at early stages of fibrosis has more possibilities to improve fibrosis score. Regarding the aim of impact clinical trial design, employing this model suggests a

sample of 65 patients to detect a change of 0.5 in fibrosis score, supposing drug effects similar to pioglitazone, using 80% power. Taking into account variability in sample size and location, and pathologist observation. This model promises to become more powerful including more data from clinical studies or combining with bridging a quantitative systems pharmacology (QSP) model that connects mechanistic drug effects to clinical outcomes (Meyer et al.).

Two reviews in this Frontiers in Medicine issue, elegantly cover fibrosis from the exploration of multi-drug treatment to animal models. Dong et al. discuss the therapeutic strategy of multi-drug combination for MAFLD-related liver fibrosis (Dong et al.). It is now accepted that liver fibrosis can be reversed by eliminating the etiological agent, but not all causes of chronic liver damage can be successfully removed, especially for MAFLD-related liver fibrosis. Approaches adopting single lifestyle interventions have not controlled the prevalence of MAFLD and in consequence, other therapeutic strategies need to be added. Approximately 40% of MAFLD patients are non-obese. For this cohort, the pharmacological treatment for MAFLD and its related liver fibrosis might be beneficial, including drugs that act on lipogenesis and fat accumulation, antioxidants, and agents that act in inflammation and extracellular matrix accumulation. However, few treatments to date have reached acceptable outcomes when assessed by liver biopsy. The exploration of multi-drug combination therapies is therefore appropriate since various mechanisms of MAFLD and fibrosis progression can be targeted. The authors analyze the possibility of combining drugs including GLP-1 receptor agonists, acetyl-CoA carboxylase inhibitors, FXR agonists, PPARs modulators, endothelial cell modulators, and natural compounds like sesquiterpene ketone, hydroxytyrosol, and vitamin E. In conclusion, since MAFLD has become the primary cause of liver fibrosis and one of the most common indications for liver transplant, it has become essential to explore multi-drug combinations targeting diverse fibrosis regression mechanisms and MAFLD pathophysiology.

Concerning fibrosis animal models, Wu et al. review the applications, advantages, and disadvantages, to select the appropriate model according to the research purpose. Independently of the five forms - chemical, dietary, surgical, transgenic, and immune – that are used to induce liver fibrosis, the molecular mechanisms are involved in largely similar pathways, including oxidative stress, inflammation, alteration in hepatic lipid metabolism, hepatocyte injury, HSC activation, and increased production of ECM (Wu et al.). One of the major decisions involved with this is the selection of species as the basis of space availability and modeling time. For example, alcohol is easily accessible, but *ad libitum* consumption time goes up to 70 weeks to produce insufficient liver injury to cause fibrotic or cirrhotic lesions. While CCl4 i.p. intoxication is a simple method of short-duration induction and causes significant hepatic pathological changes. Here, the authors also recapitulate models that include body-composition modifications where animals become obese or develop insulin resistance, reflecting the pathophysiology of MASLD and related metabolic alterations. An exception to this is the choline-deficient L-amino-defined diet-induced liver fibrosis model and the methionine-choline deficient-induced liver fibrosis model, where animals do not gain weight and lack characteristics of metabolic syndrome; however, steatohepatitis and fibrosis can be induced within shorter time. Transgenic and immune induction methods require specialized facilities and personnel, meaning these models are expensive and less used. Lately, a combination of methods is a common and effective way to model actual situations such as the high prevalence of MASLD and chronic or binge ethanol consumption. Each model has its drawbacks and advantages, and the development of such a variety of models through the years has led to a current set of experimental models that fit almost any purpose and requirement.

This Research Topic also includes a final contribution by Sergi, examining the relationship between MASLD and aspartame. Aspartame—methyl L-α-aspartyl-L-phenylalaninate- is a sweetener labeled in 2023 as a possible carcinogenic compound that can be offered as an anti-steatogenic food component. Sergi reminds us that prolonged aspartame administration leads to hepatic fibrosis, upregulating Tgfb1, Col1A1, and aSma; reducing the activation of Nrf2 and increased lipid peroxidation, which triggers NLRP3 (NOD-like receptor containing protein 3) inflammasome activation and p53 induction. Aspartame reduces PGC1α, a transcriptional coactivator, that upregulates mitochondrial biogenesis, oxidative phosphorylation, respiratory capacity, and fatty acid β-oxidation. Even though these data were obtained in experimental animals, Sergi argues that the results may be relevant in humans and linked to liver cancer through NLRP3 inflammasome activation (Sergi). Artificially sweetened soft drinks have been modeled as a healthier alternative to other drinks, however, the use of aspartame, saccharine, acesulfame-K, sucralose, and neotame is controversial, since some studies found them to be linked to some neurological effects. These perspectives motivate the realization of clinical trials to confront this theoretical conjecture, a good reminder of the importance of basic research and its translation to clinical scenarios.

This Research Topic includes contributions from experts in this area, including original research and review articles that address MAFLD from basic and clinical perspectives and update important aspects of this research. More than ever, this research is vital for treating actual and silent diseases.

Author contributions

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SYSTEMATIC REVIEW
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Effects of Telemedicine on Obese Patients With Non-alcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis

Surasak Saokaew[1,2,3,4,5,6†], *Sukrit Kanchanasurakit*[1,2,3,4†], *Chayanis Kositamongkol*[7†], *Kanyanat Chaiyo*[4], *Thirada Jirapisut*[4], *Narakorn Aomsin*[4], *Pit Leewongsakorn*[4], *Nathorn Chaiyakunapruk*[8†] *and Pochamana Phisalprapa*[7*†]

[1] Center of Health Outcomes Research and Therapeutic Safety (Cohorts), School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand, [2] Unit of Excellence on Clinical Outcomes Research and IntegratioN (UNICORN), School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand, [3] Unit of Excellence on Herbal Medicine, School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand, [4] Division of Pharmacy Practice, Department of Pharmaceutical Care, School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand, [5] Biofunctional Molecule Exploratory Research Group, Biomedicine Research Advancement Centre, School of Pharmacy, Monash University Malaysia, Kuala Lumpur, Malaysia, [6] Novel Bacteria and Drug Discovery Research Group, Microbiome and Bioresource Research Strength, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Kuala Lumpur, Malaysia, [7] Division of Ambulatory Medicine, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, [8] Department of Pharmacotherapy, College of Pharmacy, University of Utah, Salt Lake City, UT, United States

Background: Little is known of the efficacy of telemedicine on the clinical outcomes of the high-risk group of non-alcoholic fatty liver disease (NAFLD) patients, such as those with obesity. This study aimed to determine the effects of telemedicine vs. usual care for the management of obese patients with NAFLD.

Methods: Literature searches were performed from inception to 1st June 2021 in the following databases: Cochrane CENTRAL, ScienceDirect, PubMed, and Scopus. Prospective trials assessed the effects of telemedicine on obese patients with NAFLD were included. The outcomes of interest were alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride, high-density lipoprotein cholesterol levels, and body mass index, which were reported as weighted mean difference (WMD) with 95% confidence interval (CI).

Results: Four studies were examined in the systematic review, one was excluded from the meta-analysis due to an inappropriate group-comparison. In all, 285 obese patients with NAFLD were included in the meta-analysis (70% of those received telemedicine intervention). The mean ages of the patients in the telemedicine and usual-care groups were 51.78 ± 5.91 and 47.30 ± 8.14 years, respectively. Telemedicine significantly decreased ALT levels compared with usual care (WMD = −18.93 U/L [95%CI: −25.97, −11.90]; I^2 = 53.8%), and it significantly decreased AST levels (WMD = −10.24 U/L [95%CI: −13.43, −7.05]; I^2 = 0.0%). However, telemedicine did not show significant benefits for the remaining outcomes.

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Edited by:
Marcello Dallio,
University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Valerio Rosato,
Ospedale Evangelico Betania, Italy
Maria Guarino,
Federico II University Hospital, Italy

***Correspondence:**
Pochamana Phisalprapa
coco_a105@hotmail.com

†ORCID:
Surasak Saokaew
orcid.org/0000–0002–1382–0660
Sukrit Kanchanasurakit
orcid.org/0000–0002–1268–2665
Chayanis Kositamongkol
orcid.org/0000–0001–7182–0733
Nathorn Chaiyakunapruk
orcid.org/0000–0003–4572–8794
Pochamana Phisalprapa
orcid.org/0000–0003–1995–4405

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Conclusion: Compared with usual care, telemedicine significantly reduced the AST and ALT levels of obese patients with NAFLD. Further long-term studies with clinical endpoints are needed to determine the best characteristics of telemedicine and to confirm the benefits.

Systematic Review Registration: PROSPERO [CRD42020207451].

Keywords: non-alcoholic fatty liver disease, obesity, telemedicine, systematic review, meta-analysis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the diagnoses that indicate a risk of developing worsened health outcomes. The prevalence of NAFLD ranges from 6 to 33% worldwide (1–3) and its incidence is rising in every region of the world (2). In Europe, NAFLD has been reported to involve about 52 million people annually, with a disease-management cost of around €35 billion per annum (4). Patients with NAFLD are at risk of developing not only chronic liver illnesses, but also other, non-communicable, chronic diseases such as those extra-hepatic manifestations, including cardiovascular diseases and chronic kidney disease, in the future (2, 5). The all-cause mortality rate of NAFLD patients is higher than that for the general population (6, 7). Additionally, there is evidence that NAFLD patients with central obesity have a higher risk of overall and cardiovascular disease-related mortalities than non-obese NAFLD patients (7).

A definite treatment for NAFLD patients has not yet been established, and most current pharmacological treatment trials have had insufficient power to show significant benefits for NAFLD patients (8). Lifestyle modification (diet, exercise, and weight reduction) has been advocated for use in the management of NAFLD patients, and it has been reported to be effective in reducing hepatic steatosis (1). An economic evaluation also demonstrated that weight reduction could not only prevent cirrhosis and hepatocellular carcinoma occurrences, but was also a cost-saving strategy, making it the most appropriate management approach for NAFLD patients (9). However, patient adherence to lifestyle changes is critical because a long time period is required not only to permanently change routine behavior but to achieve the treatment goal. To optimize patient adherence, an intervention such as telemedicine might prove to play a vital role.

Telemedicine is the integrated use of communication technology and health information with the aim of bettering patients' health outcomes by improving the availability of medical information and treatment accessibility. Telemedicine allows healthcare professionals to utilize advanced telecommunication services in order to provide medical services to patients (10, 11). Moreover, it has proven efficacy in improving patients' health outcomes for asthma, chronic obstructive pulmonary disease, heart failure, hypertension, and type 2 diabetes mellitus (12–14).

The COVID-19 pandemic highlighted that the development and implementation of telemedicine is key to maintaining good medical care without requiring patients to unnecessarily visit high infection-risk places—like hospitals. However, the conclusive effects of telemedicine for obese patients with NAFLD are yet to be established. This systematic review and meta-analysis therefore set out to determine the effects of telemedicine for obese patients with NAFLD.

METHODS

This article was performed and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (15). This study was registered in PROSPERO (registration number: CRD42020207451).

Search Strategy

The search was performed, without language restriction, from inception to 1st June 2021 using four major electronic databases: Cochrane CENTRAL, ScienceDirect, PubMed, and Scopus. The search terms were "Non-alcoholic fatty liver disease," "NAFLD," AND "Telemedicine." The literature search was limited to research on humans. The search algorithms are provided in **Supplementary Table 1**.

Study Selection and Eligibility Criteria

The inclusion criteria for the systematic review were all prospective studies in the 4 databases that had investigated the effects of telemedicine on the surrogate outcomes of obese patients with NAFLD. Obesity was defined as per each study's inclusion criteria, given that the cutoff BMI to indicate obesity varies by ethnicity. Since current practice rarely uses a liver biopsy as a diagnostic method for NAFLD, our inclusion criteria were not limited to those studies with biopsy-proven NAFLD patients. Instead, studies were acceptable if they had enrolled NAFLD patients who had been diagnosed with a reliable method, such as an imaging test. However, the included studies were required to have measured the surrogate outcomes of interest: aspartate aminotransferase (AST), alanine aminotransferase (ALT), body mass index (BMI), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) levels.

A study was excluded if it was a review article or meta-analysis, news report, letter, poster presentation, book, or documentation. In addition, papers for which only the abstract was accessible were excluded, as were non-human studies and work that recruited participants under 18 years of age. Duplicated studies were also removed during the review process.

As to the meta-analysis, studies were excluded if they did not perform usual care (as a control group) to compare the pooled effects of telemedicine and usual care. Furthermore, included studies were required to measure the results required for analysis of the different outcomes of the 2 care strategies. On the other

hand, our eligibility criteria specified no limitations on the characteristics or durations of the telemedicine interventions.

Data Extraction

Data from the studies were independently retrieved by five authors (SK, KC, TJ, NA, and PL) using a standard extraction form. The data items were first author; publication year; country of study; study setting; study area (i.e., urban vs. rural); study type; number and characteristics of participants; NAFLD diagnostic method; details of intervention and control; and outcome measurements. The categorization of the telemedicine interventions was based on their proposed activities and their involvements in the case management, consultation, education, monitoring, and reminding processes.

Quality Assessment

The included studies were independently assessed for their methodological quality by the five aforementioned authors using

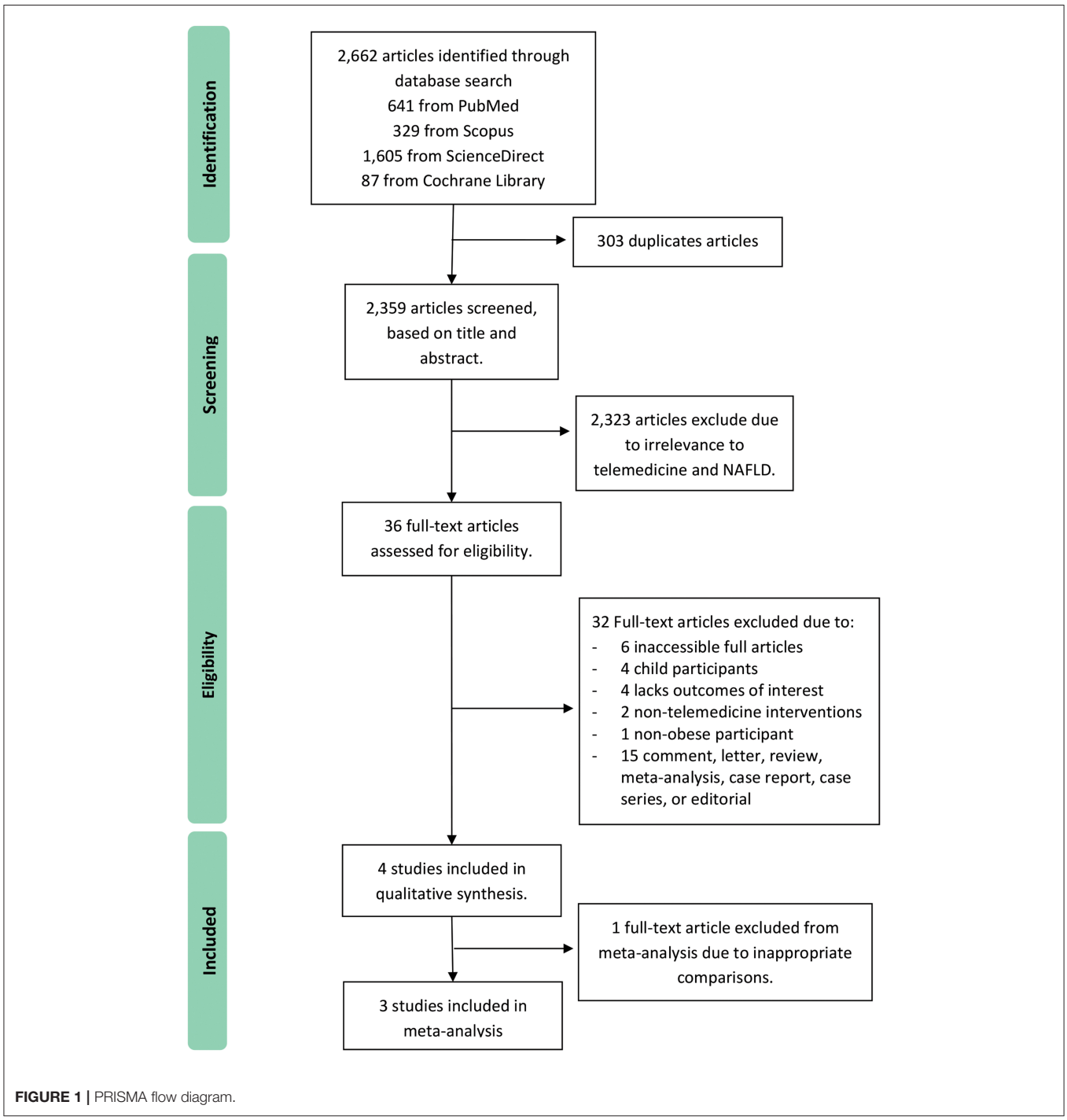

FIGURE 1 | PRISMA flow diagram.

the Cochrane Risk of Bias tool (version 2.0). The publication bias was not assessed as only a small number of studies were reviewed and pooled.

Data Analyses

A qualitative synthesis was conducted by summarizing the characteristic and outcomes of included studies. In addition, the attributes and features of telemedicine implemented in each study were extracted, described, and categorized in to 2 main types of communication, i.e., one-way and two-way communications. The results were reported in descriptive text and tables.

A meta-analysis was performed to estimate the effects of telemedicine compared with usual care. Weighted mean difference (WMD) and 95% confidence interval (CI) were used to calculate and compare those effects. The heterogeneity of the studies was assessed using the *I*-squared (I^2) statistical test (16). Subgroup analyses were also performed of the study designs (randomized controlled trial [RCT] and non-RCT) and intervention types (two-way and one-way communication).

RESULTS

The initial database search yielded 2,662 articles, of which 303 duplicates were removed. The rest were screened via their titles and abstracts, which led to the removal of another 2,323 because of their irrelevance to telemedicine and NAFLD. The remaining 36 articles were fully reviewed for their eligibility. That resulted in 32 being excluded from the systematic review due to the reasons described in **Figure 1**. In addition, another article was excluded from the meta-analysis due to inappropriate comparisons (17). A total of 4 studies (17–20) were reported in the systematic review results, and 3 studies (18–20) were included in the quantitative synthesis. A Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram is presented at **Figure 1**.

Systematic Review

All of the included studies were performed at University hospitals. Three of the four studies were conducted in western countries (17, 19, 20); the fourth was carried out in Western Asia (18). In addition, three (17–19) (75%) were conducted in urban areas, but one (20) (25%) did not identify the study area. Half of the studies were randomized controlled trials (RCTs) (18, 19), while the other half were non-RCTs (17, 20). The characteristic of the included studies is listed in **Table 1**. A total of 5 patterns of supportive management for NAFLD patients were performed, and these were able to be categorized into two types of communication: one-way and two-way. One-way communication involved text messaging (19), whereas two-way communication comprised telephone (18), on-site education (20), and web-based interventions (17, 20). The number of healthcare professionals involved in the telemedicine initiatives varied among the studies. Physicians were involved in all studies (17–20). Nutritionists/dietitians took part in two of the studies (17, 18). Nurses (20) and psychologists (17) were, each, involved in two separated studies. A variety of medical supportive management approaches were incorporated in the

TABLE 1 | Characteristics of the included studies.

References	Country	Setting	Studied area	Study type	Sample size (intervention vs. control)	Patient characteristics				Intervention	Control	Duration (months)
						Age* (year)	Male (%)	NAFLD diagnosis method	DM (%)			
Fard et al. (18)	Iran	Single school of Nursing and Midwifery	Urban	RCT	30 vs. 30	Intervention 40.3 Control 38.3	76.3%	Ultrasound	0%	Telephone	Usual care	3
Axley et al. (19)	USA	Department of Internal Medicine, University of Texas Medical	Urban	RCT	13 vs. 17	Intervention 54 ± 2.7 Control 52 ± 2.3	33%	Ultrasound, elevate liver enzyme	Intervention 38% Control 30%	Text messaging	Usual care	6
Vilar-Gomez et al. (20)	USA	4 Universities	NA	Non-RCT	157 vs. 38	Intervention 53.8 ± 8.4 Control 52.3 ± 9.5	37%	Non-invasive scores	100%	On-site education classes or via web-based	Usual care	12
Mazzotti et al. (17)	Italy	Unit of Metabolic Diseases and Clinical Dietetics, University of Bologna	Urban and rural	Non-RCT	278 vs. 438	Intervention web-treated 46.0 ± 11.5 Intervention group-treated 55.1 ± 12.3	53.5%	Ultrasound	Intervention web-treated 21.6% Intervention group-treated 40.6%	Web-based program	Group-base program	24

DM, diabetes mellitus; NA, not available; NAFLD, non-alcoholic fatty liver disease; RCT, randomized controlled trial; USA, United States of America.

**Values are presented as mean ± standard deviation.*

telemedicine activities: self-management support [2 studies (17, 20)]; monitoring patients' health status [4 studies (17–20)]; interactive communication [3 studies (17, 18, 20)]; action plan provision [3 studies (17–19)]; and education support [4 studies (17–20)]. No study utilized a reminding strategy. The duration between the provision of the individual telemedicine services ranged from daily to once a month, while the duration of the studies themselves ranged from 3 to 24 months. The features of the telemedicine initiatives and their supportive management protocols are detailed in **Table 2**. The results of the surrogate outcomes (such as weight reduction, liver enzyme decrease, and physical activity increase) of the NAFLD patients receiving telemedicine support were better than, or equal to, those of the usual-care or on-site interventions. A study by Fard et al. (18) demonstrated that telephone intervention provided by a nutritionist and a physician significantly decreased ALT and AST levels compared with those of a usual-care group ($P < 0.001$). A study by Axley et al. (19) investigated the effects of text-message management on ALT, AST, TG, HDL-C, and BMI levels. They found that the strategy significantly improved ALT and AST relative to usual care at 6 months ($P < 0.05$), but TG, HDL-C, and BMI were not affected by the intervention. Research by Gomez et al. (20) compared the effects of providing education classes on-site vs. via the Internet, and then compared both approaches with usual care. Their work revealed that both education strategies improved ALT, AST, TG, HDL-C, and BMI levels relative to usual care at 12 months ($P < 0.01$). However, the study by Gomez et al. did not report the differences between the outcomes of the on-site and web-based education approaches. This is because the 2 education groups were combined for analysis purposes, given that there were no significant differences in the values of the biochemical markers of those two groups at baseline and the conclusion of the 1-year study (21). Research by Mazzotti et al. concluded that an interactive, web-based intervention for obese patients with NAFLD was not less effective than a group-based lifestyle modification program in terms of ALT and TG reductions. Nevertheless, the effect of the web-based program on weight reduction was significantly better than that of the group-based program (17).

Meta-Analysis: the Pooled Effects of Telemedicine

Of the 4 studies, one conducted by Mazzotti et al. (17) was excluded from the meta-analysis because it did not compare the effects of telemedicine with usual care (**Table 3**). Mazzotti el al. compared the outcomes of patients who underwent telemedicine intervention, i.e., web-based program, with those patients participated in group-based program instead of usual care. Their group-based intervention provided more intensive strategy of NAFLD management such as five 120-min weekly sessions that chaired by multidisciplinary team. Psychologist was involved in one of the sessions which was a motivational session to stimulate weight reduction maintenance that usual care in other studies did not mention about. In all, the meta-analysis included 285 patients (18–20), 200 of whom were in the telemedicine group, with the remaining 85 in the control (i.e., usual care) group. The mean ages of the patients ranged from 38 to 54 years; the pooled average ages of the patients in the intervention and control groups were 51.78 ± 5.91 and 47.30 ± 8.14 years, respectively. The pooled baseline BMIs of the intervention and control groups were 37.78 ± 4.62 and 34.07 ± 5.09 kg/m^2, respectively.

Primary Outcomes

ALT: The meta-analysis pooled the outcomes of 3 studies (18–20) that investigated the effects of telemedicine on the ALT levels of obese patients with NAFLD. The results indicated that the telemedicine interventions contributed to significantly decreased ALT levels in obese patients with NAFLD relative to those receiving usual care (WMD = −18.93 U/L [95%CI: −25.97, −11.90]; I^2= 53.8%; **Figure 2A**). In the subgroup analyses, both the two-way and one-way communication interventions significantly reduced the ALT levels (two-way communication (18, 20): WMD = −19.91 U/L [95%CI: −35.17, −4.66]; I^2= 76.4%; and one-way communication (19): WMD = −19.00 U/L [95%CI: −24.48, −13.52]). The intervention-favored results were apparent in the RCT studies (18, 19) (WMD = −22.02 U/L [95%CI: −30.64, −13.40]; I^2 = 46.5%) and the non-RCT study (20) (WMD = −12.80 U/L [95%CI: −21.08, −4.52]). The subgroup analysis results are presented in **Supplementary Table 2**.

Secondary Outcomes

AST: The meta-analysis investigated 3 studies (18–20) to determine the effects of telemedicine on AST levels. The results revealed that the telemedicine interventions significantly decreased the AST levels of the obese patients with NAFLD compared with those undergoing usual care (WMD = −10.24 U/L [95%CI: −13.43, −7.05]; I^2= 0.0%; **Figure 2B**). In the subgroup analysis, both the two-way and one way-communication interventions resulted in significant reductions in AST levels (two-way communication (18, 20): WMD = −10.50 U/L [95%CI: −15.16, −5.85]; I^2= 0.0%; and one-way communication (19): WMD = −10.00 U/L [95%CI: −14.38, −5.62]). As to the RCT studies (18, 19), telemedicine showed WMDs between the groups of −10.31 U/L ([95%CI: −13.94, −6.67]; I^2= 0.0%), while the non-RCT study (20) showed a WMD of −10.00 U/L [95%CI: −16.65, −3.35]. The subgroup analysis results are detailed in **Supplementary Table 2**.

TG: The 2 studies (19, 20) that investigated the effects of telemedicine on TG levels were included in the meta-analysis. Their pooled results revealed that telemedicine did not significantly decrease the TG levels of the obese patients with NAFLD (WMD = −19.93 mg/dL [95%CI: −93.45, 53.59]; I^2= 85.4%; **Figure 2C**).

HDL-C: The 2 studies (19, 20) that examined the effects of telemedicine on HDL-C levels were analyzed. Their pooled results demonstrated that telemedicine did not significantly increase the HDL-C levels of the obese patients with NAFLD (WMD = 5.23 mg/dL [95%CI: −9.17, 19.64]; I^2= 96.8%; **Figure 2D**).

BMI: Three studies (18–20) exploring the effects of telemedicine on BMI were reviewed and pooled. The pooled results showed that telemedicine did not significantly lessen the BMI of the obese patients with NAFLD (WMD = −1.33 kg/m^2 [95%CI: −4.00, 1.34]; I^2= 77.0%; **Figure 2E**). Moreover,

TABLE 2 | Features of telemedicine and the supportive management protocols of the included studies.

References	Type of telemedicine	HCP's involvement/ device	Medical supportive managements						Duration of period between services
			Self-management support	Monitoring patients' health status	Interactive communication (Two-way communication)	Action plan provision	Education support	Reminding strategy	
Fard et al. (18)	Telephone	Nutritionist, Physician/ telephone	-	Patients were assessed evaluating the adherence to diet and physical activities.	Yes	Yes	Patients were provided dietary and physical activities education.	-	Twice a week during the first month and once a week in the second and the third months
Axley et al. (19)	Text message	Physician/ mobile	-	Patients were also asked about the challenges they face in making healthy diet changes and were provided tips for overcoming those specific challenges (e.g., not enough time, cost of healthy food and lack of knowledge).	-	Yes	Patients received the messages provided education on different domains including nutrition, exercise, and stress management.	-	1 week
Vilar-Gomez et al. (20)	On-site education classes (n = 136) or via web-based (n = 126)*	Physician or nurse practitioner/ web-based	Patients can select between two different educational modes; either via on-site education classes or via web-based	Patients were monitored glycemic and ketosis status through patient reported daily blood glucose and blood beta-hydroxybutyrate over the year.	Yes	-	Patients' dietary modifications included restricting total carbohydrate and fat, adequate intake of minerals, fluids and non-starchy vegetables	-	Daily monitoring for 1 year. On-site education was held weekly for first 12 weeks, bi-weekly for next 12 weeks, and monthly for 6 months. Recorded contents in web-based education could be independently accessed through the application.
Mazzotti et al. (17)	Web-based program	Physicians, dietitians, and psychologist	The individual sessions may be repeated without limitations.	Patients may interact with the clinical center offline, by sending food diaries or asking questions via specific tools.	Yes	Yes	The patients are provided with a series of 25–35 slides per sessions, with texts read by a voiceover and figures to support the text.	-	Sessions could be repeated without limitations. Outcomes were measured every 6 months

HCP, healthcare professional.

**No significant differences were observed in the biochemical markers of the 2 groups of patients for the different modes of education.*

TABLE 3 | Summary of study outcomes.

References	Interventions	Sample size (*N*)	Outcomes* (baseline vs. final visit)				
			ALT (U/L)	AST (U/L)	TG (mg/dL)	HDL-C (mg/dL)	BMI (kg/m^2)
Fard et al. (18)	Usual care + telephone	30	80.30 ± 38.59 vs. 36.60 ± 19.27	46.83 ± 17.64 vs. 28.80 ± 9.85	NA	NA	28.8 ± 4.32 vs. 26.36 ± 3.95
	Usual care	30	63.52 ± 23.67 vs. 65.03 ± 28.68	38.52 ± 12.90 vs. 39.79 ± 15.33	NA	NA	28.5 ± 4.28 vs. 27.92 ± 4.19
Axley et al. (19)	Usual care +text messages	13	44 ± 6 vs. 32 ± 3	46 ± 6 vs. 37 ± 4	184 ± 34 vs.167 ± 35	40 ± 4 vs. 38 ± 4	39 ± 2 vs. 38 ± 3
	Usual care	17	57 ± 12 vs. 51 ± 11	47 ± 7 vs. 47 ± 8	194 ± 37 vs. 153 ± 25	40 ± 3 vs. 40 ± 3	36 ± 2 vs. 37 ± 2
Vilar-Gomez et al. (20)	Usual care + interventions	157	39.2 ± 25.4 vs. 25.8 ± 15.4	28.5 ± 17.6 vs. 20.7 ± 8.5	196.3 ± 120.2vs. 152.8 ± 130.6	43.2 ± 13.8 vs. 49.9 ± 16.5	39.4 ± 7.2 vs. 34.3 ± 6.5
	Usual care	38	40.7 ± 23.4 vs. 38.6 ± 24.9	32.7 ± 26.1 vs. 30.7 ± 20.5	197.4 ± 100.5vs. 214.2 ± 149.8	41 ± 11.3 vs. 37.2 ± 11.4	37.6 ± 6.9 vs. 38.2 ± 8.2

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; NA, not available; TG, triglyceride.

**Values are presented as mean ± standard deviation.*

the subgroup analysis results indicated that telemedicine provided a significantly decreased BMI only for the two-way communication intervention (18, 20) (WMD = −2.53 kg/m^2 [95%CI: −4.79, −0.27]; I^2= 42.6%) and the non-RCT study (20) (WMD = −3.90 kg/m^2 [95%CI: −6.70, −1.10]). The subgroup analysis results are in **Supplementary Table 2**.

Quality Assessment

All 3 studies in the meta-analysis (18–20) had a negligible risk of bias in missing outcome data and reporting bias, but a substantial risk of performance bias blinding. Of the two non-RCTs in systematic review (17, 20), one (17) that was excluded from the meta-analysis had risks of concealment, blinding, and assessment. The risk-of-bias assessment are shown in **Supplementary Figure 1**.

DISCUSSION

Despite a few studies having been conducted to evaluate the use of telemedicine for NAFLD management, the conclusive effects of telemedicine are still to be clearly established. This is the first systematic review and meta-analysis to investigate the effects of telemedicine on the ALT, AST, TG, HDL-C, and BMI levels of obese patients with NAFLD. The addition of telemedicine was found to decrease ALT and AST levels better than that achieved via usual care alone. This evidence may guide the discovery or development of strategies that could work in conjunction with telemedicine to improve the management of NAFLD in the future. Regarding the effects of telemedicine on the management of other diseases, a systematic review and meta-analysis (22) investigated its impact on weight reduction and clinical-outcome improvement in obese patients. That work revealed that using a telephone produced a significant difference in the body weight loss achieved by members of the intervention and control groups. However, our meta-analysis showed that telemedicine did not significantly lessen the BMI of the obese patients with NAFLD.

The effects of telemedicine might depend on the characteristics and duration of the intervention. We attempted to elucidate the impact of those factors via the subgroup analyses, given that all 4 studies were conducted with various intervention durations and differing time points for outcome assessments. Unfortunately, as the number of relevant studies was too small, the only subgroup analyses that could be carried out related to evaluation of the treatment effects on ALT, AST, and BMI levels in patient subgroups defined by the study design and intervention type. The results of the subgroup analyses did not alter the results of the main analysis. Nevertheless, the results of the subgroup analyses should be interpreted with caution as they included two RCTs which examined different communication types. One critical issue is that NAFLD is a slow-progressing disease. Hence, in order to observe the final endpoints (e.g., cirrhosis, cancer, and non-liver-related outcomes), future studies should consider using a long follow-up period.

Although the reported average age of the patients in each included study was more than 18 years, the provided detail of those studies was not sufficient for us to determine if any non-adult patient was included in the cohort. This rigid

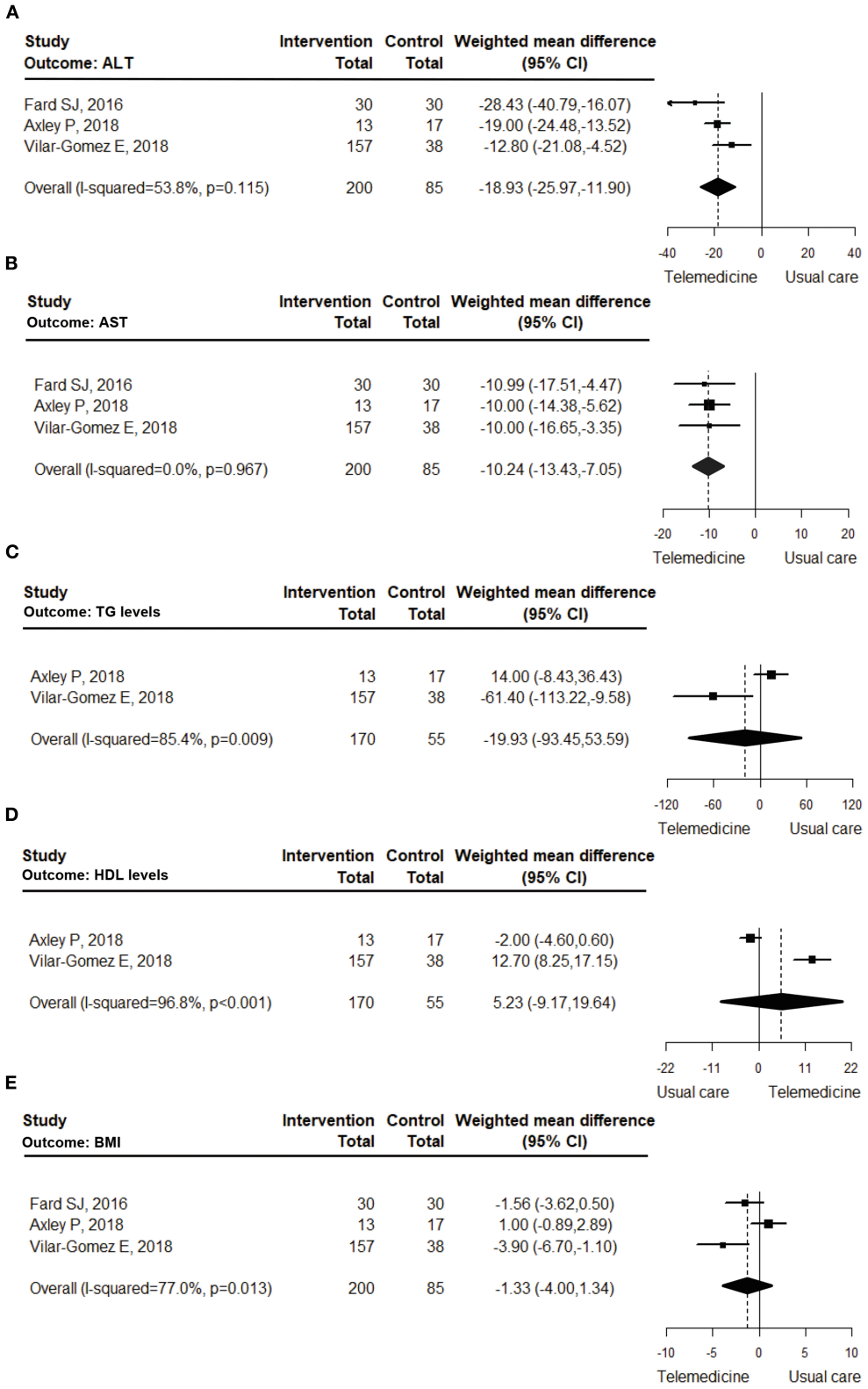

FIGURE 2 | Forest plot of the effects of telemedicine compared with usual care on the ALT **(A)**, AST **(B)**, triglyceride **(C)**, HDL-C **(D)**, and BMI **(E)** levels of obese patients with NAFLD.

inclusion of the current work is mentioned because we believe that the care of the adults and non-adults is distinctively different. Adolescents with NAFLD may need some help from their caregivers to maintain their proper diet and to manage their routine exercise, whereas adults are usually capable of self-care. Including adolescents in our review may create clinical heterogeneity from the disparity of patient groups. Furthermore, the effects of telemedicine for adolescent patients might be altered due to the additional support they receive from their caregivers, which is considered a non-telemedicine care strategy. Another point that we need to mention is that the study by Gomez et al. (20) consisted of both on-site and web-based education interventions. However, the multiple comparisons conducted by those researchers confirmed that the use of the on-site and web-based strategies resulted in no differences in their corresponding baseline and outcome biochemical markers.

The main limitations of this study are its small number of included studies and patients. These factors reduce the generalizability of the findings and make it difficult to recommend the use of tele-interventions in clinical practice. Also, none of those patients was biopsy-proven NAFLD. Nevertheless, they were diagnosed by standard imaging technique (i.e., ultrasound) and non-invasive prediction score (i.e., NAFLD liver fat score) which was proven to have a sensitivity of 86% and a specificity of 71% (23, 24). All patients with any secondary cause of hepatitis and those with significant alcohol intake were excluded. In addition, we were not able to perform the subgroup analyses of the intervention and follow-up durations because of the restricted number of included studies. This is unfortunate as the variations in the lengths of the interventions and follow-up periods of the included studies was suspected to be associated with the efficacy of telemedicine that was represented through the surrogate outcomes. Healthcare professionals who are considering the implementation of telemedicine interventions should evaluate whether their settings are comparable with those in the 4 studies. If they do decide to introduce telemedicine in their practice, they will need to determine their own follow-up period, given the absence of any current evidence for an appropriate intervention length.

In light of the COVID-19 pandemic, telemedicine is taking on an increasingly important role. It changes the traditional patterns of patient care by upgrading patient access to healthcare services, improving health outcomes, and reducing healthcare costs (25, 26). Telemedicine helps to provide patients with clinical education and supportive medical management. However, patients' inability to acquire, accept, or use the related technology could limit the use of telemedicine in practice, especially in developing countries. Although the effects of telemedicine interventions were evaluated by the present study, the challenge is to conduct research that determines the best characteristics of telemedicine for specific patient groups and analyzes the cost-effectiveness of various telemedicine strategies. Moreover, the ethical, legal, economic, and sociocultural aspects need to be considered before developing as well as implementing any strategy. Our findings provide healthcare professionals with current evidence of telemedicine's effects for NAFLD management. These findings can be used to guide the development of suitable telemedicine models for the care of obese patients with NAFLD.

CONCLUSION

In conclusions, this systematic review and meta-analysis gathered all relevant evidence and quantified pooled estimates to evaluate the effects of telemedicine on adult obese patients with NAFLD. The use of telemedicine significantly reduce AST and ALT levels in obese patients with NAFLD. Further long-term studies with clinical endpoints are needed to determine the best characteristics of telemedicine and to confirm the clinical benefits.

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 author.

ETHICS STATEMENT

The systematic review or meta-analysis is exempt from ethics approval because it is collecting and synthesizing data from previous studies. In addition, patient data is anonymized and data are available in the public domain so that ethical permission is not needed. The authors followed applicable EQUATOR Network (https://www.equator-network.org) guidelines during the conduct of research project.

AUTHOR CONTRIBUTIONS

SS, SK, CK, KC, TJ, NA, PL, NC, and PP: study concept and design. SS, SK, CK, KC, TJ, NA, and PL: acquisition of data. SS, SK, CK, KC, TJ, NA, PL, and NC: statistical analysis. SS, SK, CK, KC, TJ, NA, PL, and PP: analysis and interpretation of data and drafting of the manuscript. SS, SK, CK, NC, and PP: critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.723790/full#supplementary-material

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BRIEF RESEARCH REPORT
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Non-alcoholic/Metabolic-Associated Fatty Liver Disease and *Helicobacter pylori* Additively Increase the Risk of Arterial Stiffness

*Ji Min Choi[1], Hyo Eun Park[2], Yoo Min Han[1], Jooyoung Lee[1], Heesun Lee[2], Su Jin Chung[1], Seon Hee Lim[1], Jeong Yoon Yim[1] and Goh Eun Chung[1]**

[1] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Seoul National University Hospital Healthcare System Gangnam Center, Seoul, South Korea, [2] Division of Cardiology, Department of Internal Medicine, Seoul National University Hospital Healthcare System Gangnam Center, Seoul, South Korea

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***Correspondence:**
Goh Eun Chung
gohwom@snu.ac.kr

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Background: Non-alcoholic fatty liver disease (NAFLD) and *Helicobacter pylori* (*Hp*) infection have a close association with an increased risk of cardiovascular disease. Metabolic dysfunction-associated fatty liver disease (MAFLD) is characterized by metabolic dysfunction in NAFLD. We investigated the synergistic effects of NAFLD/MAFLD and *Hp* infection on the risk of arterial stiffness in an asymptomatic population.

Methods: We included individuals who underwent abdominal ultrasonography, anti-*Hp* IgG antibody evaluations and cardio-ankle vascular index (CAVI) during health screening tests between January 2013 and December 2017. Arterial stiffness was defined using CAVI. A logistic regression model was used to analyze the independent and synergistic effects of NAFLD/MAFLD and *Hp* infection on the risk of arterial stiffness.

Results: Among 3,195 subjects (mean age 54.7 years, 68.5% male), the prevalence of increased arterial stiffness was 36.4%. In the multivariate analysis, subjects with NAFLD but without *Hp* infection and those with both NAFLD and *Hp* infection had a significantly higher risk of increased arterial stiffness [odds ratio (OR) 1.61, 95% confidence interval (CI) 1.15–2.26, and OR 2.23, 95% CI 1.63–3.06, respectively], than subjects without *Hp* infection and NAFLD. Regarding MAFLD, *Hp* infection additively increased the risk of arterial stiffness in subjects with MAFLD (OR 2.13, 95% CI 1.64–2.78).

Conclusions: An interactive effect of *Hp* infection on the risk of arterial stiffness in individuals with NAFLD/MAFLD was observed. *Hp* infection additively increases the risk of arterial stiffness in subjects with NAFLD or MAFLD.

Keywords: *Helicobacter*, hepatic steatosis, arterial stiffness, risk, atherosclerosis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a substantial public health burden, with a prevalence of up to 25% of global population (1). NAFLD is closely associated with various metabolic conditions, including obesity, dyslipidemia, type 2 diabetes, and cardiovascular disease (2). In particular, NAFLD is known to be related to arterial stiffness, a surrogate marker of systemic atherosclerosis (3). Recently, in recognition of the close association between NAFLD and metabolic dysfunction, a new term "metabolic (dysfunction)-associated fatty liver disease (MAFLD)" has been introduced, and studies on its clinical significance have been conducted (4, 5).

Helicobacter pylori (Hp) is a gram-negative microorganism that infects more than half of the global population (6). While *Hp* is considered to cause many gastrointestinal diseases, such as chronic gastritis, peptic ulcers and gastric cancer (7, 8), its role in extragastric diseases, including metabolic syndrome and hematological and cardiovascular diseases, has also been studied (9). The association between *Hp* infection and cardiovascular risk factors or arterial stiffness has been reported (10–12). Some mechanisms, including chronic inflammation, free radical formation, and the immune response, may be the link between chronic *Hp* infection and atherogenesis (13). Also, *Hp* infection has been associated with insulin resistance (14), which is closely linked with increased arterial stiffness (15, 16).

Since both *Hp* infection and NAFLD are involved in the pathogenesis of insulin resistance and share proinflammatory conditions (17), they are known to be independently associated with arterial stiffness. Based on this background, we hypothesized that the combination of NAFLD and *Hp* infection increases the risk of arterial stiffness. Little has been reported about the association of MAFLD with arterial stiffness. Thus, we aimed to investigate the interactive effects of NAFLD/MAFLD and *Hp* infection on arterial stiffness in an asymptomatic population.

METHODS

Study Population

This retrospective cohort study included individuals who underwent routine health check-ups, including abdominal ultrasonography, anti-*Hp* IgG antibody testing and cardio-ankle vascular index (CAVI) evaluations, on the same day at the Seoul National University Hospital Healthcare System Gangnam Center from January 2013 to December 2017. The subjects were mostly symptom-free and willfully underwent examinations either voluntarily or were supported by their employers for the check-ups. Among the total eligible subjects, those who met the following criteria were excluded from the study: a prior history of ischemic heart disease, peripheral artery disease or stroke ($n = 132$), significant arrhythmia or valvular heart disease ($n = 31$), indeterminate anti-*Hp* IgG antibody results ($n = 43$), and a history of gastrectomy ($n = 20$) or *Hp* eradication ($n = 777$) (12). Finally, 3,195 subjects were included in the analysis. For the NAFLD analysis, subjects who displayed any potential cause of chronic liver disease were additionally excluded: 67 were positive for the hepatitis B virus, 29 were positive for the hepatitis C virus, and 763 had significant alcohol intake (>20 g/day). As a result, 2,357 subjects were included in the NAFLD analysis (**Figure 1**).

The study protocol followed the guidelines of the Declaration of Helsinki of 1975 and its revision in 1983. The protocol was approved by the Institutional Review Board of Seoul National University Hospital (No. 2005-051-1121). The requirement for informed consent was waived by the board, as researchers only accessed and analyzed deidentified data.

Measurement of Anthropometric and Laboratory Parameters

The methods employed in this study have been described previously in detail (18). Anthropometric and laboratory parameters were measured on the same day as the health check-ups. Body weight and height were measured using a digital scale, and body mass index (BMI) was calculated by dividing weight (kg) by the squared value of height (m^2). Waist circumference (WC) was measured at the midpoint between the lower costal margin and the anterior superior iliac crest by a well-trained person using a tape measure. Data regarding past medical history, comorbidities, and medication history were obtained using subject-recorded questionnaires. Based on smoking status, subjects were categorized as never or ever-smokers. The amount of alcohol each patient consumed was calculated. Blood pressure was measured at least twice, and mean values of the measurements were recorded. Hypertension was defined as blood pressure ≥140/90 mmHg or receiving antihypertensive medications. Diabetes was defined as a fasting blood glucose level ≥126 mg/dL or glycated hemoglobin A1c (HbA1c) level ≥6.5% or treatment with glucose-lowering agents. Dyslipidemia was defined as a total cholesterol level ≥240 mg/dL and/or triglyceride level ≥200 mg/dL and/or high-density lipoprotein (HDL) cholesterol level < 40 mg/d or the use of anti-dyslipidemic medications (19).

All blood samples were collected after a 12-h overnight fast. Laboratory tests included serum fasting glucose, total cholesterol, triglyceride, HDL cholesterol, HbA1c, and high-sensitivity C-reactive protein (hs-CRP) levels. All of these tests were performed using standard laboratory methods. The diagnosis of *Hp* infection was based on the results of a serum anti-*Hp* IgG antibody test using a commercially available chemiluminescent microparticle immunoassay kit (Immulite® 2000 CMIA, Siemens, Germany) as described previously (12). Values >1.10 IU/mL were considered positive (20). The *Hp* IgG kit has a sensitivity of 91% and a specificity of 100% (6).

Measurement of NAFLD/MAFLD and Advanced Fibrosis

Abdominal 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 individuals. 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 (21). MAFLD was diagnosed as the

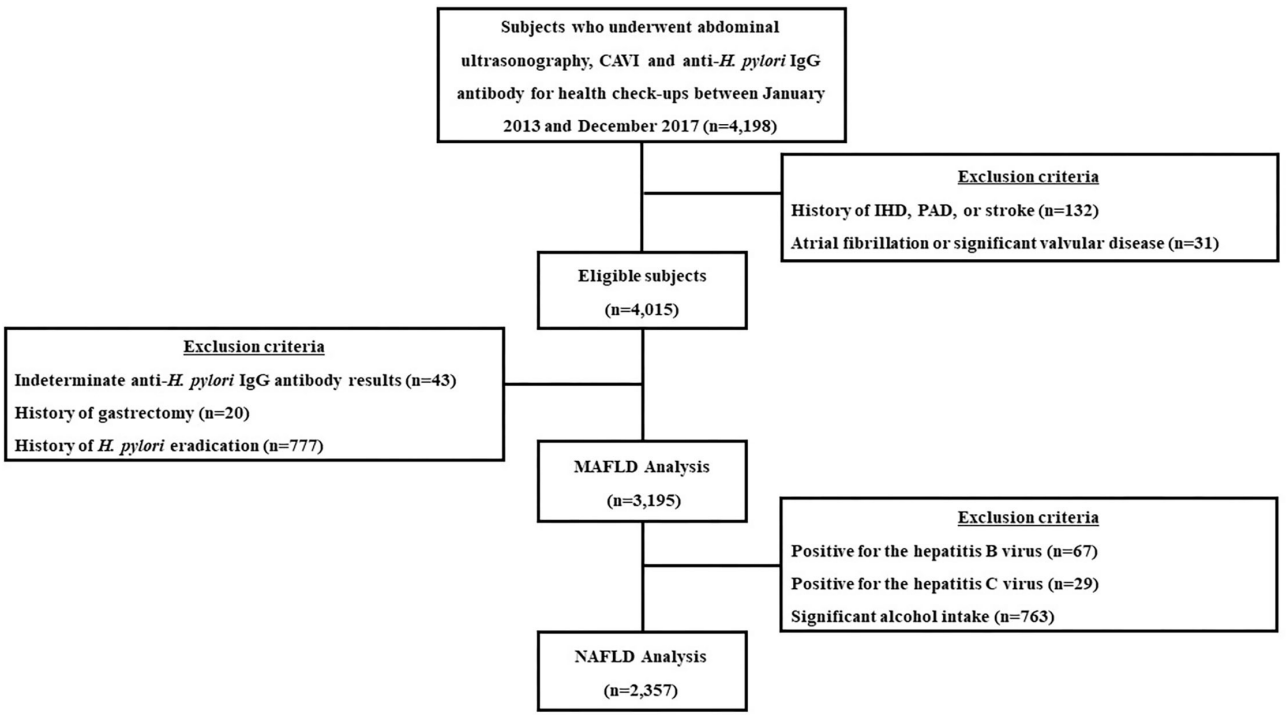

FIGURE 1 | A flow diagram of the study population. CAVI, cardio-ankle vascular index; *H. pylori, Helicobacter pylori*; n, number; IHD, ischemic heart disease; PAD, peripheral artery disease; MAFLD, metabolic-dysfunction associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease.

presence of hepatic steatosis with 1 or more of the following: (1) overweight or obese (BMI $\geq$ 23 kg/m^2) (2) diabetes mellitus (3) at least 2 metabolic risk abnormalities. Metabolic risk abnormalities consisted of (1) WC $\geq$ 90 cm for men and 80 $\geq$ cm for women, (2) blood pressure $\geq$ 130/85 mmHg or specific drug treatment, (3) fasting plasma triglycerides $\geq$ 150 mg/dl or specific drug treatment, (4) plasma HDL-cholesterol $<$ 40 mg/dl for men and $<$ 50 mg/dl for women or specific drug treatment, (5) prediabetes (fasting glucose 100–125 mg/dl or hemoglobin A1c 5.7–6.4%, (6) homeostasis model assessment of insulin resistance score $\geq$ 2.5, (7) plasma hs-CRP level $>$ 2 mg/L (5, 22).

For subjects with NAFLD or MAFLD, the Fibrosis-4 (FIB-4) index was used as a surrogate marker for advanced liver fibrosis. FIB-4 was calculated as age (years) $\times$ AST (U/L)/platelet (10^9/L) $\times \sqrt{}$ALT (U/L) and three risk categories (low, intermediate, high) for FIB-4 were based on the 2 cut points (1.30 and 2.67).We used the lower cutoff of FIB-4 index $<$1.30 to exclude advanced liver fibrosis (23).

Assessment of Arterial Stiffness Using CAVI

CAVI was measured using a VaSera VS-1000 (Fukuda Denshi Co Ltd, Tokyo, Japan) as described in previous studies to evaluate arterial stiffness (3, 12, 24). Briefly, the brachial pulse pressure was measured using an automated cuff oscillometer in seated individuals after 5 min of rest. The average value of two measurements was calculated to determine the systolic and diastolic pressures and pulse pressure. While the individuals were resting in a supine position, the cuffs were applied to ankles and both upper arms. After a 10 min rest period, the measurement was recorded. A phonocardiogram used for the detection of heart sounds was placed over the right sternum between the second intercostal spaces, and electrocardiogram electrodes were applied on both wrists. The pulse wave velocity was calculated as the vascular length (L) divided by the time (T) required for the pulse wave to propagate from the aortic valve to the ankle. Because the initiation of blood release from the aortic valve is difficult to identify based on the opening sound of the valve, T is difficult to determine; thus, the T value was calculated by summing the interval between the initiation of the brachial pulse waveform and the initiation of the ankle pulse waveform and the interval between the closing sound of the aortic valve and the notch of the brachial pulse waveform. Measurements were performed by a well-trained staff member. The CAVI was determined using the following equation:

$$\mathrm{CAVI} = a\left[\left(\frac{2\rho}{\Delta P}\right) \times ln\left(\frac{Ps}{Pd}\right) \times PWV^2\right] + b$$

where *P*s and *P*d are the systolic and diastolic blood pressures, respectively, ΔP is *Ps*–*Pd*, ρ is the blood density, and *a* and *b* are constants. The mean values of the left and right CAVI were used. We used a cutoff value of 8 to define increased arterial stiffness based on previous studies (12, 25, 26).

TABLE 1 | Comparison of baseline characteristics according to NAFLD and MAFLD.

	NAFLD analysis			MAFLD analysis		
	No NAFLD (*N* = 1,252)	**NAFLD (*N* = 1,105)**	***P*-value**	**No MAFLD (*N* = 1,699)**	**MAFLD (*N* = 1,496)**	***P*-value**
Age (years)	54.6 ± 9.9	55.5 ± 8.9	0.032	54.3 ± 9.6	55.0 ± 8.7	0.035
Male, *n* (%)	642 (51.3)	777 (70.3)	<0.001	1008 (59.3)	1179 (78.8)	<0.001
Smoker, *n* (%)	403 (32.2)	486 (44.0)	<0.001	661 (38.9)	806 (53.9)	<0.001
Hypertension, *n* (%)	437 (34.9)	600 (54.3)	<0.001	638 (37.6)	891 (59.6)	<0.001
Diabetes, *n* (%)	113 (9.0)	286 (25.9)	<0.001	151 (8.9)	400 (26.7)	<0.001
Dyslipidemia, *n* (%)	517 (41.3)	717 (64.9)	<0.001	684 (40.3)	970 (64.8)	<0.001
BMI (kg/m^2)	22.8 ± 2.9	25.5 ± 3.3	<0.001	23.0 ± 2.8	25.9 ± 3.1	<0.001
WC (cm)	82.3 ± 8.4	90.3 ± 8.7	<0.001	83.0 ± 8.3	91.3 ± 8.2	<0.001
SBP (mmHg)	123.8 ± 13.7	129.0 ± 13.5	<0.001	124.4 ± 13.5	130.1 ± 13.5	<0.001
DBP (mmHg)	80.3 ± 9.6	84.5 ± 9.3	<0.001	81.0 ± 9.6	86.0 ± 9.3	<0.001
Total cholesterol (mg/dL)	196.6 ± 37.3	199.0 ± 38.4	0.131	196.1 ± 36.6	198.0 ± 33.3	0.150
Triglyceride (mg/dL)[a]	71 (50,100)	118 (81,161)	<0.001	71 (50,102)	120 (82,165)	<0.001
HDL-cholesterol (mg/dL)	59.7 ± 15.5	50.2 ± 11.8	<0.001	59.7 ± 15.5	50.2 ± 11.8	<0.001
HbA1c (%)	5.6 ± 0.6	6.0 ± 1.0	<0.001	5.6 ± 0.6	6.0 ± 1.0	<0.001
Glucose (mg/dL)	97.9 ± 18.4	111.1 ± 29.2	<0.001	98.7 ± 17.8	112.3 ± 28.6	<0.001
Hs-CRP (mg/dL)	0.2 ± 0.6	0.2 ± 0.3	0.268	0.2 ± 0.6	0.2 ± 0.3	0.306
Helicobacter pylori infection, *n* (%)	704 (56.2)	660 (59.7)	0.086	954 (56.2)	896 (59.9)	0.033
Increased arterial stiffness, *n* (%)	402 (32.1)	455 (41.2)	<0.001	533 (32.5)	610 (40.8)	0.001

Continuous and categorical variables are shown as mean ± SD and numbers (percentages) NAFLD, nonalcoholic fatty liver disease; MAFLD, metabolic dysfunction-associated fatty liver disease; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HbA1c, glycated hemoglobin; Hs-CRP, high sensitivity C-reactive protein.

[a]*Median (interquartile range).*

Statistical Analysis

Continuous variables with a normal distribution are reported as the means ± SD or medians (with interquartile ranges) and categorical variables are reported as numbers and percentages. To test for normality, the Kolmogorov-Smirnov test and the normal Q-Q plots were used. Student's *t*-test was used when the data were normally distributed, and Mann–Whitney *U*-test was used otherwise. The differences between nominal variables were compared with the chi-square test or Fisher exact test. We divided participants into four groups according to the presence of NAFLD/MAFLD and/or *Hp* infection. A logistic regression analysis was utilized to analyze the association between NAFLD or MAFLD with *Hp* infection and increased arterial stiffness after adjusting for potential confounders. Among variables with a $P < 0.05$ in the univariate analysis, those with clinical importance were subjected to multivariate analyses. All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ were considered statistically significant.

RESULTS

Study Population

The mean age of 3,195 subjects was 54.7 years, and the proportion of males was 68.5%. The prevalence rate of increased arterial stiffness (CAVI ≥ 8) was 36.4%. **Table 1** shows the baseline characteristics of the study population according to the presence of NAFLD or MAFLD. Individuals with NAFLD or MAFLD have been observed more frequently in male (70.3 vs. 51.3% in NAFLD vs. no-NAFLD and 78.8 vs. 59.3% in MAFLD vs. no-MAFLD, respectively, $P < 0.001$), and ever smokers (44.0 vs. 32.2% in NAFLD vs. no-NAFLD and 53.9 vs. 38.9% in MAFLD vs. no-MAFLD, respectively, $P < 0.001$). In individuals with NAFLD or MAFLD, traditional risk factors of atherosclerosis were significantly more common compared to those without NAFLD or MAFLD: hypertension (54.3 vs. 34.9% in NAFLD vs. no-NAFLD and 59.6 vs. 37.6% in MAFLD vs. no-MAFLD, respectively, $P < 0.001$), diabetes (25.9 vs. 9.0% in NAFLD vs. no-NAFLD and 26.7 vs. 8.9% in MAFLD vs. no-MAFLD, respectively, $P < 0.001$), and dyslipidemia (64.9 vs. 41.3% in NAFLD vs. no-NAFLD and 64.8 vs. 40.3% in MAFLD vs. no-MAFLD, respectively, $P < 0.001$). In addition, most of the body measurements and laboratory results (including WC, BMI, systolic or diastolic blood pressure, triglyceride, HDL cholesterol, fasting glucose, and HbA1c levels) were less favorable in terms of metabolism in individuals with NAFLD or MAFLD ($P < 0.001$). The prevalence of increased arterial stiffness was significantly higher in both patients with NAFLD and MAFLD than those without NAFLD/MAFLD (41.2 vs. 32.1% in NAFLD vs. no-NAFLD and 40.8 vs. 32.5% in MAFLD vs. no-MAFLD, respectively).

Risk of Arterial Stiffness According to NAFLD/MAFLD and *Hp* Infection

We investigated the risk of increased arterial stiffness according to NAFLD/MAFLD and *Hp* infection. In the univariate analysis, subjects with *Hp* infection but without NAFLD had a significantly higher risk of increased arterial stiffness [odds ratio (OR) 1.33, 95% confidence interval (CI) 1.04–1.69] than subjects without *Hp* infection and NAFLD (used as a control group). Subjects with NAFLD but without *Hp* infection and those with both NAFLD and *Hp* infection also had a significantly higher OR for increased arterial stiffness (OR 1.47, 95% CI 1.12–1.92 and OR 1.95, 95% CI 1.53–2.48, respectively). When adjusting for multiple metabolic factors, including age, BMI, hypertension, diabetes, dyslipidemia, and smoking, the higher risk of increased arterial stiffness in the NAFLD (+) *Hp* (−) and NAFLD (+) *Hp* (+) groups remained (OR 1.61, 95% CI 1.15–2.26 and OR 2.23, 95% CI 1.63–3.06, respectively, **Table 2**). Regarding MAFLD, subjects with MAFLD but without *Hp* infection and subjects with both MAFLD and *Hp* infection exhibited significantly higher risks of increased arterial stiffness in a dose-dependent manner (OR 1.80, 95% CI 1.36–2.39 and OR 2.13, 95% CI 1.64–2.78, respectively). Meanwhile, in subjects without NAFLD or MAFLD, the risk of arterial stiffness tended to increase in the *Hp* (±) group compared to *Hp* (−), but statistical significance was not observed (OR 1.23, 95% CI 0.92–1.65, $P = 0.170$ and OR 1.16, 95% CI 0.91–1.48, $P = 0.237$, respectively).

When we performed an analysis stratified according to sex, the higher risk of increased arterial stiffness in subjects with both NAFLD and *Hp* infection persisted in both men and women (OR 2.28, 95% CI 1.52–3.42 and OR 2.18, 95% CI 1.29–3.68, respectively, **Supplementary Table 1**), and similar trends were observed for men and women subjects with MAFLD (OR 1.95, 95% CI 1.43–2.66 and OR 2.66, 95% CI 1.60–4.42, respectively).

Advanced Fibrosis, *Hp* Infection, and Increased Arterial Stiffness

Next, we performed subgroup analysis in patients with NAFLD/MAFLD for the association between advanced fibrosis, *Hp* infection and increased arterial stiffness. When participants with NAFLD/MAFLD were categorized according to the presence of advanced fibrosis using the FIB-4 index, high FIB-4 index was significantly associated with increased risk of arterial stiffness compared to low FIB-4 index in both patients with NAFLD and MAFLD (NAFLD: OR 2.53, 95% CI, 1.87–3.43, $P < 0.001$ and MAFLD: OR 2.95, 95% CI, 2.31–3.76, $P < 0.001$). *Hp* infection was independently associated with arterial stiffness in both patients with NAFLD and MAFLD (NAFLD: OR 1.36, 95% CI, 1.04–1.78, $P = 0.026$ and MAFLD: OR 1.31, 95% CI, 1.05–1.64, $P = 0.016$, **Table 3**).

TABLE 2 | Univariate and multivariate analyses of the risk for arterial stiffness in the total population.

	Univariate OR (95% CI)	*P*-value	Multivariate OR (95% CI)	*P*-value
NAFLD[a]				
Age	1.17 (1.15–1.18)	<0.001	1.16 (1.15–1.18)	<0.001
Sex	1.14 (0.96–1.35)	0.136		
Hypertension	2.96 (2.49–3.52)	<0.001	2.19 (1.76–2.73)	<0.001
Diabetes	2.78 (2.23–3.47)	<0.001	1.51 (1.14–2.00)	0.004
Dyslipidemia	1.76 (1.49–2.09)	<0.001	1.08 (0.87–1.34)	0.489
Body mass index	0.96 (0.94–0.99)	0.005	0.86 (0.83–0.90)	<0.001
Smoking	1.43 (1.20–1.69)	<0.001	1.93 (1.55–2.40)	<0.001
NAFLD and *HP*				
NAFLD (−) *Hp* (−)	1 (Ref)		1 (Ref)	0.256
NAFLD (−) *Hp* (+)	1.33 (1.04–1.69)	0.021	1.23 (0.92–1.65)	0.170
NAFLD (+) *Hp* (−)	1.47 (1.12–1.92)	0.005	1.61 (1.15–2.26)	0.006
NAFLD (+) *Hp* (+)	1.95 (1.53–2.48)	<0.001	2.23 (1.63–3.06)	<0.001
MAFLD[b]				
Age	1.16 (1.15–1.17)	<0.001	1.17 (1.15–1.18)	<0.001
Sex	1.12 (0.95–1.30)	0.181		
Body mass index	0.95 (0.93–0.98)	<0.001	0.89 (0.87–0.92)	<0.001
Smoking	1.44 (1.25–1.66)	0.011	2.07 (1.73–2.47)	<0.001
MAFLD and *HP*				
MAFLD (−) *Hp* (−)	1 (Ref)		1 (Ref)	
MAFLD (−) *Hp* (+)	1.35 (1.10–1.66)	0.004	1.16 (0.91–1.48)	0.237
MAFLD (+) *Hp* (−)	1.49 (1.19–1.87)	0.001	1.80 (1.36–2.39)	<0.001
MAFLD (+) *Hp* (+)	1.85 (1.50–2.27)	<0.001	2.13 (1.64–2.78)	<0.001

NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; CI, confidence interval; MAFLD, metabolic dysfunction-associated fatty liver disease; Hp, Helicobacter pylori.
[a]Adjusted for age, body mass index, hypertension, diabetes, dyslipidemia, and smoking.
[b]Adjusted for age, body mass index, and smoking.

TABLE 3 | Multivariate analyses of the risk for arterial stiffness in subjects with NAFLD/MAFLD.

	NAFLD[a]		MAFLD[b]	
	Adjusted OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
Hypertension	2.90 (2.19–3.84)	<0.001		
Diabetes	1.89 (1.39–2.57)	<0.001		
Dyslipidemia	1.19 (0.90–1.58)	0.229		
Body mass index	0.85 (0.82–0.89)	<0.001	0.88 (0.84–0.91)	<0.001
Smoking	1.48 (1.13–1.92)	0.004	1.38 (1.11–1.72)	0.004
High FIB-4 vs. Low FIB-4	2.53 (1.87–3.43)	<0.001	2.95 (2.31–3.76)	<0.001
Helicobacter pylori	1.36 (1.04–1.78)	0.026	1.31 (1.05–1.64)	0.016

NAFLD, nonalcoholic fatty liver disease; MAFLD, metabolic dysfunction-associated fatty liver disease; OR, odds ratio; CI, confidence interval; FIB-4, fibrosis 4 index.
[a]Adjusted for body mass index, hypertension, diabetes, dyslipidemia, and smoking.
[b]Adjusted for body mass index, and smoking.

DISCUSSION

To the best of our knowledge, our study is the first to show an interactive effect of NAFLD/MAFLD and *Hp* infection on arterial stiffness. In the present study, a significantly increased risk of arterial stiffness was observed in subjects with NAFLD/MAFLD and *Hp* infection compared with subjects without these conditions. *Hp* infection additively increased the risk of arterial stiffness in subjects with NAFLD or MAFLD.

Arterial stiffness is one of the major indicators of systemic atherosclerosis and is closely related to cardiovascular risk (27). Since increased arterial stiffness is associated with adverse cardiovascular outcomes, even in the general population (28), measurements of arterial stiffness may be helpful to identify high-risk groups for cardiovascular diseases. As a novel indicator of arterial stiffness, CAVI represents the stiffness of the entire arterial segment and is independent of blood pressure, making it highly reproducible and easy to measure (29). Thus, CAVI has been used as a screening tool to evaluate the subclinical atherosclerotic risk in asymptomatic individuals (30). In the present study, we used CAVI as a tool to measure arterial stiffness and revealed that both the presence of NAFLD/MAFLD and *Hp* infection were independently associated with increased arterial stiffness.

Previous studies have investigated the association between NAFLD and increased arterial stiffness. Arterial stiffness indicated by CAVI was associated with the ultrasonography-diagnosed presence and severity of NAFLD (3), and arterial stiffness measured using the augmentation index was associated with more severe NAFLD histology in the biopsy-proven NAFLD cohort (31, 32). NAFLD defined using controlled attenuation parameters also showed a significant association with increased arterial stiffness (24, 33). Consistent with previous results, the presence of NAFLD/MAFLD and advanced fibrosis were independently associated with arterial stiffness in our study.

Several possible mechanisms supporting the association between NAFLD and arterial stiffness are plausible. NAFLD has been recognized as a hepatic manifestation of metabolic syndrome and is closed associated with hyperglycemia, dyslipidemia, and insulin resistance (34), all of which are associated with subclinical inflammation, vascular endothelial cells damage, prothrombotic status, and hemodynamic changes that may increase the risk of atherosclerosis (35). Increased oxidative stress (36), chronic subclinical inflammation (37), reduced levels of adiponectin (38) and altered production of coagulant factors can be involved in the pathogenesis of atherosclerosis in patients with NAFLD.

On the other hand, several studies have suggested that *Hp* infection increases cardiovascular disease, including coronary artery disease and peripheral arterial stiffness (39–41). Choi et al. found that *Hp* seropositivity was significantly associated with increased arterial stiffness (12). Yoshikawa et al. reported that *Hp* infection accelerated the effects of impaired glucose metabolism and increased arterial stiffness (42). Chronic *Hp* infection has been reported to trigger an inflammatory reaction and release inflammatory cytokines, which lead to endothelial dysfunction (43). According to Yu et al. the combination of *Hp* infection and NAFLD increases carotid artery plaque formation, a surrogate marker of atherosclerosis (OR = 1.93). The risk of atherosclerosis was significantly increased in the fatty liver (±) *Hp* (±) group, but not in the fatty liver (±) *Hp* (−) group in the previous study (44). Consistently, *Hp* infection additively increased the risk of arterial stiffness in subjects with NAFLD/MAFLD, and the risk was higher than that of previous study with OR = 2.23 and 2.13 in our study. Moreover, the risk of atherosclerosis showed a dose-dependent relationship in *Hp* (−) NAFLD/MAFLD and *Hp* (±) NAFLD/MAFLD [OR (95% CI), 1.61 (1.15–2.26) and 1.80 (1.36–2.39) in Hp (−) NAFLD/MAFLD vs. 2.23 (1.63–3.06) and 2.13 (1.64–2.78) in Hp (±) NAFLD/MAFLD, respectively]. Collectively, arterial stiffness, measured using CAVI, is a novel approach in the present study, and this association was probably attributed to the synergistic effect of *Hp* infection and NAFLD/MAFLD on atherosclerosis.

Because NAFLD is a sexually dimorphic disease with respect to epidemiological and clinical features (45), we performed an analysis stratified according to sex. The increased risk of arterial stiffness in subjects with both NAFLD/MAFLD and *Hp* infection persisted in both men and women, suggesting the additive effect of *Hp* infection and NAFLD/MAFLD on arterial stiffness in both sexes.

Liver fibrosis is a crucial prognostic factor for cardiovascular outcomes in NAFLD (46). When we evaluated the association between FIB-4 index and increased arterial stiffness in subjects with NAFLD or MAFLD, high FIB-4 index was associated with increased arterial stiffness compared to low FIB-4 index in both NAFLD and MAFLD, suggesting the role of advanced fibrosis in the subclinical atherosclerosis. In line with our results, advanced fibrosis was associated with carotid atherosclerosis in patient with NAFLD (47, 48).

Interestingly, BMI showed an inverse correlation with arterial stiffness in this study. This phenomenon has also been reported in previous studies, and part of this complex association can be explained by the obesity paradox (49, 50). That is, it is explained that some of the patients with elevated BMI benefit from the preservation of arterial stiffness by increased metabolic reserves, attenuated response to renin–angiotensin–aldosterone system, greater muscular strength, potentially protective cytokines and neuroendocrine factors (51, 52). However, there are studies showing that obesity is associated with high CAVI levels and insulin resistance, an independent predictor of vascular stiffness, so additional studies are needed (53, 54).

Our study has several limitations. First, the cross-sectional nature of the study design limits the ability to assess cause and effect. Thus, we were unable to infer causal relationships from this study. Second, the NAFLD diagnosis was exclusively based on ultrasonography, but was not confirmed by liver biopsy, which is the standard diagnostic modality for confirming NAFLD. Ultrasonography has a high specificity but underestimates hepatic steatosis when the fat content is <20% and is unable to quantify fibrosis (55). However, liver biopsy is not typically performed in asymptomatic individuals, and radiographic techniques such as ultrasonography or magnetic resonance imaging are used to diagnose NAFLD in clinical practice. Third, we could not exclude patients with chronic liver disease due to causes other than viral and alcoholic hepatitis. In addition, subjects who take steatogenic drugs could not excluded from this study. However, since this study is based on health check-up examination data targeting asymptomatic adults, the prevalence of this area is thought to be low. Fourth, although the serological test does not discriminate current and past *Hp* infections (56), the *Hp* infection status was assessed only with serology and not other assessment methods, such as the urease breath test or a rapid

urease test, in the present study. Due to its cost-effectiveness and invasiveness, serology tests are a common method used in health screening centers that conduct routine blood sampling. We thoroughly investigated the history of *Hp* eradication therapy to supplement the shortcomings of serological tests and overcome this limitation. Fifth, although significant alcohol consumption is considered to be >30 g for men and 20 g for women per day according to the Korean Association for the Study of the Liver Clinical Practice Guideline for NAFLD (57), sex-specific criteria could not be applied to the amount of alcohol consumed in this study. Last, our study population of those who underwent health evaluations upon their own initiative may not represent the majority of the general Korean population, which may contribute to selection bias.

CONCLUSIONS

We demonstrated the synergistic effect of *Hp* infection and NAFLD/MAFLD on the risk of arterial stiffness among asymptomatic Koreans. *Hp* infection additively increases the risk of arterial stiffness in subjects with NAFLD or MAFLD. Therefore, evaluating *Hp* infection status in patients with NAFLD/MAFLD may be helpful in cardiovascular risk assessment. Further studies are needed to determine whether eradication of *Hp* and adequate management of NAFLD/MAFLD helps to improve arterial stiffness and prevent cardiovascular disease.

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 author/s.

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: conceptualization. GC, JC, HP, YH, JL, HL, SC, and SL: data curation. JC: formal analysis. SC, JY, and SL: supervision. JC and GC: writing—original draft preparation. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

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Terry Cheuk-Fung Yip,
The Chinese University of Hong Kong, Hong Kong SAR, China

REVIEWED BY
Anand V. Kulkarni,
Asian Institute of Gastroenterology, India
Anna Alisi,
Bambino Gesù Children's Hospital (IRCCS), Italy

*CORRESPONDENCE
Cheng Han Ng
chenhanng@gmail.com
Mark Muthiah
mdcmdm@nus.edu.sg

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work

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Depression in non-alcoholic fatty liver disease is associated with an increased risk of complications and mortality

Cheng Han Ng [1*†], Jieling Xiao[1†], Nicholas W. S. Chew[1,2†], Yip Han Chin[1], Kai En Chan[1], Jingxuan Quek[1], Wen Hui Lim[1], Darren Jun Hao Tan[1], Ryan Wai Keong Loke[1], Caitlyn Tan[1], Ansel Shao Pin Tang[1], Xin Lei Goh[1], Benjamin Nah[3], Nicholas Syn[1], Dan Yock Young[1,3,4], Nobuharu Tamaki[5], Daniel Q. Huang[1,3,4], Mohammad Shadab Siddiqui[6], Mazen Noureddin[7], Arun Sanyal[6‡] and Mark Muthiah [1,3,4*‡]

[1]Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [2]Department of Cardiology, National University Heart Centre, National University Hospital, Singapore, Singapore, [3]Division of Gastroenterology and Hepatology, Department of Medicine, National University Hospital, Singapore, Singapore, [4]National University Centre for Organ Transplantation, National University Health System, Singapore, Singapore, [5]Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan, [6]Division of Gastroenterology, Hepatology and Nutrition, Department of Internal Medicine, Virginia Commonwealth University, Richmond, VA, United States, [7]Houston Research Institute, Houston, TX, United States

Background and aims: The global prevalence of non-alcoholic fatty liver disease (NAFLD) is expected to rise continuously. Furthermore, emerging evidence has also shown the potential for concomitant depression in NAFLD. This study aims to examine the prevalence, risk factors, and adverse events of depression in NAFLD and evaluate whether treated depression can reverse the increased risks of adverse outcomes.

Materials and methods: This study analyses the 2000–2018 cycles of NHANES that examined liver steatosis with fatty liver index (FLI). The relationship between NAFLD and depression was assessed with a generalized linear mix model and a sensitivity analysis was conducted in the no depression, treated depression, and untreated depression groups. Survival analysis was conducted with cox regression and fine gray sub-distribution model.

Results: A total of 21,414 patients were included and 6,726 were diagnosed with NAFLD. The risk of depression in NAFLD was 12% higher compared to non-NAFLD individuals (RR: 1.12, CI: 1.00–1.26, $p = 0.04$). NAFLD individuals with depression were more likely to be older, females, Hispanics or Caucasians, diabetic, and have higher BMI. Individuals with depression have high risk for cardiovascular diseases (CVD) (RR: 1.40, CI: 1.25–1.58, $p < 0.01$), stroke (RR: 1.71, CI: 1.27–2.23, $p < 0.01$), all-cause mortality (HR: 1.50, CI: 1.25–1.81, $p < 0.01$), and cancer-related mortality (SHR: 1.43, CI: 1.14–1.80, $p = 0.002$) compared to NAFLD individuals without depression. The risk

of CVD, stroke, all-cause mortality, and cancer-related mortality in NAFLD individuals with treated depression and depression with untreated treatment was higher compared to individuals without depression.

Conclusion: This study shows that concomitant depression in NAFLD patients can increase the risk of adverse outcomes. Early screening of depression in high-risk individuals should be encouraged to improve the wellbeing of NAFLD patients.

KEYWORDS

depression, NAFLD, NASH, NHANES, complication, mortality

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the fastest growing cause of chronic liver disease affecting 25–33% of the prevalence and is expected to rise in conjunction with the current obesity pandemic (1–3). NAFLD comprises two subtypes including non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) (4) with the latter associated with more adverse outcomes (5). However, there are currently no Food and Drug Administration (FDA) approved medications for NASH and liver transplant (6) is reserved for patients with end-stage liver disease (7, 8).

While the presence of NAFLD has been known to be associated with a host of extrahepatic complications including increasing the risk of cardiovascular disease (9), chronic kidney disease, osteoporosis (4) and malignancies, emerging evidence has since shown the potential for NAFLD to develop depression (10, 11). A recent meta-analysis by Xiao et al. involving 2,041,752 patients found that the presence of NAFLD was associated with a 1.29 increase in the odds of depression (OR: 1.29, CI: 1.02–1.64, p = 0.03). Additionally, a longitudinal study by Labenz et al. found a 21% increase in the risk of antidepressants use in NAFLD patients. The presence of depression in the general population has been associated with a plethora of health complications such as increased tendency for development of cardiovascular diseases (CVD), metabolic syndrome and heightened risk of mortality (12–15).

Yet, the impact of depression in NAFLD has not been well-examined with only a previous analysis by Sayiner et al. showing that depression in NAFLD increases the rate of all-cause mortality (16). Here, we sought to comprehensively examine the prevalence, risk factors, and related adverse events that depression poses to NAFLD individuals in the United States with data from the National Health and Nutrition Examination Survey (1999–2018) study. We additionally sought to examine the risks of all-cause mortality, competing for risk of cardiovascular mortality, and cancer-related mortality in NAFLD individuals with depression. Lastly, we examined treating depression in NAFLD can mitigate the increased risks of adverse outcomes.

Materials and methods

Study population

This study analyses the 2000–2018 cycles of NHANES carried out by the United States National Centre for Health Statistics by the Centre for Disease Control and Prevention (CDC). The NHANES study was a cross-sectional survey platform that adopted a stratified, multistage, clustered probability sampling design. Individual representatives of the general non-institutionalized population were identified and studied. It includes a structured interview conducted in the home and a standardized health examination conducted at a mobile examination center subsequently. The standardized health examination encompasses a physical examination and laboratory tests. The complete methodology of NHANES data collection was published previously (17). National Centre for Health Statistics Research Ethics Review Board has approved the original survey. As the data used in the analysis is de-identified and publicly available, review by the Institutional Review Board was not required. The study population for this study's analysis included adult NAFLD patients, aged 18 years and older with fatty liver. Participants diagnosed with other etiologies of liver disease (alcohol, autoimmune, hepatitis B or C), retroviral disease, or participants without data on measurements of depression were excluded from the analysis. Patients with a positive human immunodeficiency virus diagnosis were also excluded from the analysis.

Definitions

The definition of NAFLD was adapted based on the American Association for the Study of Liver Disease (AASLD) guidelines for NAFLD (18) and was defined as the presence of hepatic steatosis is the absence of substantial alcohol use (≥2 drinks a day in men, ≥3 drinks a day in women). The presence of steatosis was detected by Fatty Liver Index (FLI) and the United States Fatty Liver Index (US-FLI) where possible with a cut-off of 60 (19) and 30 (20), respectively.

Overweight patients were defined as a BMI ≥ 25 for Caucasians and BMI ≥ 23 for Asians where data was available (21). Hypertension was defined as having a blood pressure of ≥140 mm Hg. Diabetes was defined as the presence of self-report presence of diabetes, glycohemoglobin ≥6.5%, or fasting plasma glucose ≥7 mmol/l. A diagnosis of depression was defined as the use of antidepressants or elevated depression scores on the Patient Health Questionnaire-9 (PHQ-9). The scoring system ranged from 0 to 27 with clinically significant depressive symptoms defined as scores ≥10 (22). The PHQ-9 adopted the modified criteria from the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV), and used categorical algorithms to diagnose psychiatric disorders (23), and questions were asked at the Mobile Examination Centre by trained interviewers in face-to-face interview (24). A PHQ-9 score of ≥10 has a sensitivity and specificity of 88% for a clinical diagnosis of depression and has been a well-validated tool for identifying major depressive disorders (22, 25, 26). The subjects responded to the frequency in which they experienced depressive symptoms over the last 2 weeks, on a scale of 0–3. These symptoms included anhedonia, sleep disturbance, fatigue, depressed mood, affected appetite, low self-esteem, suicidal ideation, concentration challenges, and suicidal ideation. Adverse outcomes were defined as events including cardiovascular disease, cancer, stroke, chronic kidney disease, and mortality. The presence of cardiovascular disease, cancer, and stroke was retrospectively reported by patients based on a formal diagnosis from a physician. The presence of chronic kidney disease was defined as an estimated glomerular filtration rate of 60 and less calculated based on the Modification of Diet in Renal Disease (MDRD) formula. Mortality was identified from supplementary data in the national death index based on ICD-9 Codes.

Statistical analysis

Analytical methods were designed with reference to previously published studies (27–29). Statistical analysis was conducted using STATA (16.1 StataCorp, Texas, United States).

TABLE 1 Summary of baseline characteristics of included population.

	NAFLD	Non-NAFLD	*P*-value
Age (Years)	52.16 (IQR: 39.00 to 65.00)	44.81 (IQR: 28.00 to 60.00)	<**0.01***
Diabetes (%)	0.29 (95% CI: 0.28 to 0.30)	0.12 (05% CI: 0.11 to 0.12)	<**0.01***
HTN (%)	0.63 (95% CI: 0.62 to 0.64)	0.41 (95% CI: 0.40 to 0.41)	<**0.01***
Body mass index (kg/m^2)	34.68 (IQR: 30.17 to 37.80)	26.92 (IQR: 22.91 to 29.52)	<**0.01***
Waist circumference (cm)	112.50 (IQR: 103.20 to 119.60)	93.60 (IQR: 82.90 to 101.50)	<**0.01***
Weight (kg)	95.81 (IQR: 81.45 to 106.20)	75.53 (IQR: 62.15 to 84.90)	<**0.01***
Platelet count	258.36 (IQR: 211.00 to 297.00)	250.11 (IQR: 206.00 to 287.00)	<**0.01***
Glycohemoglobin	6.06 (IQR: 5.40 to 6.20)	5.58 (IQR: 5.10 to 5.70)	<**0.01***
Fasting glucose (mmol/L)	6.69 (IQR: 5.44 to 6.77)	5.79 (IQR: 5.05 to 5.88)	<**0.01***
Total bilirubin (μmol/L)	10.09 (IQR: 6.84 to 11.97)	11.40 (IQR: 8.55 to 13.68)	<**0.01***
Total cholesterol (mg/dL)	198.21 (IQR: 169.00 to 224.00)	190.00 (IQR: 161.00 to 215.00)	<**0.01***
LDL-Cholesterol (mg/dL)	116.37 (IQR: 91.00 to 138.00)	111.00 (IQR: 86.00 to 133.00)	<**0.01***
Direct HDL-Cholesterol (mg/dL)	48.01 (IQR: 39.00 to 55.00)	55.52 (IQR: 44.00 to 65.00)	<**0.01***
Triglycerides (mg/dL)	194.43 (IQR: 112.00 to 235.00)	133.75 (IQR: 71.00 to 158.00)	<**0.01***
Depression score	5.29 (IQR: 2.00 to 7.00)	4.84 (IQR: 2.00 to 6.00)	<**0.01***
Antidepressants			
Antidepressants Use (%)	0.20 (95% CI: 0.19 to 0.21)	0.15 (95% CI: 0.15 to 0.16)	<**0.01***
No antidepressants use (%)	0.80 (95% CI: 0.79 to 0.81)	0.85 (95% CI: 0.84 to 0.85)	<**0.01***
Gender			
Male (%)	0.39 (95% CI: 0.38 to 0.40)	0.46 (95% CI: 0.45 to 0.47)	<**0.01***
Female (%)	0.61 (95% CI: 0.60 to 0.62)	0.54 (95% CI: 0.53 to 0.55)	
Ethnicity			
Mexican American (%)	0.17 (95% CI: 0.16 to 0.18)	0.15 (95% CI: 0.15 to 0.16)	<**0.01***
Other Hispanic (%)	0.09 (95% CI: 0.09 to 0.10)	0.10 (95% CI: 0.09 to 0.10)	
Caucasians (%)	0.44 (95% CI: 0.43 to 0.46)	0.46 (95% CI: 0.45 to 0.46)	
African American (%)	0.21 (95% CI: 0.20 to 0.22)	0.19 (95% CI: 0.18 to 0.19)	
Other race (%)	0.08 (95% CI: 0.08 to 0.09)	0.11 (95% CI: 0.10 to 0.11)	

NAFLD, non-alcoholic fatty liver disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HTN, hypertension; IQR, interquartile range; 95% CI, 95% confidence interval.
*Bolded *p*-value ≤ 0.05 denotes statistical significance.

Descriptive statistics were summarized in median and interquartile range (IQR) for continuous variables and proportions with a 95% confidence interval for binary variables. A non-parametric Wilcoxon rank sum test and chi square test were used to compare continuous variables and dichotomous variables, respectively. A generalized linear mix model with a log link, gaussian family, and robust variance estimator was used to estimate the effect size of binary events in risk ratios (RR). A risk ratio is a more robust measure of binary events when the events are uncommon as opposed to an odds ratio. Mortality outcomes were examined with a cox proportional model for hazard ratios (HR) and violations of proportionality were examined with Schoenfeld residuals and a log-log plot. A separate competing risk analysis was used to examine the risk of CVD mortality and cancer-related mortality with the fine gray sub-distribution hazard ratios (SHR). A cluster analysis was also included in both RR, HR, and SHR based on the year of study to account for heterogeneity introduced by the year of study. Subsequently, a sensitivity analysis was conducted of the included population into 3 groups: (1) no depression, (2) treated depression, and (3) untreated depression. We defined treated depression as a PHQ-9 of <10 with antidepressants and untreated depression as a PHQ-9 $\geq$ 10 without treatment.

Results

Baseline characteristics of included population

A total of 21,414 patients were included in the analysis and 6,726 were identified to have NAFLD. The summary of baseline characteristics can be found in Table 1. NAFLD individuals were significantly older with diabetes and a higher BMI measurement. The use of antidepressants was similarly more common in NAFLD compared to non-NAFLD individuals. Of the 6,726 individuals with NAFLD, 2,017 (30%, CI: 28.9 to 31.1%) individuals had either an elevated PHQ-9 score suggestive of depression or reported use of antidepressants (Figure 1). After adjusting for cofounders in a generalized linear model with variables including age, gender, race, diabetes, overweight, and a cluster variable on the year of study, the risk of depression in NAFLD was 12% higher compared to non-NAFLD individuals (RR: 1.12, CI: 1.00 to 1.26, $p = 0.04$).

Factors associated with depression

Non-alcoholic fatty liver disease individuals with depression were more likely to be older, females, diabetics, and higher BMI (Table 2). Ethnicities were also influencing factors in the diagnosis of depression in NAFLD. In multivariate analysis, an older age (RR: 1.01, CI: 1.00 to 1.02, $p < 0.01$), female gender

FIGURE 1
Proportion of NAFLD patients with depression.

(RR: 1.42, CI: 1.32 to 1.52, $p < 0.01$), and diabetes (RR: 1.10, CI: 1.32 to 1.52, $p = 0.03$) increase the risk of depression in NAFLD. A higher BMI resulted in a marginal increase in the risk of depression (RR: 1.01, CI: 1.00 to 1.02, $p < 0.01$). With Mexican Americans as a reference, other Hispanics (RR: 1.17, CI: 0.98 to 1.39, $p < 0.01$) and Caucasians (RR: 1.49, CI: 1.35 to 1.63, $p < 0.01$) resulted in an increased risk of depression amongst individuals with NAFLD. Higher total cholesterol and triglyceride levels similarly result in a marginal increase in the risk of depression in NAFLD (RR: 1.01, CI: 1.00 to 1.02, $p < 0.01$) in multivariate analysis. Multivariate adjustments for hypertension, however, resulted in a significantly higher risk of depression in NAFLD (RR: 1.21, CI: 1.12 to 1.30, $p < 0.01$).

Complications in non-alcoholic fatty liver disease

Overall analysis of depression impact in non-alcoholic fatty liver disease

A multivariate generalized linear model with a robust variance estimator adjusting for age, gender, race, BMI, and diabetes was used to compare the outcomes between individuals with and without depression in NAFLD (Figure 2). Individuals with depression were more likely to be associated with CVD (RR: 1.40, CI: 1.25 to 1.58, $p < 0.01$) and stroke (RR: 1.71, CI: 1.27 to 2.23, $p < 0.01$) but not CKD (RR: 1.05, CI: 0.92 to 1.21, $p = 0.46$) compared to NAFLD individuals without depression. A Cox proportional model was used to examine the risk of all-cause mortality, and there were no violations of the cox proportional model examined with Schoenfeld residuals and log-log plot (Figure 3). Individuals with depression were at a 50% increased risk of mortality (HR: 1.50, CI: 1.25 to 1.81, $p < 0.01$). There was no statistically significant increased risk of CVD mortality (SHR: 1.38, CI: 0.83 to 2.34, $p < 0.01$) compared to NAFLD individuals without depression. Cancer-related mortality, however, was higher in NAFLD individuals with depression (SHR: 1.43, CI: 1.14 to 1.80, $p = 0.002$).

Sensitivity analysis by treated and untreated depression

A sensitivity analysis was conducted to examine the effect of depression in a subdivided population of NAFLD including individuals without depression as a reference, treated

TABLE 2 Baseline characteristics of population with and without depression in NAFLD.

	Depression	No depression	*P*-value
Age (Years)	54.22 (IQR: 44.00 to 65.00)	51.27 (IQR: 37.00 to 65.00)	<**0.01***
Diabetes (%)	0.32 (95% CI: 0.30 to 0.34)	0.27 (95% CI: 0.26 to 0.28)	<**0.01***
HTN (%)	0.69 (95% CI: 0.67 to 0.71)	0.61 (95% CI: 0.59 to 0.62)	<**0.01***
Body mass index (kg/m^2)	35.15 (IQR: 30.50 to 38.40)	34.48 (30.00 to 37.51)	<**0.01***
Waist circumference (cm)	113.36 (IQR: 104.00 to 120.60)	112.13 (IQR: 103.00 to 119.00)	<**0.01***
Weight (kg)	95.68 (IQR: 81.10 to 106.60)	95.87 (IQR: 81.50 to 106.10)	0.88
Platelet count	263.97 (IQR: 213.00 to 302.00)	255.96 (IQR: 209.00 to 295.00)	<**0.01***
Glycohemoglobin	6.10 (IQR: 5.40 to 6.30)	6.04 (IQR: 5.40 to 6.20)	0.17
Fasting glucose (mmol/L)	6.82 (IQR: 5.44 to 6.96)	6.63 (IQR: 5.44 to 6.72)	0.41
Total bilirubin (μmol/L)	9.75 (IQR: 6.84 to 11.97)	10.23 (IQR: 6.84 to 11.97)	<**0.01***
Total cholesterol (mg/dL)	201.55 (IQR: 171.00 to 228.00)	196.78 (IQR: 167.00 to 222.00)	<**0.01***
LDL-Cholesterol (mg/dL)	116.94 (IQR: 90.00 to 139.00)	116.11 (IQR: 91.00 to 138.00)	0.48
Direct HDL-Cholesterol (mg/dL)	48.74 (IQR: 40.00 to 57.00)	47.75 (IQR: 39.00 to 55.00)	<**0.01***
Triglycerides (mg/dL)	200.03 (IQR: 119.00 to 241.00)	192.03 (IQR: 109.00 to 232.00)	<**0.01***
Depression score	10.85 (IQR: 6.00 to 15.00)	3.40 (IQR: 2.00 to 5.00)	<**0.01***
Gender			
Male (%)	0.29 (95% CI: 0.27 to 0.31)	0.43 (95% CI: 0.42 to 0.45)	<**0.01***
Female (%)	0.71 (95% CI: 0.69 to 0.73)	0.57 (95% CI: 0.55 to 0.58)	
Ethnicity			
Mexican American (%)	0.13 (95% CI: 0.11 to 0.14)	0.18 (95% CI: 0.17 to 0.20)	<**0.01***
Other Hispanic (%)	0.09 (95% CI: 0.08 to 0.11)	0.09 (95% CI: 0.09 to 0.10)	
Caucasians (%)	0.54 (95% CI: 0.52 to 0.56)	0.40 (95% CI: 0.39 to 0.42)	
African American (%)	0.18 (95% CI: 0.16 to 0.19)	0.23 (95% CI: 0.22 to 0.24)	
Other race (%)	0.06 (95% CI: 0.05 to 0.07)	0.09 (0.08 to 0.10)	

LDL, low-density lipoprotein; HDL, high-density lipoprotein; HTN, hypertension; IQR, interquartile range; 95% CI, 95% confidence interval. *Bolded p-value $\leq$ 0.05 denotes statistical significance.

depression, and untreated depression in NAFLD. Results from the multivariate generalized linear model with a robust variance estimator adjusted for age, gender, race, BMI and diabetes found a reduced magnitude of events with treated depression in NAFLD compared to untreated depression (**Figure 4**). The risk of CVD events in individuals with treated depression and untreated depression was 17% (RR: 1.17, CI: 1.10 to 1.27, $p < 0.01$) and 77% (RR: 1.58, CI: 1.33 to 1.89, $p < 0.01$) higher compared to individuals without depression. Similarly, the risk of stroke was higher in untreated depression (RR: 1.97, CI: 1.31 to 2.95, $p < 0.01$) compared to treated depression (RR: 1.38, CI: 1.00 to 1.91, $p = 0.05$) with reference to NAFLD individuals without depression. However, the risk of CKD was not increased in both treated and untreated depression (RR: 1.02, CI: 0.84 to 1.26, $p = 0.82$; RR: 1.02, CI: 0.88 to 1.18, $p = 0.76$, respectively). In the analysis of all-cause mortality, both treated (RR: 1.41, CI: 0.98 to 2.04, $p = 0.07$) and untreated depression (RR: 1.68, CI: 1.21 to 2.33, $p = 0.01$) were at increased risk of all-cause mortality. There were no violations of the cox proportional model (**Figure 5**). Additionally, CVD mortality was significantly increase in untreated depression (SHR: 1.66, CI: 0.94 to 2.93, $p = 0.08$) but not in treated depression (SHR: 0.84, CI: 0.52 to 1.35, $p = 0.46$). Cancer-related mortality was similarly increased in treated and untreated depression (SHR: 1.63 CI: 1.23 to 2.10, $p < 0.01$ and SHR: 1.61, CI: 0.93 to 2.79, $p = 0.09$).

Discussion

Main discussion

Depression is common in chronic diseases and can affect treatment compliance, prognosis, and most importantly, a holistic care for patients (30). Evidence from the previous meta-analysis (10) has found a significant association between depression and NAFLD. And the potential mechanism underlying the findings may be explained by the strong ties between hepatic steatosis and insulin resistance, which can interfere with insulin signaling in brain mechanisms (31). Inflammatory markers and pro-inflammatory cytokines, including tumor necrosis factor-alpha and interleukin-6 may also potentiate mood disorders (32). The serotonin pathway can be affected in this group of patients as the expression of monoamine oxidase-A, one of the enzymes catalyzing monoamines like serotonin, has been shown to be increased in NASH (33). Moreover, the compounded presence of

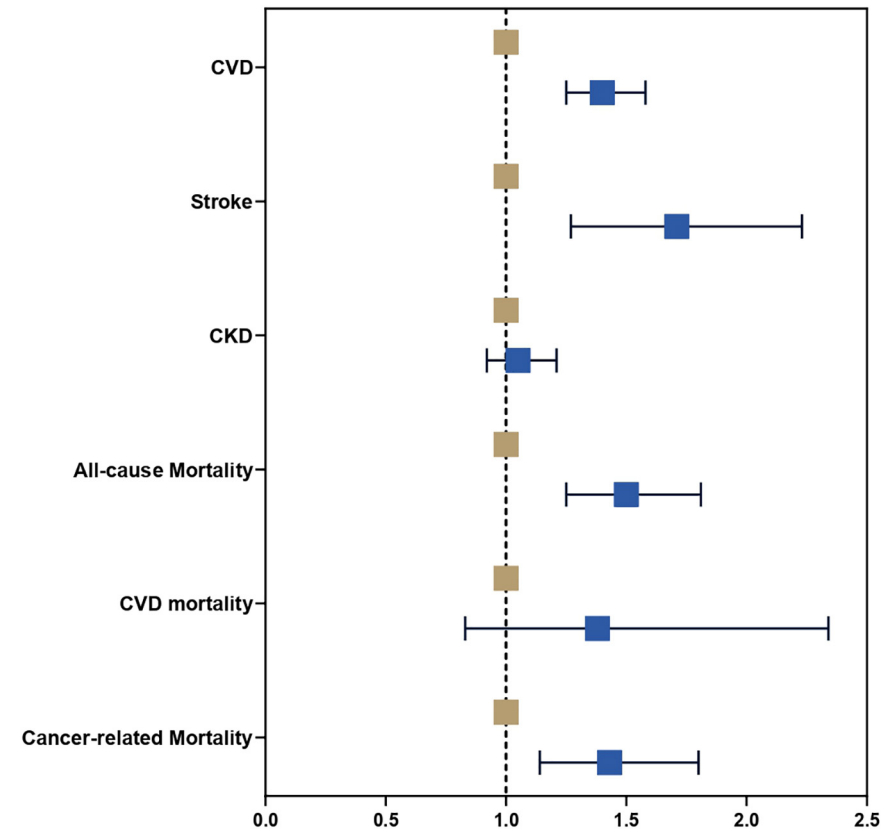

FIGURE 2
Forest plots of adverse outcomes in NAFLD with and without depression.

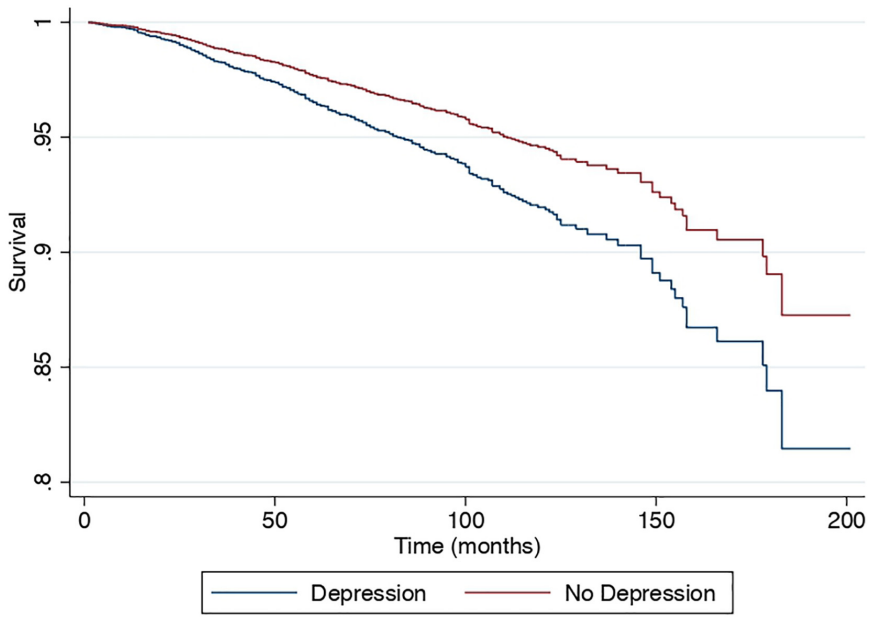

FIGURE 3
Cox proportional survival of all-cause mortality with and without depression.

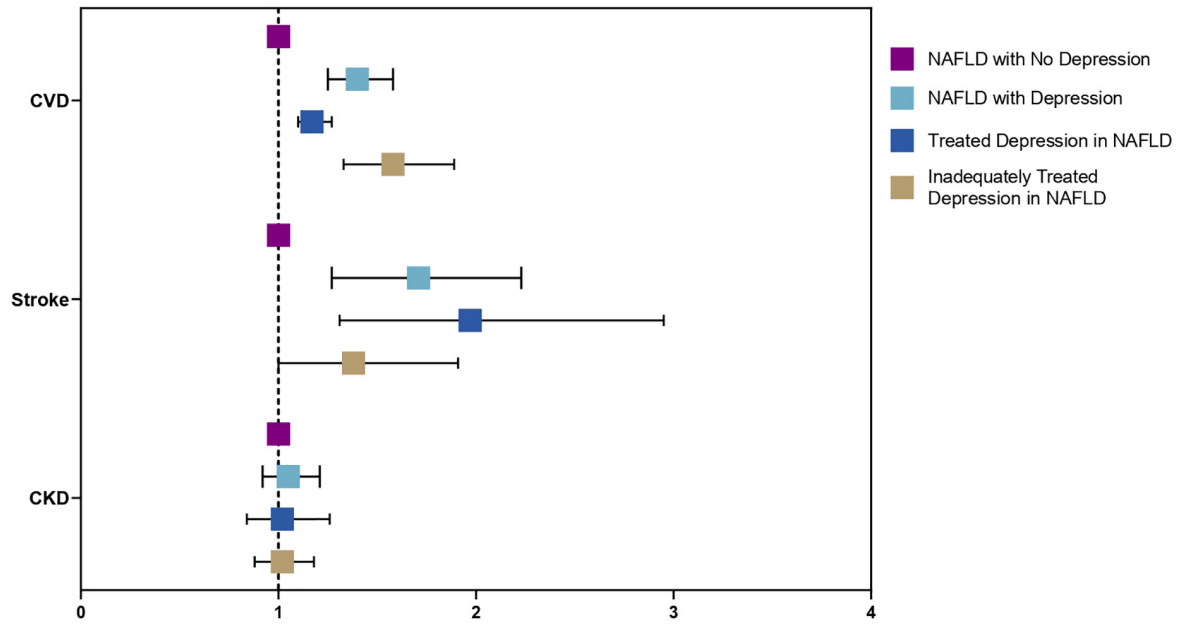

FIGURE 4
Forest plots of adverse outcomes in NAFLD with depression, without depression, treated, and untreated depression.

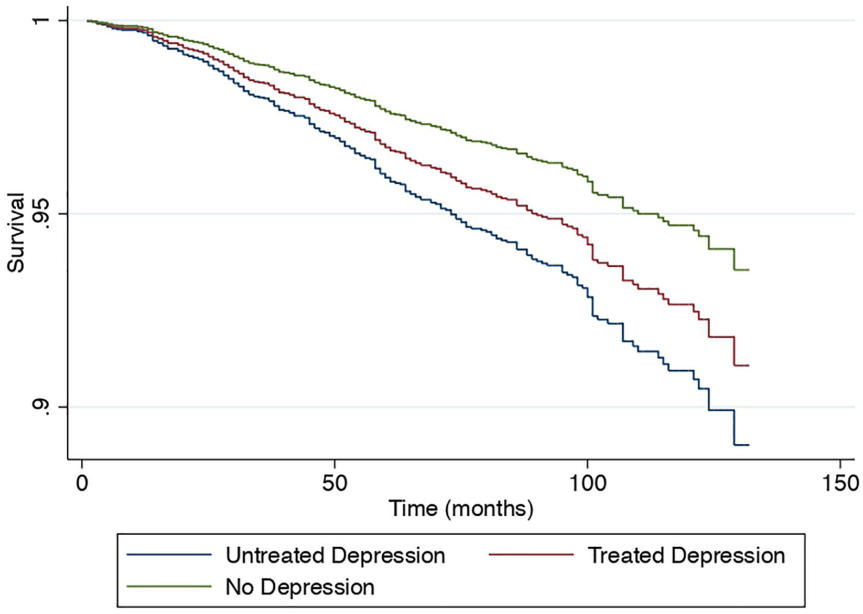

FIGURE 5
Cox proportional survival of all-cause mortality without depression, treated and untreated depression.

concomitant diabetes and obesity commonly associated with NAFLD can intensify the state of inflammation and oxidative stress resulting in a higher risk of depression (34–36).

In the current examination of the NHANES study from 2000 to 2018, we found that up to 30% of patients with NAFLD can be affected by depression with a 12% increase in the risk of depression compared to non-NAFLD individuals in multivariate analysis. Unsurprisingly, individuals who are older, females and Caucasians were at a significantly increased risk of depression in NAFLD. Previous literature has suggested that the increased susceptibility of older adults to depression might be attributed to the associated increase in the prevalence of

physical factors such as chronic diseases, organic brain diseases, malignancy, and psychosocial factors (37, 38). The higher risk for depression in females is corroborated by previous literature which highlighted the differences in hormonal changes between males and females (39, 40). In addition, Caucasians displayed a greater risk for depression as reflected in previous studies (41, 42). While some studies have reported that Hispanics and African Americans are more likely to present with depression compared to Caucasians, the difference could be due to disparities in the level of accessibility to mental health services (43). The presence of depression can significantly affect compliance (44) and prognosis in chronic disease (45). In NAFLD, Tomeno et al. found that the presence of depression resulted in a decrease benefits in standard care treatment with no reduction in steatosis markers after 48 weeks compared to NAFLD without depression (46). Also, similar to Sayiner et al., we found that NAFLD individuals with depression increase the rate of all-cause mortality (16). In addition, the cardiovascular burden was significantly higher in depressed individuals where depression increases the risk of CVD and stroke events. There is significant evidence in observational studies between NAFLD and CVD (47, 48), similarly, depression is closely associated with CVD and subsequent complications (49, 50). While the risk cannot be completely mitigated, treated depression was, however, associated with a reduced magnitude of risk of CVD events and all-cause mortality. Treated depression in fact resulted in a non-significant difference in CVD mortality, which is known to be a leading cause of death in NAFLD.

Various guidelines in chronic diseases, including diabetes, obesity, and coronary heart disease have emphasized the importance of periodic screening for depression (51–54). However, clinical practice guidelines in NAFLD have yet to emphasize the importance of psychological wellbeing and screening despite emerging evidence alluding to significant associations between the two diseases (10). Instead, a quick screening of depression can be easily conducted in clinics with widely used diagnostic tools such as the PHQ-9 scale (22, 55). Up to 56 and 90% of NAFLD may suffer from concomitant diabetes and obesity, respectively, which are risk factors in themselves for the development of depression and the risk of depression (56–59). The presence of depression also has profound implications on interventional therapies for NASH. While bariatric procedures have been proposed as a possible treatment for patients with NASH (60), these procedures themselves have also been associated with an increased risk of suicide, postulated to be due to the inability to rely on the coping mechanisms of overeating in some of these patients after bariatric procedures (61). Hence, a multidisciplinary team committed to NAFLD (16), therefore, is essential to allow for the encapsulation of all aspects of care beyond medical needs, and a greater emphasis on psychosocial wellbeing should be emphasized in future guidelines.

Strength and limitations

The present study provides a comprehensive analysis of the impact of depression on NAFLD. However, there are several limitations. The current analysis is observational in nature and cannot be used to draw causality inferences but rather to show the association between diseases. Nevertheless, it has been described that the association between the two disease entities is complex and likely bi-directional due to the central and peripheral inflammatory response of both diseases (10). The current limitations within the NHANES 2000–2018 dataset have also introduced some limitations. Firstly, diagnosis of hepatic steatosis was limited to FLI and US-FLI as imaging-based diagnosis was unavailable for the NHANES 2000–2018 dataset. Still, the FLI has been widely employed and validated (62) in population-based studies for liver steatosis (36, 63, 64). In addition, the use of antidepressants for a diagnosis of depression in a proportion of the population could result in the presence of a string bias in the evaluation of the results.

Conclusion

Depression is a major issue in NAFLD patients but has tended to be overlooked and forgotten whose presence can increase the risk of all-cause mortality and cardiovascular disease. While treatment of depression does not result in a complete elimination of risk, there was a reduction in the magnitude of the effect associated with treated depression. Early screening of depression in high-risk individuals should be encouraged to improve the wellbeing of individuals with NAFLD.

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

CN and NC: conceptualization and design. CN, JX, NC, and YC: acquisition of data and analysis and interpretation of data.

CN, JX, NC, YC, KC, JQ, WL, DT, RL, CT, AT, XG, BN, NS, DY, NT, DH, MS, MN, MM, and AS: writing – original draft and writing – review and editing. MM: guarantor of the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of interest

Author AS was President of Sanyal Biotechnology and has stock options in Genfit, Akarna, Tiziana, Indalo, Durect, and Galmed. He has served as a consultant to Astra Zeneca, Nitto Denko, Enyo, Ardelyx, Conatus, Nimbus, Amarin, Salix, Tobira, Takeda, Jannsen, Gilead, Terns, Birdrock, Merck, Valeant, Boehringer-Ingelheim, Lilly, Hemoshear, Zafgen, Novartis, Novo Nordisk, Pfizer, Exhalenz, and Genfit. He has been an unpaid consultant to Intercept, Echosens, Immuron, Galectin, Fractyl, Syntlogic, Affimune, Chemomab, Zydus, Nordic Bioscience, Albireo, Prosciento, Surrozen, and Bristol Myers Squibb. His institution has received grant support from Gilead, Salix, Tobira, Bristol Myers, Shire, Intercept, Merck, Astra Zeneca, Malinckrodt, Cumberland, and Norvatis. He receives royalties from Elsevier and UptoDate. Author MN has been on the advisory board for 89BIO, Gilead, Intercept, Pfizer, Novo Nordisk, Blade, EchoSens, Fractyl, Terns, Siemens, and Roche diagnostic; MN has received research support from Allergan, BMS, Gilead, Galmed, Galectin, Genfit, Conatus, Enanta, Madrigal, Novartis, Pfizer, Shire, Viking, and Zydus; MN was a minor shareholder or has stocks in Anaetos, Rivus Pharma, and Viking.

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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EDITED BY
Giovanni Tarantino,
University of Naples Federico II, Italy

REVIEWED BY
Valentina Cossiga,
University of Naples Federico II, Italy
Cristiane Nogueira,
Federal University of Rio de Janeiro, Brazil
Hanqing Chen,
Guangzhou First People's Hospital, China

*CORRESPONDENCE
Negar Azarpira
negarazarpira@gmail.com

†These authors have contributed equally to this work and share first authorship

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The role of FGF21 and its analogs on liver associated diseases

Kimia Falamarzi[1,2†], Mahdi Malekpour[1,2†], Mobin Fallah Tafti[1,2], Negar Azarpira[2*], Mehrdad Behboodi[1,2] and Mohammad Zarei[3,4]

[1]Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran, [2]Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, [3]Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States, [4]John B. Little Center for Radiation Sciences, Harvard T. H. Chan School of Public Health, Boston, MA, United States

Fibroblast growth factor 21 (FGF21), a member of fibroblast growth factor family, is a hormone-like growth factor that is synthesized mainly in the liver and adipose tissue. FGF21 regulates lipid and glucose metabolism and has substantial roles in decreasing lipogenesis and increasing hepatic insulin sensitivity which causing lipid profile improvement. FGF21 genetic variations also affect nutritional and addictive behaviors such as smoking and alcohol consumption and eating sweets. The role of FGF21 in metabolic associated diseases like diabetes mellitus had been confirmed previously. Recently, several studies have demonstrated a correlation between FGF21 and liver diseases. Non-alcoholic fatty liver disease (NAFLD) is the most prevalent type of chronic liver disease worldwide. NAFLD has a wide range from simple steatosis to steatohepatitis with or without fibrosis and cirrhosis. Elevated serum levels of FGF21 associated with NAFLD and its pathogenesis. Alcoholic fatty liver disease (AFLD), another condition that cause liver injury, significantly increased FGF21 levels as a protective factor; FGF21 can reverse the progression of AFLD and can be a potential therapeutic agent for it. Also, NAFLD and AFLD are the most important risk factors for hepatocellular carcinoma (HCC) which is the fourth deadliest cancer in the world. Several studies showed that lack of FGF21 induced oncogenic condition and worsened HCC. In this review article, we intend to discuss different aspects of FGF21 in NAFLD, AFLD and HCC; including the role of FGF21 in pathophysiology of these conditions, the effects of FGF21 mutations, the possible use of the FGF21 as a biomarker in different stages of these diseases, as well as the usage of FGF21 and its analog molecules in the treatment of these diseases.

KEYWORDS

fibroblast growth factor 21 (FGF21), NASH, NAFLD, HCC, MAFLDs, FGF21 polymorphism, FGF21 analogs

Introduction

Fibroblast growth factor 21 (FGF21) is an endocrine regulating factor that is produced mainly in the liver and adipose tissues (1, 2). FGF21 can improve many critical liver-associated diseases by contributing to metabolic pathways. Reducing lipogenesis, inducing fatty acid β-oxidation, increasing hepatic insulin sensitivity,

decreasing very-low-density lipoprotein (VLDL) transmission to the liver and subsiding the hepatic endoplasmic reticulum (ER) stress are the major mechanisms of FGF21 to improve fatty liver diseases (3–5). It is also reported that FGF21 could be a protective factor against lipotoxicity (1). Furthermore, FGF21 can induce insulin sensitization and increase the glucose uptake in white adipose tissues (1, 3, 4, 6). In addition, FGF21 may reduce the risk of atherosclerosis due to lowering inflammation, regulating of lipid metabolism and its effect on adiponectin expression (7).

The liver is one of the most important organs in the body because of its crucial role in several processes including detoxification, anabolism and catabolism, immune factors production and lipids metabolism regulation. The liver's functions can be affected by several diseases. Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. Insulin resistance, lipid metabolism dysfunctions and inflammation are the major causes of NAFLD (8). Decreased mitochondrial fatty acid oxidation, increased hepatic lipogenesis and decreased lipid export from hepatocytes are the mechanisms that may lead to hepatic steatosis (9). NAFLD is defined by fatty infiltration of the liver in more than 5% of hepatocytes and in 20% of patients progressed from Nonalcoholic Steatohepatitis (NASH) to liver fibrosis and eventually cirrhosis (10, 11). Patients with NAFLD and NASH have a high risk for developing cardiovascular diseases, cirrhosis and hepatocellular carcinoma (HCC) (12, 13). Some studies found an association between FGF21 levels in serum and the amount of fatty contents in the liver (14). Therefore, FGF21 can be used as a diagnostic biomarker for NAFLD (8, 14). FGF21 can modulate oxidative and ER stress, decrease fat synthesis and the levels of inflammatory cytokines (15–18) and enhance the expenditure and catabolism of stored lipids (19).

NAFLD is the leading risk factor for HCC (20). HCC is the most common primary liver cancer which is fatal due to its late diagnosis (21, 22). The 5-year average survival rate of HCC is <10% (22). Because FGF21 rise at an early stage of HCC, it can be used as a diagnostic factor for HCC (23–25). However, the FGF21 levels were decreased when HCC is well-developed (26, 27). Lack of FGF21 can accelerate the progression of NAFLD to HCC *via* induction of inflammation and accumulation of lipids in the liver (28, 29). Overexpression of FGF21 likely delays the development of adenomas at an early stage of carcinogenesis (30).

FGF21 protects the liver not only from NAFLD and NASH but also from alcoholic fatty liver disease (AFLD). About 50% of cirrhosis-related death is attributed to alcohol consumption (31). Chronic alcohol consumption may lead to the accumulation of lipids in hepatocytes and liver injury (32). Previous studies show that alcohol usage can increase FGF21 serum levels (33). FGF21 may ameliorate AFLD by improving hepatomegaly, reducing lipid synthesis, enhancing mitochondrial oxidative function and decreasing the production of reactive oxygen species (32–35).

In this review article, we attempt to concisely explain the role of FGF21 and its mutations and analogs on liver disorders. Further studies will be required to determine the effectiveness and accuracy of FGF21 and its analogs in targeted therapy to cure and diagnose hepatic disorders.

FGF21

FGF21 mechanism of action

FGF21 is a hormone-like growth factor composed of 209 amino acids (1). In humans, FGF21 (gene ID: 26291) is on chromosome 19 (19q13.33) and contains 3 exons that encode this protein (36). FGF21 physiology is somewhat complex mainly because it is secreted from different organs and affects various organs (36). FGF21 is secreted predominantly from the liver and adipose tissues (1, 2) even though there are many other sites in which FGF21 is synthesized such as the pancreas, skeletal muscles and cardiac endothelium (3, 37). FGF21 can be released from the site where synthesized into the bloodstream to act as an endocrine hormone (4). FGF21 is also detectable in human cerebrospinal fluid (2, 5). FGF21 binds to fibroblast growth factor receptors (FGFRs) with extremely low affinity and since it lacks a heparin-binding domain, the presence of a co-receptor called β-klotho is required for improving the affinity of FGF21 binding (1, 3, 36). β-klotho is a transmembrane protein that is necessary for FGF21 signaling and its activities on target tissues (1, 36). β-klotho is expressed in specific metabolic tissues such as the liver, pancreas and adipose tissues which determines the FGF21 target organs while FGFRs are expressed in various tissues and cells such as kidney, liver, adipose tissues, skeletal muscle and etc but mainly in liver and adipose tissue (1, 3, 13). In addition, hepatic FGF21 expression is strongly controlled by peroxisome proliferator-activated receptor α (PPARα), a transcription factor activated by the non-esterified fatty acids released from adipocytes which decreases the lipogenesis and increases fatty acid β-oxidation (38).

Several studies confirmed that FGF21 has an important role in lipid and carbohydrate metabolism as well as energy and nutrient homeostasis (39). Hence, FGF21 could be considered a potential diagnostic biomarker and therapeutic agent for metabolic diseases such as obesity, type 2 diabetes mellitus (T2DM), and fatty liver (4).

FGF21 increases hepatic insulin sensitivity, decreases lipogenesis, triggers fatty acid β-oxidation, reduces hepatic ER stress, and diminishes VLDL delivery to the liver (through down-regulation of VLDL receptor expression in hepatocytes) (3–5). FGF21 could also decrease postprandial triglycerides (TGs) and facilitate fatty acid storage in adipose tissue (40). These actions eventually result in lipid profile

improvement, weight loss, decreased hepatic triglyceride content, and ameliorate fatty liver, NASH, and metabolic-related diseases (3, 39).

FGF21 levels are positively correlated with obesity, body mass index (BMI) and hepatic fat accumulation (1, 36). It is demonstrated that increased FGF21 levels could be an adaptive mechanism to protect the body against lipotoxicity (1). Several studies argued that FGF21 administration increased the browning of white adipose tissue and activation of brown adipose tissue; therefore resulting in increased energy expenditure, maintained body temperature during cold exposure and ultimately weight loss (4, 41).

FGF21 level increased in T2DM and is positively correlated with hyperglycemia, insulin resistance and inflammatory processes (1). FGF21 increased glucose uptake in white adipose tissue through induction of glucose transporter1 (GLUT1) expression which is and independent process from insulin (3, 4, 6). Previous studies demonstrated that FGF21 could lead to a rapid insulin sensitization within 1 hour (1, 3). Administration of FGF21 or its analogs significantly increased plasma adiponectin levels (1, 3, 4). Adiponectin is an insulin-sensitizing factor which mainly secreted from adipocytes and has anti-inflammatory and anti-sclerotic effects and it mediates FGF21 impacts on energy metabolism and insulin sensitivity (4, 38). FGF21 increased adiponectin gene expression. The secretion of adiponectin is from adipocytes through a Peroxisome proliferator-activated receptor γ (PPARγ)-dependent mechanism (4). PPARγ is a transcription factor that its activation increases FGF21 effects such as reduction of fat and lipidemia, improvement of tissue insulin sensitivity and increase of lipogenesis in white adipose tissue (1, 5).

In contrast to mouse models, short-term fasting or ketogenic diets do not increase FGF21 levels in humans (2). Long-term starvation (about 7–10 days), high sugar intake and protein restriction in diet cause FGF21 elevation in humans (3, 42). FGF21 mutations also are associated with macronutrient preference in humans independently of BMI (2, 6, 43). These mutations are believed to have associations with increased sweet taste preference in humans (3). Further studies showed that, the administration of an FGF21 analog to obese individuals can

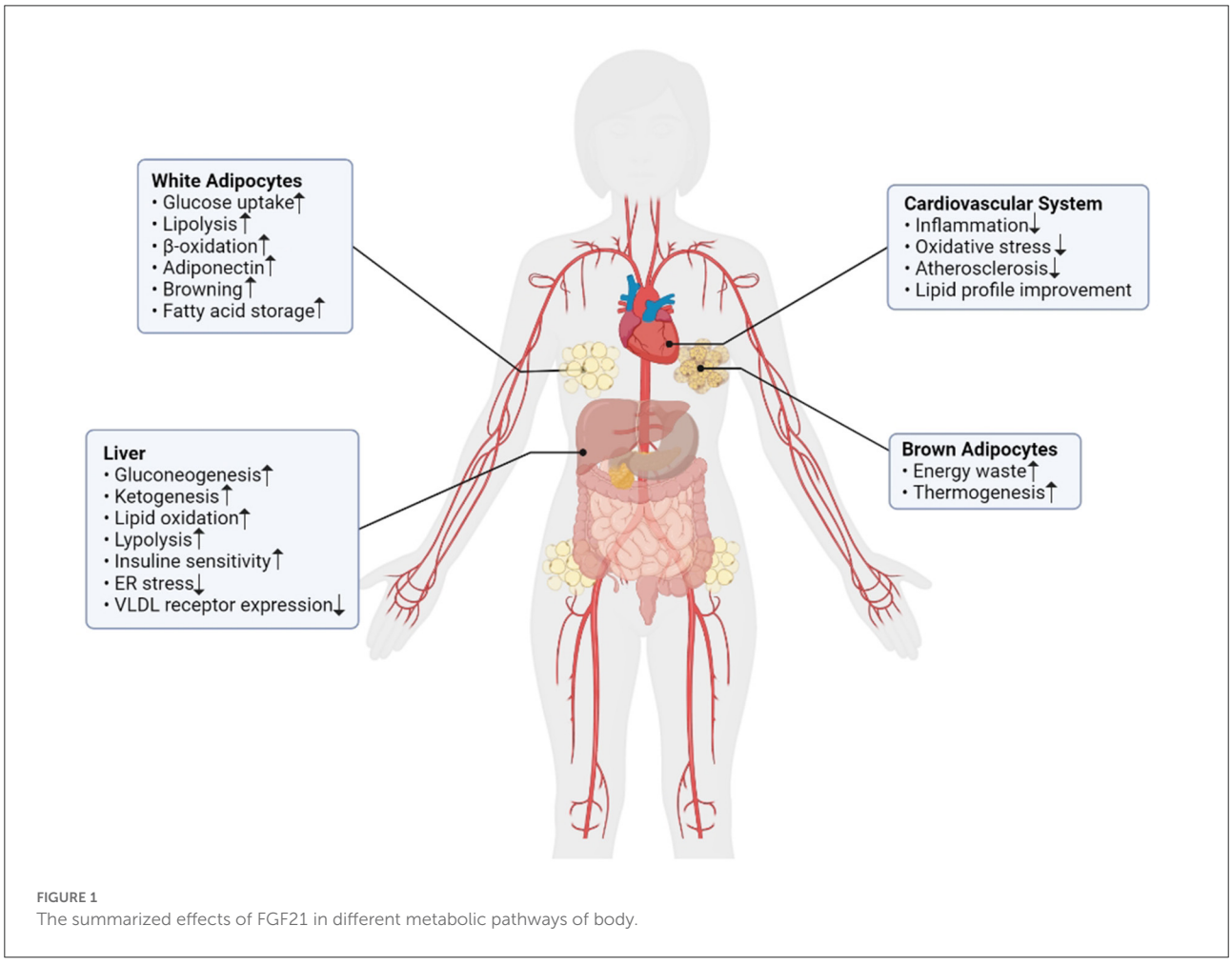

FIGURE 1
The summarized effects of FGF21 in different metabolic pathways of body.

decrease the preference for sweet-tasting food and carbohydrate intake (3, 44).

FGF21 also is elevated in patients with atherosclerosis (1, 7). Several studies indicated that FGF21 by its anti-oxidative and anti-inflammatory effects and its influences on lipid profile and adiponectin expression could, directly and indirectly, decrease atherosclerosis incidence (7).

These findings suggested that FGF21 increased in obesity and other related conditions such as T2DM, metabolic syndrome, fatty liver disease, etc. FGF21 has several important roles in lipid and carbohydrate metabolism and energy homeostasis which it's important roles summarized in Figure 1. Considering these facts, FGF21 could be a potential agent for the diagnosis and treatment of metabolic-related diseases. Further studies will be required to determine its effectiveness and accuracy.

FGF21 genetic variations

To the best of our knowledge, there are not enough studies about the association of genetic variations of FGF21 with hepatic diseases such as NAFLD, NASH, and HCC. While FGF21 can affect the pathomechanism of these diseases, it can also play an important role as a biomarker for NAFLD and AFLD and show significant results in curing fatty liver and metabolic associated diseases. In a study done on a Han Chinese non-diabetic population the association of four Single Nucleotide Polymorphisms (SNPs) with NAFLD was investigated (45). They found that rs499765 was associated with serum levels of FGF21 and can be a predicting factor for measuring the risk of NAFLD (45). Besides, they found that rs2071699 and rs838136 correlate with aspartate aminotransferase serum concentration and rs838136 is associated with alanine aminotransferase levels (45).

Another study was done in order to discover the association between genetic variations of FGF21 and Metabolic Associated Fatty liver diseases (MAFLDs) suggesting that rs838136 could be a risk factor for MAFLDs *via* changes in folding and stability of FGF21 mRNA (46).

Furthermore, previous research found that rs838133 is correlated with behaviors such as alcohol and candy consumption and also daily smoking. This study implies that the liver can regulate eating and lifestyle habits *via* producing different hormones like FGF21 (47). The other studies confirmed the role of FGF21 SNPs in addictive behaviors like eating habits and the amount of coffee consumption (48–51). Previous studies also showed the importance of FGF21 genetic variations in obesity (rs11665896) (50), fat and macronutrient intake (rs838147) (51), renal function in diabetic patients (rs2071699, rs838136, and rs499765) (52), and alcohol dependence (rs11665896) (53) which indirectly can have effects on steatosis of liver.

Earlier studies also have suggested that FGF21 serum level could be a biomarker for dysregulated metabolic pathways and also the level of fat accumulation in the liver (14, 54). This upregulation of FGF21 could also help to prevent fat deposition in the liver resulting in reduced inflammation and fibrosis of the liver (55). Moreover, the results of a cohort study demonstrated that a higher serum level of FGF21 could be a prognostic factor for HCC (23). Consequently, due to the importance of circulatory FGF21 concentration the SNPs regulating the serum level of FGF21 are considerably crucial in liver diseases. Previously a Genome-Wide Association Study (GWAS) reported the most important SNPs which regulate FGF21 serum levels. These SNPs were rs12565114, rs9520257 and rs67327215 (56). Investigations on the role of these SNPs and the susceptibility of people to having liver diseases may help to personalized the cure of these diseases.

At last, according to the significant role of the FGF21 gene in metabolic associated diseases and behavioral habits related to these diseases and additionally the direct influence of FGF21 variations on MAFLDs as well as the heritability pattern of fatty liver diseases (57, 58), there should be further studies for investigating the association of FGF21 genetic variations and MAFLDs.

The relation between FGF21 and liver associated diseases

FGF21 in alcoholic fatty liver disease

Alcohol-related deaths have the third rank among the most common preventable causes of death after smoking and hypertension (59). More than 200 diseases and a range of injuries have a link to the consumption of alcohol, such as cardiovascular diseases, cirrhosis, and several cancers (60). Liver diseases particularly cirrhosis has the largest alcohol-attributable fraction; Almost 50% of cirrhosis-related mortality is caused directly or indirectly by alcohol (31). In 2010, almost half a million deaths occurred due to alcohol-related cirrhosis (61).

Alcoholic liver diseases consist of a spectrum of pathologies ranging from alcoholic hepatitis to cirrhosis, and cirrhosis complications (2). AFLD is one of the major causes of mortality in the United States, with nearly 250,000 deaths due to AFLD in 2010 (62).

Among the risk factors of AFLD, the amount and duration of an individual's alcohol consumption are the most important factors (60). Also, it has been demonstrated that gender is another risk factor for AFLD because the relative risk of AFLD is higher in women than men (63). Also, chronic viral hepatitis such as hepatitis C and genetic and epigenetic factors have been suggested as the risk factors for AFLD (60, 64).

Accumulation of alcohol in hepatocytes as a result of chronic alcohol consumption can induce liver injury (32). Alcohol-induced fatty liver injury is reversible at the initial stages but AFLD can develop more severe forms of liver injury such as alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma as long as the individual continues alcohol consumption (32). Alcohol also affects the liver through nutritional disturbance as a result of its metabolism process in the liver (65).

Acute alcohol consumption increases FGF21 levels in both humans and mice (66). Previous studies demonstrated that FGF21 levels significantly increased more than 3 fold by acute ethanol intake (67) and alcohol exposure lead to increased hepatic FGF21 expression and its circulating level (68). Additionally, patients with alcoholic steatohepatitis had FGF21 levels 6 times greater than non-drinking healthy subjects without any liver diseases (68). Chronic alcohol exposure results in FGF21 up-regulation in mice and the absence of FGF21 causes substantial liver pathologies (69). Chronic alcohol consumption could cause hepatic lipogenesis and impaired fatty acid β-oxidation by hepatic factors dysregulation such as PPARα, Sirtuin 1, and Adenosine monophosphate-activated protein kinase (AMPK) (68).

Adenosine monophosphate-activated protein kinase (AMPK) is a metabolic regulator which senses the oxidative stress and reduced energy charge of body. Several energy-generating pathways such as glycolysis and fatty acid oxidation are up-regulated by AMPK (32). AMPK also inhibits the activity of several energy-demanding processes, including fatty acid, cholesterol, and protein synthesis (32). Several studies have demonstrated that the activity of AMPK is decreased in ethanol-fed rodents (70, 71), as a result, fatty acid synthesis is promoted in these rodents, whereas the fatty acid oxidation pathway is blocked (71). In conclusion, the pathogenesis of AFLD is associated with AMPK inactivation (32). FGF21 also regulates energy homeostasis in adipocytes by activating AMPK and Sirtuin 1, which results in enhanced mitochondrial oxidative function (35). Intracellular reactive oxygen species production induced by alcohol in hepatic cells can remarkably decrease by FGF21 (32).

Besides, alcohol exposure may lead to hepatic fat accumulation, hepatic ER stress and inflammation which results in FGF21 production (68). Several studies demonstrated that FGF21 expression induced by alcohol is a hepatic adaptive response due to lipid dysregulation (68). Lipid synthesis can be inhibited due to an increase in FGF21 serum levels (72). The mRNA expression of lipogenic genes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase 1 and 2 (ACC1 and ACC2) are significantly suppressed by FGF21 (73). The role of FGF21 in ameliorating lipid metabolism has also been demonstrated (73). The rise in FGF21 expression leads to increased fatty acid oxidation and limited lipid accumulation (69). Loss of FGF21 in mice leads to worsening of alcohol-induced steatohepatitis and liver injury which is due to increased activation of genes involved in lipogenesis and decreased expression of genes involved in fatty acid oxidation (68). Also, FGF21 Knocked Out mice showed an enhanced hepatic inflammation due to alcohol exposure (68, 74). These results suggested that, the protective effects of FGF21 in alcoholic liver disease might be associated with de novo lipogenesis and fatty acid catabolism and also its role as an anti-inflammatory factor (2, 68). Some studies showed that FGF21 administration could ameliorate alcoholic liver disease in mice (32, 68). These studies suggested that FGF21 had positive effects on serum lipid profile, decreased hepatocytes lipid accumulation and reduced oxidative stress in mice with alcoholic fatty liver disease (32). Additionally, FGF21 related treatments could prevent fatty liver progression and reverse the development of AFLD in mice (68). The results of the experiments on the plasma of ethanol-fed rodents have demonstrated a significant increase in FGF21 serum level, therefore alcohol consumption can increase FGF21 gene expression (72). FGF21 also plays a preventable role against hepatomegaly and can reduce the swelling of the liver, which can improve AFLD (32).

According to these findings, FGF21 might be a potential target for AFLD treatment and further trials are required to investigate its effects as a therapeutic agent in humans.

FGF21 in NAFLD and NASH

Non-alcoholic fatty liver disease is nowadays the most common chronic liver disease in the world. It affects 1.8 billion people worldwide with a prevalence of about 30% of the adult population (75, 76). NAFLD is the main risk factor for the development of hepatocellular carcinoma. NAFLD is also associated with obesity, diabetes mellitus and metabolic syndrome and it can be a hepatic manifestation of metabolic syndrome (18). NAFLD is defined as the accumulation of lipid in more than 5% of total liver weight when there are no secondary causes such as excessive alcohol intake, infections, autoimmune diseases, or any other etiologies of liver diseases (8). The comparison table between different aspects of AFLD and NAFLD can be seen in Table 1.

The pathophysiology of NAFLD is still unknown; however, insulin resistance, lipid metabolism dysfunctions and inflammation are established as the main pathogenic pathways for developing NAFLD (8). Steatosis occurs when there is an imbalance between the input and export of hepatocellular fat. The sources of hepatic fat can come from dietary intake, fatty acid flow to the liver from adipose tissue and hepatic *de-novo* lipogenesis (9). Insulin resistance is another important factor for lipogenesis in NAFLD. Other mechanisms that cause hepatic steatosis are decreased mitochondrial fatty acid oxidation, increased hepatic lipogenesis and decreased lipid export from hepatocytes (9). Fat accumulation can lead

TABLE 1 The comparison table between NAFLD and AFLD.

	NAFLD (77)	AFLD (78, 79)
Prevalence	30% (About 1 billion people worldwide)	4%
Risk factors	Diabetes, obesity, metabolic syndrome, age, male gender	Amount and duration of alcohol consumption, genetic factors, female gender
Pathophysiology	Insulin resistance, hepatic fat accumulation, inflammation	Hepatic fat accumulation, hepatic ER stress, inflammation
Complications	NASH, cirrhosis, HCC	Chronic fibrosis, cirrhosis, HCC
Treatment	No specific pharmaceuticals are currently FDA approved	Cessation of alcohol consumption, no specific medical treatment approved
FGF21	Elevated expression and serum FGF21	Elevated expression and serum FGF21

to lipotoxicity, oxidative stress, immune cell, and satellite cell activation which result in hepatic inflammation and fibrosis (9).

The pathological spectrum of NAFLD ranges from simple hepatic steatosis to non-alcoholic steatohepatitis and hepatic fibrosis and cirrhosis (15, 76). Even simple steatosis can put individuals at risk for developing NASH (8). NASH has a global prevalence of 1.5–6.5% in adults and patients with NASH have an increased mortality rate compared to the general population due to an increased rate of cardiovascular diseases, cirrhosis, and hepatocellular carcinoma (12). NASH is currently the second leading cause of cirrhosis among adults who are waiting for liver transplantation. About 20% of NASH patients develop liver cirrhosis (11). The inability of hepatocytes to regulate fatty acids overload may lead to NASH development. Fatty acid excess can cause lipotoxicity, mitochondrial dysfunction, ER stress, activation of inflammatory pathways, cell injury and cell death. Those changes will eventually induce fibrosis, cirrhosis and HCC (80). Inflammation and cell injury are important factors that define NASH and when these mechanisms took place, simple steatosis turns into NASH (17).

FGF21 has significant roles in lipid and glucose metabolism and energy homeostasis. Single nucleotide polymorphisms of FGF21 were associated with the pathogenesis of NAFLD (15). Previous studies found that serum FGF21 was elevated in NAFLD and significantly correlated with hepatic fat content (or intrahepatic TG content) (14). Consequently, FGF21 could be a potential diagnostic marker for NAFLD (8, 14). A meta-analysis published in 2017 suggested that FGF21 showed excellent performance to distinguishing NASH from hepatic steatosis and it performed well in identifying NASH; however, its ability to confirm the diagnosis was inadequate due to the fact that the number of studies included was very few (this biomarker was modestly sensitive and specific, with pooled values of 62 and 78%, respectively) (81, 82). Another study suggested that FGF21 had sensitivity and specificity of 72.6 and 85.1% for diagnosis of NAFLD as well as sensitivity and specificity of 53.7 and 71.9% for diagnosis of NASH (83).

Free fatty acids stimulated FGF21 expression *via* PPARα activation (19, 76, 84). In previous human studies a significant association of both serum concentration and liver mRNA expression of FGF21 with hepatic fat and TG content was found (76). Previous investigations demonstrated that FGF21 levels increased in mild and moderate NAFLD patients but as hepatic fat content increased and severe NAFLD occurred FGF21 concentration decreased. This happened due to hepatic cell injury or death caused by lipotoxicity and hepatic inflammation in severe NAFLD or NASH patients so that the remaining hepatic cells were unable to produce as much FGF21 as needed (54, 76, 85). Accordingly, FGF21 might be sensitive in diagnosing mild or moderate steatosis and predicting the onset of simple steatosis (76). While for severe NAFLD and NASH diagnosis combining FGF21 with other circulating markers like cytokine 18 (CK-18) seems more preferable with an overall specificity of 95% and a positive predictive value of 90% (2). Hence, FGF21 is better for predicting the onset of simple steatosis, while other markers (such as CK-18) are better for predicting the prognosis of NAFLD (2).

FGF21 is one of the most potent insulin sensitizers (3) and since insulin resistance is one of the most important factors in NAFLD development, FGF21 with this mechanism can ameliorate NAFLD (18). Besides, intact insulin signaling is necessary for most of the FGF21 effects on lipid metabolism (16).

FGF21 also modulates the process of oxidation stress, ER stress, mitochondrial dysfunction and inflammation to slow the progression of NAFLD (15). FGF21 level increase in NAFLD, in order to sustain homeostasis against lipotoxicity, oxidative stress, and ER stress (14). FGF21 decreased gene expression related to fat synthesis such as FAS, acetyl ACC1 and ACC2, and significantly increased gene expressions related to energy expenditure (18). *In vitro* experiences also revealed a crucial role of FGF21 in fat metabolism and hepatic lipid regulation (10). These functions resulted in decreasing lipogenesis, increasing lipolysis of lipid droplets, the clearance of fatty acids, and the enhanced expenditure of the stored lipid energy by enhanced mitochondrial substrate oxidation, catabolism and uncoupling (19). Previous studies found that FGF21 analogs can also improve mitochondrial functions in a way that mitochondria could manage excessive fatty acids without producing reactive oxygen species (17). Preclinical studies have demonstrated that FGF21 has anti-inflammatory, anti-diabetic and hypolipidemic roles. Therefore, the administration of FGF21 analogs has been shown to reverse hepatic steatosis in both mice and

humans (8). In NASH mouse models using leptin-deficient mice and methionine and choline-deficient diet, FGF21 analogs reversed hepatic inflammation and fibrosis (15–17). It can also reduce hepatic inflammation and immune cell infiltration in mice (8). Inflammation can suppress β-klotho expression and impair FGF21 signaling leading to FGF21 resistance (8). The elevation of FGF21 levels observed in individuals with NAFLD is likely due to a compensatory response to FGF21 resistance. FGF21 also reduced the level of the inflammatory cytokines such as Interleukin-18 (IL-18) and Tumor necrosis α (TNF-α) (16, 17). Autophagy is an important mechanism in recycling cytoplasmic components and damaged organelles (86). Defective hepatic autophagy results in abnormal accumulation of hepatic TG, insulin resistance, fatty liver and ultimately more serious hepatic conditions like HCC (18). Hepatic expression of autophagy components and autophagy gene activators are decreased in NAFLD patients (86). FGF21 significantly increases the expression level of genes related to autophagy (18, 86).

In the end, considering FGF21 roles in glucose and lipid metabolism and also in energy balance and according to its effects on NAFLD and NASH, FGF21 could be a potential biomarker for diagnosis of NAFLD and NASH and it might also be a target for the treatment of these conditions. Hence, further studies and trials are needed to identify FGF21 and its mutations' roles in NAFLD and NASH development and also FGF21 analogs' effectiveness in NAFLD and NASH treatment.

FGF21 in HCC

HCC is the most common primary liver cancer which is the fifth most common cancer in men and the seventh in women (21, 87). HCC is the 4th deadliest cancer in the world and its mortality and morbidity rates have been increasing over the past decades (21, 88, 89). Also, HCC has a very poor prognosis because of the late diagnosis of the disease. The 5-year average survival rate of HCC is <10% (22). Therefore, studying the risk factors and molecular mechanisms of HCC can continue the progression of understanding the disease.

HCC is a multi-stage cancer that is induced by many factors. Viruses, aflatoxin, alcohol usage, lack or mutations in some regulatory genes such as FGF21 and many other factors that cause hepatic injury, can stimulate a preceding process of HCC (90). Hepatic injury may trigger the proliferation and regeneration of hepatocytes (90). The following hepatic inflammation and the presence of several cytokines, growth factors, chemokines and oxidative stress components play a major role in making an environment wherein hepatocytes can alter phenotypically (90). Also, NAFLD and NASH are the leading causes of HCC (20). Fatty acids concentrating in the liver can induce steatosis and inflammation. Inflammatory cytokines and infiltrating macrophages may cause chronic inflammation and liver cells death subsequently. These reactions along with some factors such as transforming growth factor β (TGF-β) and IL-18 extremely increase the risk of liver cancer (20). In addition, the nodules that appeared in cirrhosis also can provide a condition that transforms the normal hepatocytes into dysplastic hepatocytes (90). Hepatocyte injury, inflammation and proliferation of liver cells and subsequent fibrosis and cirrhosis predispose the liver to cancer.

FGF21 is a liver-derived factor that regulates lipid and glucose metabolism (38, 91). Lack of FGF21 could induce inflammation and lipid accumulation in the liver (29). In the absence of FGF21 the production of Interleukin-17A (IL-17A), a critical factor for NASH development, is highly increased (29). IL17-A recruits macrophages and neutrophils into the inflammation area (29). In addition, FGF21 reduces lipid concentration in the liver by activating of sirtuin 1 pathway and preventing lipolysis (28, 29). FGF21 also regulates the inflammatory cytokines due to its negative impact on the NF-κB (nuclear kappa light chain enhancer of activated B cells) mediated TGF-β signaling pathway (92).

Some viruses such as Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV) can be major risk factors for HCC (93). FGF21 is a novel diagnostic biomarker to monitor the progression of chronic hepatitis B (CHB) (24) and chronic hepatitis C (CHC) (94, 95). Also, Obesity and diabetes mellitus have a crucial role in developing HCC (21). A study reveals that exogenous FGF21 can decrease blood glucose and serum triglycerides to the normal amount in obese or diabetic mice (96). Alcohol intake also can be a contributing factor to liver cancer (21, 93). Alcohol consumption can increase FGF21 levels (66, 68). The role of diet in HCC is still controversial. Some studies reported that foods containing milk, wheat, vegetable, fish and fruit have reduced the risk of HCC but other studies disclosed no association (21). However previous studies report the role of FGF21 in human diet preferences (48–51).

To the best of our knowledge, few studies reported an association between HCC and the expression of FGF21. FGF21 is induced by liver injury and stress and can be a prognostic biomarker to monitor the carcinogenesis of the liver and established as an early diagnostic biomarker for HCC (23–25). A study reported that higher levels of FGF21 related to worse survival in HCC patients (23). Finn et al. discovered that higher levels of FGF21 associated with shorter overall survival in HCC patients regardless of treatment (97) and they suggested that FGF21 might be an independent prognostic factor for overall survival in HCC (97). P53 is a transcription factor that controls FGF21 expression in some abnormal hepatic functions. P53 is a stress regulator that decreases FGF21 expression in hepatic cells. Also, a study reported that the haploinsufficiency of p53 can progress to carcinogenesis and has a significant effect on increasing FGF21 expression (25). This study showed that the FGF21 levels are significantly increased before the HCC becomes clinically obvious (25). Besides, Liang et al. demonstrated that CHB patients who developed HCC experienced elevated

levels of FGF21 (24). Also, another study mentioned that FGF21 was increased in liver cancer and regeneration after partial hepatectomy in a genetic model mouse (30). Although overexpression of FGF21 delays the emergence of adenomas at early stages *via* activating of hepatocyte FGFR4, it accelerates the progression of tumors to HCC by interacting with FGFR1 (30).

The deficiency of FGF21 appears to have a role in the progression of NAFLD to HCC (29, 98). Zhang et al. first demonstrated that diminished FGF21 levels were associated with cancerous hyper-proliferation and atypical oncogening signaling in the liver (27). It appears that the level of FGF21 indicates the amount of triglyceride accumulation in the liver (26, 98). In a study by Garima et al. FGF21 deficient mice were found to have significantly more accumulation of hepatic lipids in comparison with the wild type (WT) mice with the same high fat, high sucrose (HFHS) diet (98). The sinusoidal fibrosis which can develop to HCC was significantly higher in FGF21 KO mice than WT mice with the same diet (98). 78% of FGF21 KO mice on HFHS diet in comparison to 6% of WT mice represent 1–3 large liver nodules which can lead to HCC histologically. Remarkably, HCC was developed without cirrhosis in their study (98). Another study exhibited that lack of FGF21 could worsen the metabolic disorders in NASH and provide the microenvironment, wherein inflammation, regenerating proliferation of hepatocytes and fibrosis may happen (26). This condition which contains many inflammatory and mitotic factors has a high risk to progress to HCC in diabetes mice (26).

A study identified that FGF21 levels were increased at the early stage of hepatic stress in a genetic model mouse presenting diabetes-steatohepatitis (27); however, the reduction in FGF21 levels was reported when HCC was well-developed. This may show that the early rise in FGF21 expression can indicate its protective role of it and the late decrease in expression of FGF21 may refer to chronic hepatic disorders comprising liver cancer (26, 27). Three reasons for the downregulation of FGF21 have been suggested before. First, the expression of the FGF21 gene has a negative association with the concentration of liver triglyceride. Because of the major role of high hepatic lipid concentration in HCC development, FGF21 levels were reduced in carcinogenesis. Second, G9a, a factor that suppresses FGF21 expression epigenetically, modifies the process of HCC. Lastly, hypoxia can reduce the FGF21 mRNA level (99); because of the hypoxic condition of the most solid tumors, the FGF21 levels can be decreased (27). Unlike the early rise of FGF21 in a pre-cancerous liver, due to the lipid accumulation in the liver, the G9a factor and the hypoxic condition of the liver, the level of FGF21 is decreased in well-developed HCC.

In conclusion, FGF21 has a protective and diagnostic role at the early stage of HCC. FGF21 can delay the conversion of adenomas to malignant tumors by regulating inflammation and lipid concentration in the liver. Note that the level of FGF21 seems to be decreased at the late stage of carcinogenesis. This reduction may probably relate to the deteriorating effect of FGF21 on the progression of HCC at an advanced stage of tumorigenesis. Further studies will be needed to find the exact role of the FGF21 in liver cancers.

FGF21 as a drug

According to the important effects of FGF21 on metabolic associated diseases some clinical trials have been established in order to assess the safety and therapeutic efficacy of human FGF21 analogs and FGF21 receptor agonists (4). Nevertheless, there were some challenges in the use of FGF21 as a drug such as its poor pharmacokinetic characteristics including short half-life (0.5–2 h), poor instability and bioavailability (80, 100). This resulted in the development of FGF21 analogs applying polyethylene glycosylation (PEGylation) or fusion to antibody fragments (80). The summarized table of the FGF21 analogs and clinical trials can be seen in Table 2. In these studies different effects of FGF21 analogs were discussed such as glycemic and lipid profile of blood, fibrosis reduction of livers (*via* Pro-C3 biomarker reduction measurement) and bodyweight.

LY2405319, a glycosylated FGF21 variant, in a randomized, double-blind, placebo-controlled study demonstrated significant improvements in lipid profile, body weight and adiponectin level in obese and T2DM patients (101). The main effects of LY2405319 treatment were reduction in low-density lipoprotein (LDL) cholesterol and TGs levels and increased high-density lipoprotein (HDL) (101). Additionally, a prominent decrease in mean fasting insulin levels was observed. This finding is consistent with the potential improvement in insulin sensitivity (101). Daily administration of LY2405319 for 28 days resulted in a less atherogenic lipoprotein profile (13, 101, 109). However, in contrast to the glucose-lowering effect of LY2405319 in monkeys and mice, there were no statistically significant changes in human fasting glucose (4, 101).

PF-05231023, another FGF21 long-acting analog, considerably reduced body weight, plasma TGs and LDLs and increased HDLs and adiponectin in overweight or obese subjects with T2DM (13, 34). However, PF-05231023 did not show any significant effects on glycemic control (4, 34). Kim et al. showed pulse rate, systolic and diastolic blood pressure increased after administration of PF-05231023 in a dose- and time-related manner (110). Additionally, modest changes in bone absorption and resorption markers were observed during PF-05231023 administration (34, 110). Although it is not clear whether it is a direct effect of FGF21 or an indirect effect on bone turnover due to weight loss (111).

A PEGylated FGF21 analog called Pegbelfermin (formerly BMS-986036) was tested in biopsy-confirmed NASH patients previously (12). This study showed daily or weekly administration of Pegbelfermin reduced TG and LDL while

TABLE 2 The summarized table of the FGF21 analogs and the effect of the analogs on the body.

	Study groups	Hepatic fat content	Hepatic inflammation/ fibrosis	Blood tests	Body weight	Reference	FGF21 analog
1	AFLD mice	Suppress hepatocyte lipid droplet accumulation	Decrease oxidative stress	Decreased TG, TChol, LDL	–	Zhu et al. (32)	Recombinant FGF21
2	Alcohol-treated HepG2 cells	Reduced fat accumulation	Suppressed intracellular ROS products	–	–	Zhu et al. (32)	Recombinant FGF21
3	Alcohol-fed Wild type mice	Decrease hepatic fat accumulation	Decrease inflammation	Reduced ALT, AST and plasma TG	–	Liu et al. (68)	Recombinant FGF21 (rhFGF21)
4	Obese cynomolgus monkeys	–	–	Decrease TG- increase adiponectin	Decreases Body Weight and Food Intake	Saswata Talukdar (34)	PF-05231023
5	Obese/overweight humans withT2DM	–	–	Decrease TG and LDL- increase HDL and adiponectin	Decrease body weight	Saswata Talukdar (34)	PF-05231023
6	Obese and T2DMpatients	–	–	Decrease in LDL, TG and increase in HDL and adiponectin	Decrease body weight	Gaich G,2013 (101)	LY2405319
7	Diet-induced obese (DIO) mice	–	–	Decrease plasma glucose	Decrease body weight	Kharitonenkov et al. (102)	LY2405319
8	Patients with biopsy-confirmed NASH	Significant decrease in absolute hepatic fat fraction	Decrease Pro-C3	Decrease ALT, AST, TG and LDL- increase HDL and adiponectin	No substantial changes in bodyweight	Sanyal et al. (12)	Pegbelfermin
9	Patients with obesity and T2DM	–	Decrease Pro-C3	Decrease TG, increase in HDL and adiponectin	No statistically significant decrease in weight	Charles et al. (103)	Pegbelfermin
10	Patients with NASH	Significant reduction in hepatic fat fraction	Decrease Pro-C3	Decrease in AST, ALT, ALP, HbA1C, fasting glucose, cholesterol and LDL- increase HDL and adiponectin	Decrease body weight	Harrison et al. (104)	Efruxifermin
11	Patients with obesity and NAFLD	Decrease hepatic fat content	Decrease Pro-c3	Decrease TG, LDL- increase HDL and adiponectin	Transient body weight reduction	Negi et al. (105)	BFKB8488A
12	Obese cynomolgus monkey	–	–	Increase adiponetin	Decrease weight and food intake	Baruch et al. (44)	BFKB8488A
13	Insulin resistant, obese subjects with increased liver fat	Decrease hepatic fat content	–	Decrease HbA1C, TG, LDL- increase HDL	–	Depaoli et al. (106)	MK-3655,
14	Obese and mildly hypertriglyceridemic adults	Decrease hepatic fat content	Decrease Pro-C3 and improved liver fibrosis	Decrease in AST, ALT, ALP, cholesterol and LDL-increase HDL	–	Rader et al. (107)	LLF580
15	Mice on methionine and choline-deficient diets	Decrease hepatic TG	Decrease inflammation and fibrosis	Decrease in ALT and free fatty acid	No change in body weight	Fisher et al. (108)	Recombinant FGF21

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increasing HDL and adiponectin (12, 13). Additionally, Pegbelfermin administration caused a significant reduction in hepatic fat content and a prominent decrease in plasma markers of liver injury [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and fibrosis (4, 12). Another 12-week phase 2 study with the administration of Pegbelfermin in patients with obesity and T2DM demonstrated a remarkable increase in HDL and adiponectin levels but no statistically significant decrease in weight, fasting insulin and HbA1C levels were observed (103, 109). It is reported that Pegbelfermin induces anti-pegbelfermin and anti-FGF21 antibodies, which may cross-react with the endogenous FGF21 (80).

Efruxifermin, a long-acting Fc-FGF21 fusion protein was assessed in a trial for the treatment of NASH (104). This study showed that treatment with Efruxifermin for 12 weeks was associated with an absolute reduction in hepatic fat fraction and this reduction in hepatic fat content was followed by a rapid and marked decrease in liver stress and injury markers (such as ALT and AST and pro-C3) (13, 104). Reduced TG, LDL, fasting glucose and fasting insulin were reported in this study while increasing HDL and adiponectin levels were observed (104). Efruxifermin also improved glycemic control by a substantial reduction in HbA1C (13, 104).

BFKB8488A is a humanized antibody that specifically activates the FGFR1/β-Klotho complex (112). In phase 2 clinical trial consisting of NAFLD patients, 12 weeks of administration of BFKB8488A diminished liver fat, serum TG and pro-C3 while increasing adiponectin and HDL (13, 105, 112).

MK-3655, a monoclonal antibody targeted to β-klotho and FGFR1c, in a clinical trial in phase 2 showed improvements in glycemic control and reduction in liver fat content while decreasing serum TG and LDL (13, 105).

LLF580, a genetically engineered variant of human FGF21, demonstrated beneficial effects on serum lipids profile (caused a decrease in total cholesterol and LDL, and an increase in HDL level) in obese and mildly hypertriglyceridemic adults (107). This study also showed a substantial reduction in hepatic fat content and improvement in liver function tests and biomarkers of liver injury suggesting that LLF580 may be beneficial for the treatment of metabolic disorders such as NAFLD (107).

These clinical trials on FGF21 analogs and mimetics showed that they could be used as potential therapeutic agents for metabolic and liver disorders. Besides, safety concerns for instance cardiovascular side effects and possibility of bone loss raise the questions like will FGF21 analogs be safe in chronic treatments and how would potential side effects including anti-drug antibodies (82) influence the development of FGF21 analogs. Another challenging issue is FGF21 resistance which was discussed by Fisher et al. (113). According to their investigations, diet-induced obese mice had an elevated endogenous level of FGF21 and responded poorly to acute exogenous FGF21 administration (113). Also, a study on NAFLD model of mice revealed that expression level of β-klotho was negatively correlated with plasma FGF21, intrahepatic triglyceride and body weight which suggested a resistance to FGF21 (14). However, other authors did not find any evidences of FGF21 resistance in obese mice (114) and as mentioned above FGF21 analogs are able to decrease body weight, plasma lipids and improve insulin sensitivity in obese patients with no obvious evidence of FGF21 resistance (115). Admitting these facts, FGF21 resistance due to its co-receptor alteration is a controversial issue to be considered during the further investigations of FGF21 as a novel pharmacological agent (14).

Therefore, larger and long-term trials should be established in order to assess their safety and efficacy.

Conclusion

Taken together our review suggested that FGF21 has a major role in the pathophysiology and treatment of AFLD, NAFLD and HCC. Administrating of FGF21 analogs and mimetics has demonstrated therapeutic benefit in human and rodent models of metabolic diseases, but still more studies and clinical trials will be required to prove the efficacy of these treatments. FGF21 is also used as a diagnostic biomarker for metabolic associated diseases of the liver. Also, despite the few studies that have been done on the genetic variations of FGF21, significant results have been obtained regarding the direct and indirect effects of these variations on metabolic diseases. In summary, the present study suggests that FGF21 administration can ameliorate fatty liver diseases and HCC furthermore studies are needed.

Author contributions

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

Conflict of interest

The 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.

Publisher's note

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EDITED BY
Ana Sandoval-Rodriguez,
University of Guadalajara, Mexico

REVIEWED BY
Moshe Levi,
Georgetown University, United States
Rohini Mehta,
Quest Diagnostics Nichols Institute,
United States

*CORRESPONDENCE
Thomas P. Burris
✉ burris.thomas@ufl.edu

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Development of LXR inverse agonists to treat MAFLD, NASH, and other metabolic diseases

Kristine Griffett[1] and Thomas P. Burris[2]*

[1]Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, AL, United States, [2]The University of Florida Genetics Institute, Gainesville, FL, United States

Activation of LXR activity by synthetic agonists has been the focus of many drug discovery efforts with a focus on treatment of dyslipidemia and atherosclerosis. Many agonists have been developed, but all have been hindered due to their ability to efficaciously stimulate *de novo* lipogenesis. Here, we review the development of LXR inverse agonists that were originally optimized for their ability to enable recruitment of corepressors leading to silencing of genes that drive *de novo* lipogenesis. Such compounds have efficacy in animal models of MAFLD, dyslipidemia, and cancer. Several classes of LXR inverse agonists have been identified and one is now in clinical trials for treatment of severe dyslipidemia.

KEYWORDS

MAFLD, NAFLD, liver X receptors, dyslipidemia, hypercholesterolemia, cirrhosis, hepatocellular carcinoma, pharmacology

1. Introduction

Metabolic-associated fatty liver disease (MAFLD) is a relatively new classification of disease to incorporate the metabolic dysfunction that often occurs within patients presenting with non-alcoholic fatty liver disease (NAFLD) (1). NAFLD has been deemed the world's leading chronic liver disease leading to liver transplantation or death (2). Currently, there are no universally approved therapies for NAFLD, and its heterogenous pathology often makes it difficult to identify and treat. The incorporation of metabolic dysfunction (e.g., high plasma triglycerides, prediabetes/diabetes, and increased blood pressure) into the definition of NAFLD has a widespread impact on patients and physicians alike and will improve treatment options for those with the disease (1, 3, 4). The current requirements for the diagnosis of MAFLD includes: 1) hepatic steatosis and 2) overweight/obesity, type 2 diabetes mellitus (T2DM), or metabolic dysfunction. Thus, MAFLD diagnosis partly overlaps with NAFLD but is independent of alcohol intake and co-existing causes of liver diseases. MAFLD is considered independent of other liver disease etiologies and allows for the identification of fatty liver in patients displaying other metabolic disorders (2, 3, 5). This review describes some of the processes that contribute to the development of MAFLD, processes that are involved in both MAFLD and NAFLD, and the novelty of targeting the liver X receptor (LXR) pathway using tissue selective inverse agonists to alleviate this disease.

2. Pathophysiological processes in the development of MAFLD

The pathophysiology of chronic liver diseases is quite complex, and with the multitude of factors than may contribute to the development of fatty liver, the term MAFLD allows physicians to distinguish between the term "non-alcohol" and be inclusive of key metabolic factors contributing to the disease and potential therapeutic options (6, 7).

2.1. Genetic factors involved in MAFLD development

Scientific evidence suggests that genetic factors strongly influence the development of MAFLD, and these factors overlap with those identified as factors for NAFLD and NASH (non-alcoholic steatohepatitis) (1, 5, 8). The patatin-like phospholipase domain-containing protein 3 gene (*PNPLA3*), Membrane bound O-acyltransferase domain containing 7 gene (*MBOAT7*), transmembrane 6 superfamily member 2 gene (*TM6SF2*), and glucokinase regulator gene (*GCKR*) have been the most recognized genes involved in the pathogenesis of fatty liver diseases (9, 10). *PNPLA3* was the first NAFLD-related genetic variant (rs738409; I148M) identified that displays a robust association with the development and severity of NAFLD. This gene is highly expressed in the liver and white adipose tissues and is regulated by insulin signaling via LXR and sterol regulatory binding protein 1c (SREBP1c) pathways (5, 9, 11, 12). Normally, this protein hydrolyzes triglycerides and retinyl esters, however the genetic variant results in impairment of the hydrolase activity, leading to hepatic lipid accumulation.

MBOAT7 functions to remodel phosphatidyl inositol with arachidonic acid and is primarily expressed in the liver (13–15). The rs641738 mutation (C > T), increases the risk of developing not only MAFLD, but an entire spectrum of liver diseases (NASH, cirrhosis, and hepatocellular carcinoma) (16, 17). The TM6SF2 protein facilities hepatic secretion of triglyceride-rich lipoproteins through the VLDL secretion pathway. The variant associated with the development of MAFLD/NAFLD involves a C-to-T substitution at nucleotide 499, which causes a glutamate to lysine change and results in decreased expression of TM6SF2 (18–20). Reduced expression of TM6SF2 results in an increase in hepatic lipid content and is associated with increased hepatic fibrosis in patients.

GCKR regulates glucose influx into hepatocytes to control *de novo* lipogenesis (9). There have been several variants of *GCKR* associated with the development of liver diseases, and the most severe variants lead to overexpression of GCKR, enhancement of hepatic glucose uptake, and increased hepatic lipogenesis (21, 22). Interestingly, this often leads to reduced serum glucose levels (attributed to the enhanced hepatic uptake), and while it may be beneficial by lowering T2DM risk, can alter insulin signaling and further contribute to the progression of MAFLD (16, 23). While these are not the sole genetic factors that contribute to MAFLD and liver disease development, they are currently the most common and well-characterized of the genetic factors. Interestingly, a commonality among these factors is that they either enhance or inhibit pathways involved with insulin and glucose signaling, *de novo* lipogenesis, or lipoprotein secretion and/or packaging.

2.2. *De novo* lipogenesis in MAFLD

Because of metabolic dysfunction during MAFLD progression, adiponectin levels are often decreased which leads to the decrease in free fatty acid (FFA) oxidation, which can stimulate *de novo* lipogenesis (DNL) in the liver. DNL is the metabolic pathway that synthesizes saturated fatty acids and monounsaturated fatty acids (MUFAs) from acetyl-coA (5, 19). In patients with MAFLD, the rate of hepatic DNL in greatly increased due to enhanced expression of DNL pathway enzymes that are regulated by the transcription factors sterol regulatory element binding protein-1 (SREBP1) and carbohydrate response element-binding protein (ChREBP) (19). These transcription factors can be activated via glucose flux and insulin signaling, demonstrating how metabolic dysfunction caused by hyperglycemia and/or hyperinsulinemic conditions promotes DNL and steatosis in MAFLD.

Within hepatocytes, FFAs can be esterified to produce TGs, which are either stored as lipid droplets in the liver or packaged as VLDLs into circulation. Because of this, MAFLD patients often present with pro-atherogenic lipid profiles (e.g., low HDL-C and elevated LDL-C, TG, and apolipoprotein B) (11, 19, 22, 24). Humans have a compensatory mechanism to reduce hepatic fat content through the activity of cholesterol ester transfer protein (CETP), which exchanges TG and cholesterol esters between VLDL, HDL, and LDL cholesterol (25–27). However, this mechanism often results in abnormally high HDL cholesterol metabolism and leads to undesirable alterations in lipid profiles in patients. Like NAFLD, dyslipidemia is not constant across the stages of MAFLD (19). Typically, circulating levels of VLDL and LDL are increased in earlier stages, then as MAFLD progresses, patients will develop hepatic fibrosis and circulating levels of apoB-containing lipoproteins decrease (4, 11, 25, 28). Therefore, research indicates that dyslipidemia in MAFLD appears the most pronounced at the earlier stages of the disease.

While DNL contributes to hepatic steatosis, it also is linked to very low-density lipoprotein (VLDL) production via ChREBP activation of microsomal triglyceride transfer protein (*MTTP*) and *TM6SF2*. Several studies have described an increase in VLDL particle size and number due to increased ChREBP and SREBP1 activity, which was observed due to pharmacological activation of the nuclear receptor LXR, a known regulator of DNL (29, 30). Stimulation of the DNL pathway and an increase in the quantity and particle size of VLDL via LXR activation was further confirmed in a study that investigated the stearoyl-CoA desaturase (SCD) enzyme which is involved in the synthesis of MUFAs (29, 30). In MAFLD patients, SCD activity is increased, leading to increased VLDL secretion as well as increased plasma and hepatic triglyceride (TG) levels. Inhibition of SCD, which is a direct target gene of LXR, can suppress hypertriglyceridemia (5, 22).

Other enzymes within the DNL pathway have been identified as targets for alleviating dyslipidemia and MAFLD. The inhibition of fatty acid synthase (FASN), which is the enzyme that synthesizes palmitate from acetyl-CoA and malonyl-CoA, can suppress hepatic steatosis in a variety of mouse models of fatty liver disease (5, 31, 32). While it has yet to be determined whether specifically

inhibiting FASN is a valid approach for the treatment of MAFLD, it is also a direct target gene of LXR, suggesting that targeting this nuclear receptor for MAFLD will have beneficial effects in several physiological pathways involved in the pathogenesis of this disease.

2.3. Altered lipoprotein processing in the liver attributes to MAFLD

Lipoprotein processing and signaling play an important role in the development of metabolic dysfunction and MAFLD. As mentioned earlier, genetic anomalies and altered DNL processes can contribute to lipoprotein processing defects in MAFLD (5, 16, 19). For example, alterations in VLDL secretion leads to increased lipid content in hepatocytes. However, one area that should be discussed is the role of lipoprotein receptors in the development of MAFLD. The major receptor for cholesterol enriched APOB containing lipoproteins is the LDL receptor (LDLR) (33, 34). Decades of studies have demonstrated that the functional loss of this receptor induces severe hypercholesterolemia and plays a key role in the development of several cardiovascular diseases including atherosclerosis (35–37). *Ldlr* knockout rodents are prone to develop hepatic steatosis particularly when fed a western or high fat diet (38, 39). The role that this receptor plays in MAFLD in humans is unclear, but mutations in the *LDLR* gene are relatively common (40).

ApoE-deficient rodents are another model of hypercholesterolemia and cardiovascular disease commonly used to evaluate therapeutics for atherosclerosis (41–43). Like the *Ldlr*-deficient mice, *ApoE*-deficient mice and rats also exhibit steatohepatitis regardless of diet and are likely an important model of MAFLD for drug discovery. APOE in human and mice, affects hepatic lipid balance via VLDL secretion. This altered balance signals for the activation of resident Kupffer cells and infiltration of peripheral macrophages, leading to progression of MAFLD and hepatic fibrosis. As VLDL balance is implicated in the development of MAFLD, there is a role for the VLDL receptor (VLDLR) in this disease as well. VLDLR is typically expressed at low levels in healthy liver and mediates the clearance of triglyceride-rich particles. During MAFLD development, in both mouse models and humans, the expression levels of VLDLR in liver increases enhancing the development of the disease. Recent data demonstrated that in mice lacking the proprotein convertase subtilisin/kexin type 9 (PCSK9) protein have increased expression of VLDLR, LDLR, and fatty acid transporters including CD36, which enhance the development of MAFLD. Loss of *PCSK9* also results in increased hepatic lipid accumulation, impaired beta cell function, and decreased plasma insulin levels.

MAFLD is a highly complex and systemic disease associated with a variety of metabolic changes and has similarities in the development of progression between mouse models and humans. Like NAFLD, MAFLD often begins with the accumulation of lipids in the hepatocytes, driven by a variety of biological factors (e.g., genetic alterations, nutrition, etc.) which is often mediated by VLDL secretion, fatty acid and lipoprotein uptake and processing, and DNL. These altered metabolic signaling processes and downstream effects on insulin signaling/regulation share features with NAFLD, cardiovascular diseases, and T2DM. While there is likely no single therapeutic target that can fully alleviate the complex metabolic dysfunction occurring in MAFLD, the nuclear receptors have proven to be a rich target class for targeting metabolic and cardiovascular diseases.

3. Nuclear receptors

Nuclear receptors (NRs) are ligand-regulated transcription factors that orchestrate numerous physiological processes including metabolism, immunity, and development (44). In humans, there are 48 members of the NR superfamily, which include receptors for steroid hormones, retinoic acid, thyroid hormones, fatty acids, and cholesterol metabolites or oxysterols (44–46). Many of the NRs are categorized as orphans since their natural ligands are not yet known. These signaling molecules regulate target gene transcriptional activity through a common mechanism enhanced by their modular structures. NRs have a highly conserved N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) connected by a variable (in size and sequence) hinge region. While the LBD is involved in determining ligand specificity, this region also contains a ligand-dependent transactivation function 2 (AF-2) domain, which allows the NRs to recruit co-factors for transcriptional regulation of target genes. These transcriptional co-factors include coactivators that mediate activation of transcription as well as corepressors that mediate silencing of target gene transcription (47–49). While many receptors are considered either exclusively activators (recruit coactivators) or repressors (recruit corepressors) of transcription, several receptors can recruit either coactivators or corepressors depending on the context of a physiological situation.

Several NRs respond to changes in cellular levels of lipids and other metabolic signals including LXRs, farnesoid X receptor (FXR), and peroxisome proliferated-activated receptors (PPARs), and have been identified as therapeutic targets for a variety of metabolic diseases (44, 50). FXR ligands (obeticholic acid, bile acid analogs, etc.) have therapeutic potential for the treatment of NASH with fibrosis (51). PPAR agonists (PPARα and PPARγ) have been clinically used for many years as treatments for diabetes and dyslipidemia while mixed (PPARα/δ/γ) agonists have been evaluated for efficacy against NASH (52–55). Here, we will focus on the LXRs, as they are master regulators of hepatic lipogenesis and are intricately involved in a variety of processes that lead to the development of MAFLD.

3.1. Liver X receptors

LXRα and LXRβ were originally identified as orphan members of the NR superfamily (56, 57). Both isoforms form heterodimers with obligate partner Retinoid X Receptor (RXR) and share the conserved domain structure with other NR members including a central DNA-binding domain (DBD) and carboxy-terminal ligand-binding domain (LBD). LXRα is primarily expressed in the liver, kidneys, intestines, and adipose tissues while LXRβ is widely expressed (56, 57). LXRs function as ligand-dependent transcription factors and bind directly to specific DNA sequences known as LXR 4esponse elements (LXREs). Following the discovery of the LXRs in the 1990s,

oxysterols were identified as the direct ligands for both receptor proteins (56, 57). Since oxysterols are metabolites of cholesterol and have been shown to be key signaling molecules that indicate sterol levels, it has been elucidated that LXRs function as cholesterol sensors. LXRs can detect relative cholesterol levels through oxysterol metabolites and alter cell physiology as appropriate. LXRs have been shown to regulate cholesterol efflux and transport, as well as regulate lipogenesis and glucose metabolism. Synthetic LXR agonists (T0901317, GW3965) have been shown to display anti-atherogenic properties due to their effects on reverse cholesterol transport mediated by increased cholesterol efflux from peripheral tissues (58–60). However, the activation of LXR by synthetic ligands results in deleterious effects due to increased hepatic lipogenesis and the development of hepatic steatosis (61, 62). This has led to significant difficulties in the development of tissue selective LXR agonists for the treatment of atherosclerosis. The stimulation of hepatic lipogenesis by LXR agonists is due to the increased expression of lipogenic enzymes including FASN, SCD1, and SREBP1c that are direct target genes of LXR (61). LXR expression has been correlated with the degree of hepatic lipid accumulation, as well as hepatic fibrosis and inflammation in patients with liver diseases.

LXRs have a significant role in the regulation of physiological processes involved in the development of MAFLD. As described earlier, numerous physiological pathways can contribute to MAFLD in both mice and humans. This disease is systemic in its development and pathogenesis, beginning with altered lipid storage and metabolism, and progressing in part, due to abnormal metabolic functioning in a variety of tissues and cell types (i.e., T2DM, obesity, inflammation, lipoprotein processing, etc.). Here, we will focus on the physiological processes that LXR regulates, that are distinctively known for enhancing the development and progression of MAFLD.

3.2. LXRs are involved in cholesterol and fatty acid metabolism

The role of LXRs functioning as "cholesterol sensors" was confirmed utilizing *Lxrα*-null mice, which accumulated significant amounts of cholesterol in the liver when challenged with a high cholesterol diet due to their inability to activate an LXR-dependent mechanism for excess cholesterol to be converted to bile acids (63). Subsequent studies have identified that LXRs enhance hepatobiliary cholesterol excretion through the direct activation of target genes, *Abcg5* and *Abcg8* (64). Reverse cholesterol transport (RCT) is the process by which excess cholesterol in the periphery is transferred to HDL and transported to the liver for bile acid synthesis and excretion. This process is mediated by the ATP-binding cassette transporter ABCA1 and ABCG1 in macrophages, both of which are direct target genes of LXR (65). Activation of LXRs also induces the expression of several apolipoproteins and genes involved in lipoprotein remodeling including phospholipid transfer protein (PLTP), lipoprotein lipase (LPL), and CETP (66–70). LXR's role in regulating cholesterol homeostasis via cholesterol transport into and out of the liver has a significant physiological impact on the development of MAFLD. Interestingly, this is not the only component of cholesterol metabolism that is regulated by LXR and has a direct effect on the pathogenesis of MAFLD and other dyslipidemic diseases.

It is well known that high levels of LDL cholesterol (LDL-C) contribute to the development of cardiovascular diseases including atherosclerosis. LDLR is responsible for the uptake of LDL-C and maintenance of systemic cholesterol levels and can be regulated at both the transcriptional and post-transcriptional levels. The sterol regulatory element-binding protein 2 (SREBP2) is the main transcription factor that regulates the expression of LDLR and is activated in response to low cholesterol levels in the cells. LXR however, can control the post-transcriptional regulation of LDLR through its direct target gene, *inducible degrader of LDLR* (*IDOL*) (71–73). The IDOL protein functions as an E3 ubiquitin protein ligase that directly leads to the degradation of LDLR, as well as VLDLR and other related proteins. Studies have shown that treatment with LXR agonists (T0901317 or GW3965) reduces LDLR expression and raises LDL-C plasma levels through an IDOL-dependent mechanism in humans and non-human primates. Genome-wide association studies have identified polymorphisms in the LDLR locus that leads to severe forms of statin-resistant hyperlipidemia (Familial hypercholesterolemia; FH). Patients with FH often are also diagnosed with some form of fatty liver disease.

LXRs are not only important in maintaining cholesterol homeostasis, but they are intricately involved in the regulation of DNL in the liver. Numerous studies have demonstrated the enhancement of fatty acid biosynthesis and VLDL secretion due to LXR agonist treatment. LXR directly controls the transcription of SREBP1c, FASN, and SCD1, and modulates the expression of ChREBP, all of which are directly involved in the pathogenesis of MAFLD and have been discussed earlier.

3.3. Synthetic LXR modulators

LXRs are master regulators of lipid and cholesterol metabolism and have remarkable anti-inflammatory activities. Because of their multiple roles, they are very interesting drug targets. The major classes of LXR modulators are agonists and antagonists. Agonists bind the LBD of the receptor and recruit coactivator proteins leading to receptor activation and increased expression of downstream target genes. Three LXR agonists are currently in clinical trials for the treatment of atopic dermatitis and advanced solid tumors and lymphoma. LXR antagonists block the binding of agonists and have yet to demonstrate therapeutic utility. A third type of modulator is LXR inverse agonists that was first developed by our group. LXRs have been demonstrated to recruit either coactivators or corepressors depending on the physiological context. We envisioned that development of a LXR ligand that bound to the LBD and selectively enhanced the ability of the receptor to recruit corepressor and suppress the expression of LXR target genes, such as those encoding the DNL enzyme genes, would have the potential to be used in the treatment of metabolic disorders such as MAFLD. The LXR antagonist scaffold (74) was used as an initial point to develop and optimize two novel LXR inverse agonists, SR9238 and SR9243 (**Figure 1**), that display potent activity for both LXRα and LXRβ and function to very efficaciously recruit corepressor proteins (75–77).

SR9238 exhibits the ability to suppress basal transcriptional activity of LXRα (IC_{50} = 210 nM) and LXRβ (IC_{50} = 40 nM) in a co-transfection assay with a multimerized LXRE luciferase reporter in HEK293T cells (76). In biochemical assays, SR9238 binding to LXRα or LXRβ resulted in recruitment of corepressor NCoR CoRNR

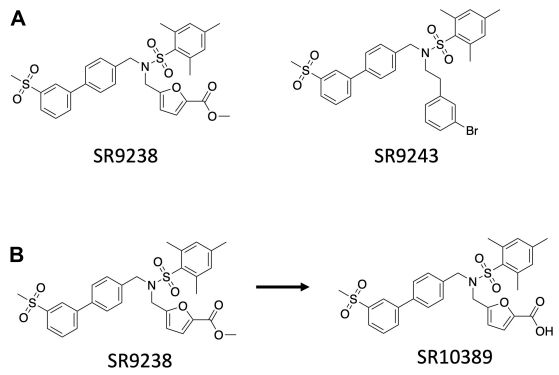

FIGURE 1
Structure of the SR series of LXR inverse agonists. **(A)** Chemical structure of SR9243 and SR9238. **(B)** Rapid metabolism of the ester functionality of SR9238 to the acid yields a liver specific LXR agonist.

box peptides consistent with the ligand functioning as an inverse agonist. Although both NCoR ID1 and ID2 peptides were recruited in a SR9238-dependent manner, there was clear preference for the ID1 peptide for both receptors (76). Treatment of HepG2 cells with SR9238 resulted in significant decreases in the expression of *FASN* and *SREBF1c*, which are key drivers of DNL (76).

Our goal was to develop a LXR inverse agonist that could be used *in vivo* to test our hypothesis that such compounds may hold utility in treated NAFLD. However, a significant concern we had about this type of compound was that if it may decrease RCT even though it may have a beneficial effect on hepatic DNL. Thus, we designed SR9238 with this in mind and SR9238 is a compound with a labile ester group that is rapidly metabolized to a carboxylic acid (Figure 1B). We had noticed previously that certain LXR antagonists would display significant activity with an ester substitution that was lost with hydrolysis of the ester to an acid (78). We observed a similar paradigm with SR9238 as its acid analog (SR10389) was inactive (76). When administered i.p. SR9238 displayed intestinal and hepatic exposure, but no SR9238 was detected in the plasma, skeletal muscle, or brain. Thus, SR9238 provided a tool to assess the ability to target the liver without adversely affecting LXR target genes in peripheral tissues that drive RCT.

When administered to diet induced obese (DIO) mice, SR9238 (i.p.) drove a decrease in expression of *Fasn*, *Srebf1c,* and *Scd1*, which was associated with a significant reduction in hepatic steatosis (76). The reduction in hepatic fat accumulation was accompanied by a decrease in expression of inflammatory genes including *Tnfa* and *Il1b* and a decrease in hepatic F4/80 + cells was also noted. Markers of hepatocellular injury in the plasma (ALP, ALT, and AST) were also significantly reduced consistent with SR9238 improving hepatic function.

The classic DIO mouse provides a model of NAFLD, but hepatic fibrosis (associated with NASH) is not typically noted in this model. However, mice provided a diet high in cholesterol, fructose and trans-fat do develop NASH (79) and we examined the effect of SR9238 in this model as well. This NASH model has been utilized in both C57Bl6 mice and the *ob/ob* leptin deficient mice with similar results, but the disease is accelerated in *ob/ob* mice possibly due to their increased intake of the diet. In the *ob/ob* mice provided this NASH diet, we observed similar effects of SR9238 as we did in the DIO mice with decreased expression of genes encoding DNL enzymes and decreased hepatic steatosis (80). Hepatic weight was decreased, and plasma liver enzymes were also substantially decreased. Importantly, hepatic inflammation was significantly suppressed and hepatic fibrosis decreased by 75% as assessed by collagen staining (80).

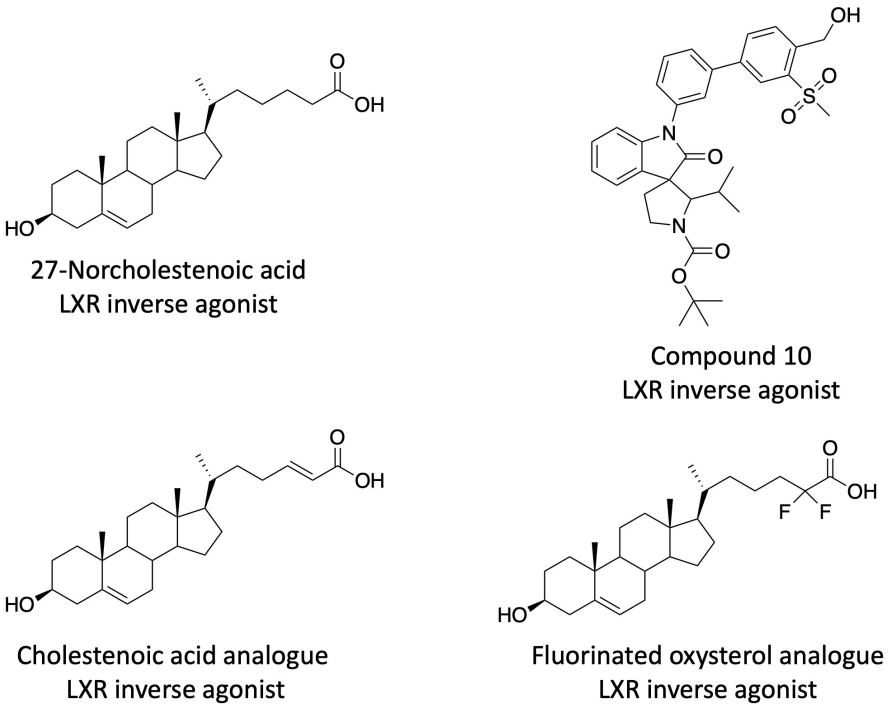

FIGURE 2
Examples of additional steroidal and non-steroidal LXR inverse agonists.

Although the mechanism of suppression of hepatic fibrosis is not clear, we hypothesized that this was due to the reduction of hepatic steatosis due to suppression of *de novo* lipogenesis leading to reduced inflammation and thus, reduced fibrosis. One interesting point that we noticed in both the NAFLD, and NASH models was that plasma LDL-cholesterol levels (LDL-C) were lowered significantly. In the DIO mice there was a ∼20% decrease whereas in the NASH model there was a ∼50% decrease. We also observed that SR9238 analog, SR9243, that has systemic exposure also displayed similar effects on plasma LDL-C in mice on a normal chow diet (∼50% decrease) (81). At this time, we did not have a proposed mechanism underlying the reduction in LDL-C, but interestingly, a later study of a LXR agonist showed an increase in LDL-C in both non-human primates and in clinical studies (82). This suggested that the LXR inverse agonist mediated decrease in LDL-C we observed in mouse models may be clinically relevant. An independent group assessed the activity of SR9243 in distinct models of NASH including the bile-duct ligation and carbon tetrachloride treatment (83). Huang et al. observed that SR9243 treatment reduced hepatic fibrosis and liver enzymes in both models (83). LDL-C levels were also substantially reduced.

Alcohol consumption is another major driver of liver disease and ethanol also induces hepatic DNL leading to inflammation and fibrosis. Chronic ethanol consumption by mice (Lieber-DiCarli (LD) diet) leads to substantial hepatic steatosis but does not lead to significant hepatic fibrosis. However, addition of "binge" ethanol doses near the end of the chronic ethanol consumption does lead to fibrosis and is a model of alcoholic hepatosteatosis (ASH). We assessed the effects of SR9238 in both models and observed that the drug reduced both fat content in the liver and inflammation (and fibrosis in the ASH model) (84). Like in the NASH model, SR9238 treatment resulted in substantial decrease in the expression of *Srebpf1c* and *Fasn*. Interestingly, treatment also led to an increase in expression of ethanol metabolizing enzymes *Cyp2e1*, *Adh2,* and *Adh3*, suggesting that not only did the LXR inverse agonist suppress DNL but also increased ethanol clearance (84). Given that many patients with steatohepatitis that is driven by both a high fat diet and ethanol consumption, we developed a diet that is composed of both the high cholesterol/trans-fat/fructose and ethanol (WASH diet – western diet and alcohol steatohepatitis). We found that the high cholesterol/trans-fat/fructose diet synergistically acted with the ethanol to enhance hepatic steatosis, inflammation, and fibrosis (85). Importantly, SR9238 treatment was able to suppress the severity of the effects on the liver (85).

As indicated above, one interesting observation we had made with any of the LXR inverse agonists we had tested *in vivo* was that there was a significant decrease in LDL-C. When examining the expression of intestinal genes that changed with SR9238 or SR9243 treatment (i.p.) we found that sterol O-acyltransferase 2 (*Soat2*) gene expression was suppressed by ∼95% (86). This intrigued us given that SOAT2 has been a target for development of drugs to treat hypercholesterolemia and atherosclerosis (87). SOAT2 is an enzyme that converts cholesterol to cholesterol esters and drives intestinal cholesterol absorption (88). Mice with an intestine specific KO of *Soat2* are resistant to development of elevated plasma LDL-C on a high cholesterol diet (89) suggesting that targeting intestinal SOAT2 function or expression may be sufficient to provide this benefit. With our knowledge that SR9243 had no significant oral bioavailability we treated *Ldlr* null mice on a high cholesterol diet and observed that even though there was no liver or plasma exposure when SR9243 was administered orally, LDL-C was substantially decreased and was associated with repression of intestinal *Soat2* expression and increased fecal cholesterol elimination (86). These data provided us with a clear mechanism that was driving the reduction in plasma LDL-C that we consistently observed as well as suggested that such compounds may hold utility in treatment of hypercholesterolemia, particularly in individuals that have mutations in the LDL receptor driving familial hypercholesterolemia.

After the description of the SR9238/SR9243 series of LXR inverse agonists additional chemical scaffolds with similar pharmacological profiles have been described. Burton et al. discovered that several cholestenoic acid analogs displayed LXR inverse agonist activity (**Figure 2**) (90, 91). These compounds showed the ability to suppress basal transcription in LXR cotransfection assays as well as suppress the expression of LXR target genes (*Fasn*, *Srebf1c*, and *Abcg1*) in HepG2 cells. These compounds drove the recruitment of corepressor proteins to the LXRs, but they did not appear to be very potent as doses greater than 1 μM were required for activity) (90, 91). This group also identified certain fluorinated oxysterol agonists as LXR inverse agonists based on their activity in LXR cotransfection assays in HEK293 cells, but these were also relatively low potency (**Figure 2**) (92). Chen et al. identified several non-steroidal LXR inverse agonists based on a screen of a compound library that was designed based on co-crystal structures of LXRβ in complex with spiro[pyrrolidine-3,3′-oxindole] agonists (93). These compounds displayed a significant degree of LXRβ selectivity (as much as 100-fold) and the most potent compound was approximately 3.5-fold less potent than SR9238 in a LXRβ cotransfection assay (93). Their most potent compound, 10rr (**Figure 2**), effectively suppressed *SREBF1c*, *ACC*, *FASN,* and *SCD1* expression in both 3T3-LI adipocytes and HepG2 cells. Compound 10rr suppressed DNL in HepG2 cells consistent with the effects on gene expression and suppressed hyperlipidemia in the Triton WR-1339 induced mouse model (93). Working from the SR9238/SR9243 scaffold, Phenex Pharmaceuticals, developed additional LXR inverse agonists based on a published patent application (94). This intellectual property was licensed by Orsobio, Inc., and a compound (TLC-2716) is currently in phase I clinical trials for treatment of severe dyslipidemia (ClinicalTrials.gov NCT05483998). The structure of TLC-2,716 is not directly disclosed.

4. Conclusions and perspectives

LXRs have long been the focus of synthetic ligand development for the purpose of treating dyslipidemia and atherosclerosis. However, this focus has almost entirely been on the development of LXR agonists attempting to drive RCT. It was rapidly determined that such compounds had limiting on-target toxicity associated stimulating DNL resulting in hepatic steatosis and hypertriglyceridemia. Work from our lab focused on utilizing this observed side effect to develop and characterize LXR inverse agonists that actively silence LXR target genes, particularly those that drive *de novo* lipogenesis. With these compounds in hand we were able to demonstrate that were effective in treatment of NASH, ASH, hypercholesterolemia, and cancer in animal models. Several LXR inverse agonist chemical scaffolds have now been identified that display similar pharmacology and even one has entered phase I clinical trials for treatment of severe dyslipidemia.

Author contributions

KG and TB wrote and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

Publisher's note

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EDITED BY
Juan Armendariz-Borunda,
University of Guadalajara,
Mexico

REVIEWED BY
Renata Heisler Neves,
Rio de Janeiro State University,
Brazil
Leticia Bucio Ortiz,
Autonomous Metropolitan University,
Mexico

*CORRESPONDENCE
Yan He
✉ helen-1101@hotmail.com
JianMing Wang
✉ wjm18jgm@liyun.com

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An update on animal models of liver fibrosis

ShuTing Wu[1], XinXin Wang[1], WenBo Xing[1], FenYao Li[1], Ming Liang[1], KeShen Li[1], Yan He[1]*and JianMing Wang[1,2]*

[1]Institute of Regenerative and Translational Medicine, Tianyou Hospital, Wuhan University of Science and Technology, Wuhan, China, [2]Department of Hepatobiliary and Pancreatic Surgery, Tianyou Hospital, Wuhan University of Science and Technology, Wuhan, China

The development of liver fibrosis primarily determines quality of life as well as prognosis. Animal models are often used to model and understand the underlying mechanisms of human disease. Although organoids can be used to simulate organ development and disease, the technology still faces significant challenges. Therefore animal models are still irreplaceable at this stage. Currently, *in vivo* models of liver fibrosis can be classified into five categories based on etiology: chemical, dietary, surgical, transgenic, and immune. There is a wide variety of animal models of liver fibrosis with varying efficacy, which have different implications for proper understanding of the disease and effective screening of therapeutic agents. There is no high-quality literature recommending the most appropriate animal models. In this paper, we will describe the progress of commonly used animal models of liver fibrosis in terms of their development mechanisms, applications, advantages and disadvantages, and recommend appropriate animal models for different research purposes.

KEYWORDS

liver fibrosis, liver injury, inflammation, *in vivo*, animal models

1. Introduction

For a long time, advances in biomedical research have often relied on the use of animal models as the basis for experimental and clinical hypotheses. The occurrence and development of various human diseases are very complex, and it is impossible and not allowed to conduct experimental research on human body to explore the pathogenesis, prevention and treatment mechanism of diseases. Therefore, animal models are frequently used to simulate and understand the underlying mechanisms of human disease. Organ fibrosis is the characteristic of the progression of chronic inflammatory diseases, which account for 45% of global all-cause mortality (1). Equally, the development of fibrosis primarily determines quality of life and prognosis in the liver (2). Liver fibrosis animal models are indispensable tools for studying the pathogenesis of liver fibrosis and developing therapeutic drugs. Although organoids can be used to simulate organ development and disease, they have wide applications in basic research, drug development and regenerative medicine (3–5). However, obtaining freshly isolated human hepatocytes is very limited and maintaining cultures in spinner flasks can be cost prohibitive, and hepatocyte maturation, culture longevity, and large-scale production of pure cultures remain challenges (6). Therefore, animal models are still irreplaceable at this stage.

At present, there are five types of *in vivo* models of liver fibrosis: chemical, dietary, surgical, and transgenic and immune (7) (Figure 1). Animals commonly used to prepare models are mainly mice (8), rats (9), rabbits (10), Ossabaw pigs (11), macaques (12) and zebrafish (13). There are various animal models of liver fibrosis with different efficacy, which have different effects on

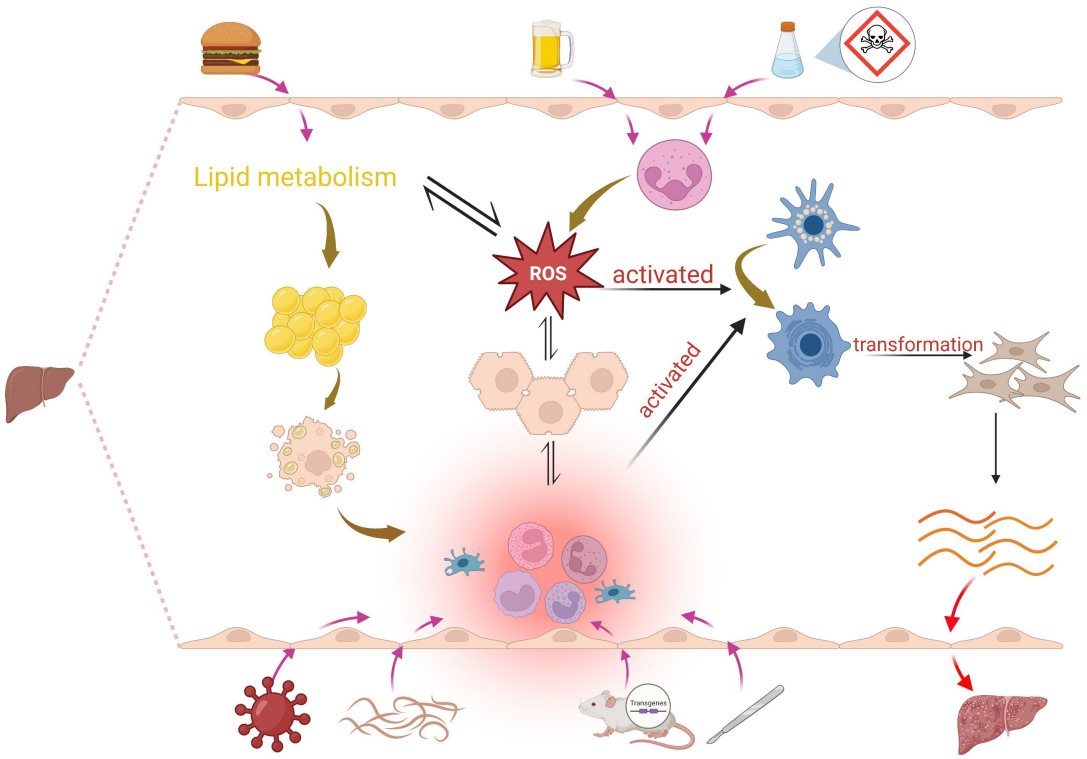

FIGURE 1
Mechanisms of induction in the liver fibrosis model: Chemical induction mainly leads to massive infiltration of neutrophils to produce ROS, which induces hepatocyte injury and activation of HSCs, and also interferes with lipid metabolism; hepatocyte injury significantly increases the production of immune cells such as cytokines, chemokine, Kupffer cells, B cells, T cells and ROS, and this pro-inflammatory environment and ROS activates HSC, promotes the conversion of HSC into myofibroblasts, and increased production of ECM proteins, which subsequently leads to liver fibrosis. Dietary induction mainly affects lipid metabolism, which leads to ROS as well as fat accumulation, and excessive accumulation of lipids in hepatocytes also activates inflammation. Surgery, transgenic, and immune induction induce liver fibrosis mainly by causing inflammation.

the correct understanding of the disease and the effective screening of therapeutic drugs. There is currently no high-quality literature recommending the most appropriate animal model. This article will describe the research progress of commonly used animal models of liver fibrosis from the aspects of the development mechanism, application, advantages and disadvantages of animal models (Table 1) and recommend suitable animal models for different research purposes.

2. Chemical induction methods

The chemical injury liver fibrosis model is used to induce the formation of liver fibrosis by using chemical drugs to enter hepatocytes to produce toxic metabolites that cause persistent liver injury. Currently, this model preparation method mainly uses ethanol, carbon tetrachloride (CCl_4), thioacetamide (TAA), dimethylnitrosamine (DMN), diethylnitrosamine (DEN) or other liver toxins to induce liver fibrosis models.

2.1. Alcohol-induced liver fibrosis model

The liver is the main organ involved in alcohol metabolism. Fibrosis associated with alcoholic liver disease is caused by multiple mechanisms, including acetaldehyde accumulation, reactive oxygen species (ROS) and hepatic overload of endogenous lipopolysaccharide (LPS) (14). Related research has shown that chronic alcohol abuse leas to overproduce of ROS and interferes with lipid metabolism in the liver, resulting in ROS-mediated liver injury (15). It is supposed that fibrosis is promoted by neutrophils through ROS production inducing hepatocyte injury and hepatic stellate cells (HSC) activation (16). Moreover, alcohol-stimulated liver fibrosis is the result of strong immune response involving many types of hepatocyte and different signal transduction pathways (17). Alcohol-induced liver injury significantly increases the production of cytokines, chemokines, other soluble mediators and components of the innate immune system, this pro-inflammatory environment leads to the activation of HSC and myofibroblast, increases the production of extracellular matrix (ECM) proteins, which can subsequently induce fibrosis in the liver (18).

Alcohol *ad libitum* feeding model is one of the earliest animal models used for alcoholic liver disease research in rodents (19). The concentration of ethanol solution is at 10–40% (v/v), and the alcohol administration cycle used in different groups is from 8 weeks to 70 weeks, there is no significant change in mortality (20). In most studies, models of alcohol *ad libitum* feeding can sufficiently induce liver injury, and accompanied by significant steatosis and exaltation in aspartate aminotransferase and alanine aminotransferase, but no more advanced fibrotic or cirrhotic lesions (20, 21). Because mice are naturally adverse alcohol; methods of feeding alcohol-containing liquid food are greatly limited. At present, the more commonly used method is alcohol combined with chemical gavage, which replicates the alcoholic liver fibrosis model

TABLE 1 Induction method, modeling time, and liver fibrosis in animal models of liver fibrosis.

Model	Induction method	Species	Method	Periodicity (weeks)	Liver injury	Inflammation	Fibrosis
Chemical							
Ethanol	Ethanol	rat/mouse	i.g.	8 ~ 70	Y	↑	↑
CCl_4	CCl_4	rat/mouse	i.p.	4 ~ 6	Y	↑↑	↑↑
TAA	TAA	rat	i.p.	12 ~ 13	Y	↑↑	↑↑
		macaque	s.c.	8	Y	↑↑	↑↑
		marmoset	s.c.	11	Y	↑↑	↑↑
Nitrosamines	DMN	rat	i.p.	4	Y	↑↑	↑↑↑
	DEN	rat/mouse	i.p.	4 ~ 6	Y	↑↑	↑↑↑
Diet							
MCD	MCD	mouse	p.o.	6 ~ 8	Y	↑↑	↑
HFD	HFD	mouse	p.o.	24 ~ 25	Y	↑	↑
	WD	Ossabaw pig	p.o.	16	Y	↑↑	↑
	FFD	mouse	p.o.	30	Y	↑↑	↑↑
CDAA	CDAA	rat/mouse	p.o.	12	Y	↑↑	↑
CDAHFD	CDAHFD	rat/mouse	p.o.	6 ~ 9	Y	↑↑	↑↑
Surgical							
BDL	BDL	rat/mouse	p.o.	4 ~ 5	Y	↑↑	↑↑
Transgenic							
Transgenic	Gnmt-	mouse	knockout	12	Y	↑↑	↑↑
Transgenic	$Mdr2^{-/-}$	mouse	knockout	8 ~ 14	Y	↑↑	↑↑
Immunity							
Schistosoma	Schistosoma j	mouse	s.c.	8	Y	↑↑	↑↑
Virus	HBV	mouse	i.v.		Y	↑↑↑	↑↑
PS	PS	rat	i.p.	16 ~ 24	Y	↑↑	↑
Con A	Con A	mouse	i.v.	4 ~ 8	Y	↑↑	↑
Composite							
Chemical +Chemical	CCl_4 + Ethanol	mouse	i.g. + i.p.	7	Y	↑↑↑	↑↑
Chemical +Diet	STAM	mouse	s.c. + p.o.	3 ~ 8	Y	↑↑	↑↑↑
	HFD + Ethanol	mouse	i.g. + p.o.	12	Y	↑↑	↑↑
	CCl_4 + WD	mouse	i.p. + p.o.	12	Y	↑↑	↑↑↑
	TAA + FFD	mouse	i.p. + p.o.	8	Y	↑↑	↑↑↑
Transgenic +Diet	*ob/ob* + HFD	mouse	p.o.	20	Y	↑	↑↑
	adropin-KO + MCD	mouse	p.o.	4	Y	↑↑↑	↑↑
	adropin-KO + WD	mouse	p.o.	16	Y	↑↑↑	↑↑

"i.g." represents intragastric administration; "i.p." represents intraperitoneal injection; "s.c." represents subcutaneous injection; "p.o." represents per os; "i.v." represents intravenous injection. "Y" means yes, the "↑." indicates mild, "↑↑" indicates moderate, and "↑↑↑" indicates severe.

while controlling the diet, this model has the advantages of simple operation, short cycle, high forming rate (22).

2.2. CCl_4-induced liver fibrosis model

CCl_4 has been widely used to induce mice liver injury and fibrosis for decades (23). High dose (≥1 ml/kg) of CCl_4 can lead to reproducible acute liver injury. Toxicity of CCl_4 is dependent on the P450-catalyzed metabolism to the reactive metabolite trichloromethyl radical (CCl_3), and CCl_4 is converted into $\cdot CCl_3$ to bind to proteins, deoxyribonucleic acid (DNA) and lipids, which can cause mitochondrial damage and oxidative stress. $\cdot CCl_3$ can also react with O_2 to form trichloromethylperoxy radical (CCl_3OO), thereby initiating lipid peroxidation chain reaction and destroying cell membrane (24).

Liver fibrosis is induced by intraperitoneal injection of CCl_4 administered 2–3 times a week for 4–6 weeks in most research protocols. Bubnov et al. (9) injected freshly prepared 50% CCl_4 hydrated olive oil solution into the rat intraperitoneally. On the 8th week after injection observed ultrasound manifestation of advanced liver fibrosis, including hepatosplenomegaly, portal hypertension, demonstrating that Carbohydrate tetrachloride induces injury of liver parenchyma evoking fast and severe liver fibrosis. CCl_4 treatment increased serum aspartate aminotransferase and alanine aminotransferase levels, produced hepatic oxidative and nitrative stress, and evoked profound expression of pro-inflammatory cytokine expressions in liver tissue (25). Moreover, the animals of CCl_4 treatment exhibited higher apoptosis and showed obvious fibrosis in animal liver (25). Research showed that the non-specific liver inflammation triggered by CCl_4 recruited high numbers of $CD4^+$ T, $CD8^+$ T and B cells, and elevated the expression of proinflammaitory cytokines in mice, further breaking liver tolerance and inducing autoimmune response, Autoimmune hepatitis inflammation and liver fibrosis in the presence of CYP2D6 antigen mimicry (26).

The advantages of the CCl4-induced fibrosis model are the relatively low cost of development, the relatively simple method of implementation, the short duration of induction, and the significant pathological changes in the liver tissue, which can be reversed even after cessation of drug administration (23). This model is a representative and reproducible model of liver fibrosis and is frequently used in the research of liver fibrosis development and the research of liver repair mechanism. However, the disadvantage of this model is that the animals cannot become obese or develop insulin resistance (IR), which is different with pathophysiological features of non-alcoholic fatty liver disease (NAFLD) patients induced by metabolic disorder (27). Furthermore, CCl_4 is highly toxic and volatile, requiring researchers to take appropriate safety measures.

2.3. TAA-induced liver fibrosis model

TAA is a classic liver toxin and also a potent carcinogen and mutagen, which can induce oxidative stress and sterile inflammation, leading to acute and chronic liver injury (28, 29). TAA induces hepatotoxicity in mice and rats at doses ≥100 mg/kg. It is converted to metabolites TAA S-oxide and S, S-dioxide by cytochrome P450 enzymes and S, S-dioxide initiates toxicity by binding to lipids and proteins (24). TAA-induced liver injury is mainly caused by reaction metabolites secreted by TAA, which not only activate HSC, but also produce fibrinogen and growth factors, aiming to promote acute liver injury and chronic liver fibrosis (29).

TAA-induced liver fibrosis is a widely used model, and TAA can be administered orally or by intraperitoneal injection. But intraperitoneal injection provides more consistent results. Many researches have used Sprague–Dawley (SD) rats to induce liver fibrosis by intraperitoneal injection of TAA at a dose of 200 mg/kg twice a week for 12–13 weeks (30, 31). Matsuo et al. (12) used healthy macaca fascicularis to induce fibrosis model, dissolving TAA in normal saline and administrated three times a week at a dose of 100 mg/kg, and obtained that the TAA-induced model was superior to the CCl_4 model. It both induced liver fibrosis progression and worsened residual liver function, but there were also individual differences in the effect of the reagent and the inability to assess whether reversal of fibrosis would occur after cessation of the reagent. Inoue et al. (32) have developed a marmoset hepatic fibrosis model for regenerative medicine research. The female marmosets were administered TAA at a dose of 2.5–40 mg/kg two or three times a week, lasting 11 weeks, the results suggest that continuous TAA administration induces persistent hepatic fibrosis in the common marmoset and this nonhuman primate hepatic fibrosis model have the possibility to evaluate the therapeutic effects of test samples to ameliorate hepatic fibrosis.

TAA-induced liver fibrosis is very similar to human liver fibrosis in terms of hemodynamic, morphological and biochemical metabolism (33). TAA disrupts DNA, RNA and protein synthesizing enzymes in hepatocytes, leading to metabolic disturbances and hepatocyte necrosis, a distinctive feature of this model compared to the CCl4 model, whose fibrosis remains stable for several weeks after TAA withdrawal. However, TAA is a carcinogen, which is both toxic and volatile (34).

2.4. Nitrosamines-induced liver fibrosis model

2.4.1. DMN-induced liver fibrosis model

DMN is a powerful liver toxin, which can lead to liver injury, and provides a suitable experimental rat modeling reagent for liver fibrosis. The metabolic activation and detoxification of DMN cause hepatocyte injury, inflammation, neutrophil infiltration, and massive hepatic necrosis, which results in oxidative stress and production of ROS. These processes induce activation of hepatic stellate cells and increased synthesis of connective tissue components, especially collagens that end up in hepatic fibrosis (35). DMN not only induces liver fibrosis, but also can lead to cirrhosis due to repeated exposure to low doses in animals (36).

Many studies induced fibrosis in male SD rats by intraperitoneal injection of DMN at a dose of 1 ml per 100 g body weight per week, 3 days per week for 4 weeks (37–39). Repeated exposure to low doses of DMN results in subacute or chronic liver injury with varying degrees of necrosis, fibrosis, and nodular regeneration (40). DMN can cause acute liver injury in rats and reproduce the characteristics of human liver fibrosis and cirrhosis, as well as collagen accumulation, hepatocyte apoptosis, elevated oxidative stress and lipid peroxidation (41).

DMN-induced liver fibrosis rat model is a commonly used animal model to study liver injury diseases. Due to its short modeling time and low mortality, the formation of liver fibrosis is stable and is very similar to the characteristics of early changes and collagen fibrosis deposition of human liver fibrosis, and it is not easy to spontaneously resolve and recover after the cessation of exposure, so it is one of the classic animal models for studying the pathological mechanism, serum marker evaluation and drug therapy of liver fibrosis (42). However, researchers should ensure appropriate safety measures are in place due to the toxicity of nitrosamines.

2.4.2. DEN-induced liver fibrosis model

DEN is considered to one of the most toxic drugs, which can result in various forms of necrosis and subsequent fibrosis (43). DEN has been shown to induce severe liver injury by inducing mutant DNA damage and upregulating ROS production (44). Furthermore, DEN administration results in excessive deposition of ECM protein

(collagen) in rat liver and seems appropriate to study early events associated with the development of liver fibrosis (45). Some studies induced liver fibrosis by intraperitoneal injection of DEN in rats once a week for 4–6 weeks (46, 47).

DEN, a known carcinogen that leads to significant oxidative stress and DNA mutations, enhances lipotoxicity and accelerates the progression of fibrosis and cirrhosis, has long been used in hepatocellular carcinoma (HCC) models (48). Chen et al. (49) studied a DEN-induced cirrhosis mouse model, in which male C57BL/6 mice were given 0.014% DEN in drinking water 6 days a week, 1 day interval from normal drinking water, for 15 weeks. In this model, all mice given oral DEN developed liver fibrosis, cirrhosis, and HCC, and the histological pattern in the model was similar to that described in humans. DEN-induced rat HCC, which presents a stepwise histopathological progression similar to human HCC, was used to analyze different stages of inflammation, fibrosis, and cancer. Ding et al. (50) injected DEN in rats at a dose of 30 mg/kg body weight twice a week for 11 weeks and the animals were observed until week 20. The results suggested that the model characterized resulted in three stages: the inflammation stage (week 2–6), the fibrosis stage (week 8–12), and the HCC stage (week 14–20).

2.5. Other liver toxins

Other liver toxins such as arsenic (As), acetaminophen (APAP), and d-galactosamine (D-GalN) can also induce liver fibrosis.

As is an environmental toxicant and human carcinogen, and the liver is the main target organ for arsenic toxicity. As and its metabolites are toxic to hepatocytes, causing DNA damage and generating several free radicals. Free radicals subsequently induce lipid peroxidation, which may lead to cellular dysfunction or directly attack cells, triggering their damage (51). Repeated damage and repair of hepatocytes leads to liver fibrosis (52). As exposure causes liver injury in rats and liver fibrosis increases with increasing dose and time (53). Arsenite-induced liver fibrosis is a slow disease process in which many cellular and inflammatory factors are involved, including hepatocyte water degeneration, hepatocyte balloon formation, hepatocyte necrosis (inflammatory infiltration), hepatocyte regeneration, fibrous tissue proliferation, and liver fibrosis (53). Arsenite exposure induced HSC activation and extracellular matrix deposition, and long-term exposure to arsenite induced liver damage, inflammation, and fibrosis in mice or rats (54–56). Wang et al. (52) fed SD rats at a high dose of 100 mg/kg and exposed to sodium arsenite, cell swelling, inflammatory cell infiltration, and fibrous proliferation were evident.

APAP is a major cause of hepatic failure (57). The vast majority of ingested APAP is glucuronidated or sulfated and rapidly excreted. However, a small fraction is metabolized by hepatic cytochrome P450 enzymes to the highly reactive intermediate N-acetyl-p-benzoquinone mine (NAPQI), which is usually detoxified by glutathione (GSH)-coupled detoxification. In the initial stages of APAP liver injury, NAPQI depletes GSH stores and adds sulfhydryl adducts to cellular proteins (58). The resulting oxidative stress, mitochondrial uncoupling, adenosine triphosphate (ATP) depletion and c-Jun N-terminal (JNK) activation eventually lead to hepatocyte necrosis (58, 59). Related studies have shown that administration of repeated doses of APAP induces liver fibrosis (60, 61).

Acute co-injection of LPS/D-GalN is a widely used experimental model for acute liver injury, while long-term and low-dose treatment with LPS/D-GalN induces a chronic inflammatory response similar to that of liver fibrosis (62). Liver injury caused by a large depletion of uracil nucleotides, resulting in reduced RNA and protein synthesis, is mostly used to induce acute liver injury with a high degree of fibrosis, mostly in stages III to IV, with high similarity to human liver fibrosis and good reproducibility, but the disadvantage is the high time and cost consumed by modeling (63).

3. Diet induction methods

Many diseases are influenced by dietary factors, and simulating daily meals helps prepare animal models that are more closely related to the clinical manifestations of human diseases. The model preparation methods mainly include methionine choline-deficient diet (MCD), high-fat diet (HFD), Western diet (WD), choline-deficient, l-amino acid-defined (CDAA), and choline-deficient, l-amino acid-defined, high-fat diet (CDAHFD).

3.1. MCD-induced liver fibrosis model

A standard MCD contains 40% high sucrose and 10–20% fat. The deficiency of two essential nutrient, choline and methionine, lead to impaired fatty acid β oxidation and impaired production of very low density lipoprotein particles (64). In addition, choline deficiency leads to impaired hepatic very low density lipoprotein secretion, resulting in hepatic fat accumulation, hepatocyte death, oxidative stress, and changes in cytokines and adipokines, but causes only slight hepatic inflammation and fibrosis (65). After addition of methionine deficiency, there will be more pronounced inflammation and early development of fibrosis (after 8–10 weeks) (64).

Dietary animal models are widely used to research nonalcoholic steatohepatitis (NASH) pathogenesis, and mice fed the MCD diet are the preferred method (66). Feeding mice with the MCD diet is a mature nutritional model of NASH, which elevates serum transaminases, and liver histological changes similar to human NASH, including hepatic steatosis, lobular inflammation, and pericellular fibrosis (67). This model provides Histological marker of NASH because it is prone to transition from simple steatosis to steatohepatitis and can reach fibrosis stages (68). Many studies induce NASH (69, 70) and dietary liver fibrosis (71) by feeding mice MCD diet for 6–8 weeks. The gene expression of inflammatory markers in the MCD diet animal model occurs much earlier than that in the HFD animal model and can spontaneously develop liver injury characterized by fibrosis patterns within a short period of time (72). Furthermore, the MCD diet is able to induce significant changes in the expression of genes that encode proteins involved in the fibrogenesis pathway much earlier than HFD and most of the related genes, such as COL1A1, COL1A2, MMP-9, MMP-13, TIMP-1, and TGF-β, were upregulated within 2 weeks of feeding with the MCD diet (72).

The advantage of MCD dietary model is that it is more efficient and reproducible for inducing severe liver injury and progressive fibrosis; this dietary approach, which mimics a subgroup of NASH patients with advanced histological NASH, is ideal for studying the mechanisms driving NASH-associated inflammation/fibrosis and

strategies for inhibiting these processes (71) and can be used to screen drugs that directly target liver fibrosis (73), and it is widely available. Moreover, steatohepatitis and fibrosis was induced in a shorter time (less than 10 weeks) than HFD model, increased pro-inflammatory cytokine levels and oxidative stress (74). But the MCD diet also has certain drawbacks, as it leads to weight loss and does not induce characteristics of metabolic syndrome, which is an important risk factor for NAFLD (75). Although a non-physiological diet low or deficient in certain essential amino acids promotes more severe fibrosis, it also leads to significant weight loss, making these NASH models more suitable for detecting the effects of drug therapy on liver injury and regeneration (76).

3.2. High-fat diet-induced liver fibrosis model

3.2.1. HFD-induced liver fibrosis model

Many diet-induced obesity models mimic the natural history of NASH and show relatively good clinical translatability in terms of key metabolic and hepatic pathological changes in mild to moderate liver fibrosis, so these models are increasingly used in preclinical drug development (76). The use of high fat content alone is often referred to as the HFD model (77). Animal HFD usually include 45% energy-supplying high-fat diets and 60% energy-supplying high-fat diets. A HFD enhances glycolysis and accelerates NAFLD fibrosis progression by downregulating geranylgeranyl diphosphate synthase (GGPPS) expression; chronic HFD overload decreases GGPPS expression in mice, thereby shifting fuel preference from fatty acids to glucose; liver-specific GGPPS deficiency drives the Warburg effect by impairing mitochondrial function, which then induces liver inflammation, thereby exacerbating fibrosis (78). Transcription and protein levels of IL-1 were significantly increased in the liver of HFD-fed mice, and excessive accumulation of lipids in hepatocytes activates inflammation. The inflammatory process leads to an increased level of TGFβ and activation of β-catenin signaling pathways promoting epithelial-mesenchymal transition, which leads to acquisition of mesenchymal features and induces hepatic fibrosis (79).

Most studies about animals fed an HFD diet for less than 4 months showed that no significant changes in gene expression of proteins involved in fibrogenesis pathway, but it found that these have significant changes in studies with longer HFD exposure (24–25 weeks) (80, 81). HFD animal models require prolonged feeding with HFD to stimulate the progression of steatosis to mild steatohepatitis (72). Although long-term HFD feeding caused obesity and IR in mice, two key risk factors of NASH (82), it only mimicked the gene expression profile and histopathology of simple steatosis, not stimulated the gene expression profile and histopathology of NASH (83, 84). HFD-fed animal models can mimic metabolic abnormalities of NAFLD, other spectrums of oxidative stress and inflammation, but fail to reach advanced stages, such as fibrosis and cirrhosis (68). It is well known that only HFD diet feeding mice will cause a lot of steatosis, but little liver fibrosis.

3.2.2. WD-induced liver fibrosis model

A recent mouse model combining long-term administration of a "Western diet" with high saturated fat and cholesterol content was able to replicate NASH with increased but not inflated fibrosis markers (85). The WD is a diet rich in saturated fats, trans fats and table sugar (86) and represents a cholesterol-added HFD that mimics the fast-food diet (FFD) associated with the pathogenesis of NASH in humans (80).

Panasevich et al. (11) fed juvenile female Ossabaw pigs with WD and developed severe NASH after 16 weeks with hepatic steatosis, hepatocyte ballooning, inflammatory cell infiltration and fibrosis, histological inflammation and fibrosis after 36 weeks of WD feeding further deteriorated. The WD model mimics the vast majority of obese NAFLD/NASH patients who typically have IR and metabolic syndrome but relatively mild liver damage. Therefore, the WD model should be the first choice for studying how NAFLD/NASH affects systemic metabolic and cardiovascular risk of tissue complications with type 2 diabetes and atherosclerosis (71). Diet induced obese mice fed with WD are attractive because they summarize the natural history of NASH, and traditional obesogenic HFD promotes dyslipidemia, fatty liver, and mild NASH in rodents without significant fibrosis (87).

The lack of high levels of fructose in the Western diet may be physiologically important because adding high fructose content to a diet high in saturated fat and cholesterol has been thought to reproduce all the characteristics of NASH. Tsuchida et al. (8) developed a new rodent model of NASH fibrosis based on a "fast food" (high cholesterol, high saturated fat and high fructose) diet administered for 6 months, outlining the characteristics of metabolic syndrome and NASH with progressive fibrosis in C57BL/6 mice. After Xin et al. (88) gave mice a high-fat, high-carbohydrate diet for 30 weeks, mice exhibited significant hepatic fibrosis, hepatic steatosis, ballooning degeneration and inflammation. Feeding C57BL/6 J mice a high-fat, high sucrose, high-cholesterol diet has been shown to induce features of human liver fibrosis such as steatohepatitis, hepatocyte ballooning, and progressive fibrosis (80). However, a major challenge in high-fat, carbohydrate diet models is the long dieting period (usually >20 weeks) required for the progression of steatohepatitis disease to hepatic fibrosis.

3.3. Choline-deficient L-amino-defined diet-induced liver fibrosis model

3.3.1. CDAA-induced liver fibrosis model

Another formulation of the MCD diet is a CDAA diet. Like the MCD diet, the CDAA diet induced hepatic triglycerides accumulation by inhibiting the liver output of very low density lipoprotein and impairing fatty acid oxidation in hepatocytes and these inhibitory effects on lipid disposal are sufficient to increase lipid synthesis and oxidation and endoplasmic reticulum stress to stimulate hepatitis cell infiltration and HSC activation (89).

Related studies showed that C57BL / 6 J mice fed with CDAA diet gained the same or more weight than mice on a standard diet (90). The CDAA diet induces changes similar to human NASH in rats, such as steatohepatitis, fibrosis, cirrhosis and HCC, but has minimal effects on body weight and glucose metabolism compared to semi-purified MCD diet (91). CDAA diet-fed rats lack obesity and IR (92), and CDAA diet-fed mice exhibited obesity and IR develops limited liver fibrosis (93). Exogenous LPS administration exacerbates pericellular fibrosis in CDAA-mediate steatohepatitis in mice. Nakanishi et al. (94) fed C57BL/6 J mice a CDAA diet to induce NASH and intraperitoneally

injected low-dose LPS (0.5 mg/kg) three times a week, LPS challenge potentiated CDAA-diet-mediated insulin resistance, hepatic steatosis with upregulation of lipogenic genes, and F4/80-positive macrophage infiltration with increased proinflammatory cytokines. LPS administration extensively promoted HSC activation in mice fed on a CDAA diet, thereby promoting pericellular fibrosis. Tølbøl et al. (95) have described a new rat NASH model of cholesterol-supplemented CDAA diet with severe fibrosis, which reflected the human NASH phenotype and disease progression, and stably induced the phenotype in short period of time. The CDAA diet had resulted in significant hepatomegaly and fibrosis after 4 weeks of feeding, with further development of collagen deposition and fibrogenesis-related gene expression during 12 weeks of feeding. Cholesterol supplements enhanced the stimulating effect of the CDAA diet on transcripts of genes associated with fibrogenesis without significantly increasing collagen deposition.

3.3.2. CDAHFD-induced liver fibrosis model

CDAHFD is composed of 60 kcal % fat and 0.1% methionine by weight (96). Mice are largely resistant to the CDAA diet (97), but Chiba et al. (98) recently developed a modified CDAA diet that effectively induced NASH in mice by adding lard to reduce methionine and increase fat mass. Mice fed a 60% fat CDAA diet exhibited steatohepatitis with dietary fat-driven dysregulation of lipid metabolism-related genes, progressive fibrosis, and HCC (89).

Some study protocols induced rapid liver fibrosis development of NASH by feeding C57BL/6 mice with a CDAHFD diet for 12–15 weeks (99–101). After feeding a CDAHFD diet, mice showed higher serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase levels, significantly increased serum CK18 levels, and also enhanced the pathological features of steatohepatitis and liver fibrosis (101). This model can rapidly and consistently develop liver fibrosis, steatosis and inflammation. Zhou et al. (102) fed adult male Wistar rats a CDAHFD diet for 9 weeks, and the model successfully induced fibrosis and steatosis in the rat liver. It has been reported that CDAHFD dietary models developed steatosis, steatohepatitis and liver fibrosis faster and more severe than traditional models and prevent weight loss in mice, but CDAHFD dietary models do not develop obesity (96).

4. Surgical induction methods

Bile duct ligation (BDL) is the most widely used and longest used experimental model for cholestasis because of its high reproducibility. This technique requires a mid-abdominal laparotomy and isolation of the common bile duct above the duodenum, followed by double ligation and dissection of the bile duct to produce a model of obstructive cholestasis (103). It induces proliferation of intrahepatic biliary epithelial cells, proliferation of myofibroblast differentiation of portal vein fibroblasts surrounding biliary epithelial cells, resulting in high reproducibility, high expression and deposition of ECM (104, 105). The BDL model shows liver injury manifested by histological changes and elevations in serum biochemistry, ductal reactions, fibrosis, and inflammation, leading to activation of Kupffer cells and recruitment of immune cells, possibly triggering an inflammatory response through activation of the NF-κB pathway (106).

Common BDL in rats or mice is a classic method to produce an animal model of liver fibrosis (107). The application of this model in rats and mice is popular among scientists who aim to understand the pathogenesis of liver inflammation and fibrosis. BDL in mice is a model widely used to induce biliary inflammation, fibrosis and cholestatic liver injury (108). Meier et al. (109) anesthetized male DBA-1 mice with isoflurane, performed midline laparotomy, dissected common bile duct and cut between four ligations under anatomical microscope to induce fibrosis in mice. It was reported that sinusoidal and portal fibrosis had fully developed on days 10 and 20 after BDL surgery in mice, respectively (103). Significant bile duct proliferation and dilated portal fibrosis were observed in all mice included in the study 5 weeks after BDL surgery in mice (110). Matyas et al. (111) demonstrated that BDL-induced advanced liver fibrosis is a suitable mouse model to study the pathophysiology of cirrhosis and cardiomyopathy at the preclinical level, as it resembles the characteristics of the clinical syndrome in patients. BDL induced massive inflammation, oxidative stress, microvascular dysfunction, and fibrosis in the liver, and these pathological changes were accompanied by impaired diastolic, systolic, and macrovascular functions, cardiac inflammation, and oxidative stress. Schewe et al. (112) induced liver fibrosis in male SD rats by BDL surgery for 4 weeks. After BDL surgery, the liver showed low fibrosis and severe bile duct proliferation, accompanied by overall parenchymal fibrosis and moderately inflammatory fibrous septum. These modifications were typical features of BDL and were characteristic of liver fibrosis (113).

The BDL model is mainly used to evaluate the study of cholangiocyte proliferation, apoptosis and portal fibrosis due to extrahepatic cholestasis (114). Because fibrogenesis and liver regeneration proceed simultaneously in the BDL model (115), this model is also an ideal tool to evaluate the protective effect of liver regeneration on fibrosis. Marques et al. (113) suggested that BDL was considered a safer method to induce cirrhosis in rats compared with the use of CCl_4, inducing cirrhosis after 4–6 weeks. However, mortality due to bile leakage and gallbladder (or mouse gallbladder) rupture that may occur during BDL is relatively high (11), and BDL is much more painful than CCl_4-induced liver injury (116).

5. Transgenic induction methods

A number of transgenic animal models have been developed for the study of liver fibrosis based on the different pathogenesis of liver fibrosis and the key functional genes regulated by liver fibrosis. Sterol regulatory element-binding protein-1c transgenic mice developed severe IR and NASH, with perivenular and pericellular fibrosis, but reduced adipose tissue volume (64). Gnmt-deficient (Gnmt) mice characterized by elevated SAME levels spontaneously developed liver fibrosis at 3 months of age and HCC at 8 months of age (117). Zhang et al. (118) developed the Liver-specific O-linked β-N-acetylglucosamine (O-GlcNAc) transferase-KO (OGT-LKO) model, in which OGT-LKO mice exhibit hepatomegaly and ballooning degeneration at an early stage and progress to hepatic fibrosis and portal inflammation at 10 weeks of age, which can potentially be used as a novel, effective mouse model of liver fibrosis with broad translational implications for the screening and evaluation of anti-fibrotic drugs. $Mdr2^{-/-}$ is also a widely used mouse model for the study of cholestatic liver fibrosis and cirrhosis. Deficiency of Mdr2 (a tubular

phospholipid flipping enzyme) disrupts the secretion of biliary phospholipids, leading to increased bile secretion. Potentially toxic bile acids, which induce hepatocellular damage and cholestasis, are characterized by peribiliary inflammation and onion skin-type periductal fibrosis, similar to the pathology of primary sclerosing cholangitis (119). However, such transgenic/knockout mice can determine the role of the gene in liver fibrosis, but they are long in development, expensive and less used.

6. Immune induction methods

Autoimmune hepatitis can induce immune cells to attack their own hepatocytes under the influence of immunity or viral infection and other factors, resulting in inflammatory necrosis of the liver, followed by the development of liver fibrosis and cirrhosis. The model preparation method mainly includes schistosomiasis, virus, pig serum (PS), concanavalin A.

6.1. Schistosoma-induced liver fibrosis model

The main species of schistosomiasis that infect humans include *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum* (120). Infection by *S. japonicum* is a routine model for mechanistic or drug research purposes in liver fibrosis-related studies (121), and after infection, liver fibrosis is the main pathological manifestation of the disease. Schistosomiasis is a serious parasitic infection caused by the *S. haematobium*. Liver fibrosis in schistosomiasis occurs in the development of a complex series of hepatology involving immune inflammation, granuloma formation and liver injury (122). During schistosomiasis, where parasites deposit eggs in the host liver, inflammatory granulomas initially form around schistosomiasis eggs, and granulomatous reactions appear during the egg-laying period approximately 5–6 weeks after infection. As the granuloma matures, fibroblasts that lead to the production of extracellular matrix and collagen fibers are recruited in the outer zone of the granuloma, and dormant HSC are activated by various cytokines and transformed into myofibroblasts, leading to fibrosis (122–125).

Some studies selected mice percutaneously infected with cercariae of *S. japonicum* to establish a liver fibrosis model (126, 127). The results of the study (127) showed that compared with uninfected mice, mice infected with *S. japonicum* developed severe granulomatous inflammation and tissue fibrosis in the liver, spleen and large intestine 8 weeks after infection, the number of eosinophils was significantly increased by immunohistochemical staining with hematoxylin and eosin staining and CD68 macrophage-positive areas. CD4 helper cells, including Th1, Th2, Th17 and Treg cells, are also known to be involved in schistosomiasis egg-induced liver granulomatous inflammation and fibrosis. Lei et al. (128) found in mice that CD1d expression on hepatocytes was significantly reduced after infection with *S. japonicum*, accompanied by an increase in NKT cells, and an upregulation of Th1 and Th2 responses. During schistosomiasis infection, the eggs were trapped in the host liver and egg-derived products induce a polarized Th2 cell response leading to granuloma formation and eventual fibrosis (129). The proportion of γδ T cells producing and secreting IL-17A was significantly increased in the livers of mice infected with *S. japonicum*. In this mouse model of schistosomiasis infection, γδ T cells may promote liver fibrosis by recruiting CD11bGr-1 cells (130). In these models, the inducing mechanism of injury and the nature of the response, even if it leads to fibrosis, are of specific inflammatory and immune types, and the results may not be replicated in other fibrosis models. However, they highlighted the importance of the immune component in liver fibrosis (131).

6.2. Virus-induced liver fibrosis model

Human hepatitis B virus (HBV) belongs to the family hepatoviridae and is a small, enveloped, partially double-stranded DNA virus. Chronic HBV infection remains a major cause of liver injury and fibrosis. Individuals chronically infected with HBV can develop a range of liver diseases, ranging from liver fibrosis to cirrhosis to HCC. HBV infection leads to inflammatory changes followed by the release of different cytokines and chemokines such as IL-1 and IL-8, INF-γ and TNF-α. These cytokines and chemokines kill HBV-associated CD8+ cytotoxic T cells, this type of hepatic oxidative stress leads to activation of Kupffer cells, and then activation of HSC leads to fibrosis by triggering different genes (132, 133). Hepatitis C virus (HCV)-induced liver fibrosis mechanism is also one of the main causes of liver fibrosis. Hepatocyte specificity (CREBH) was identified as a key positive regulator of TGF-β2 transcription in HCV-infected cells. TGF-β2 released from infected cells may promote the cross-induction of TGF-β in an autocrine manner through its own signaling pathway, leading to increased fibrotic responses in adjacent HSCs (134).

Ye et al. (135) developed a mouse model of chronic HBV infection using adeno-associated virus serotype 8 (AAV8)-mediated delivery of the 1.2 kb HBV genome, which induces persistent HBV infection with hepatic fibrosis in immunocompetent mice; no animal model currently exists to mimic hepatic fibrosis during long-term HBV infection in immunocompetent mice. Therefore, this model can be used as a model to study the exact mechanism of liver fibrosis after chronic HBV infection and the potential development of new therapies. To closely mimic chronic hepatitis, Li et al. (136) used a replication-deficient recombinant adenoviral vector to deliver recombinant covalently closed circular DNA (rcccDNA) of HBV with site-specific DNA recombination to the liver and found a persistent necroinflammatory response and fibrosis in the mouse liver, with dysplastic lesions usually visible in the late stages of viral persistence, resembling the progressive pathology of clinical chronic hepatitis. HBV transgenic mice provide a reliable HBV replication model for studying the molecular mechanism of liver disease. However, viral genomes integrated into the host genome and in the immune system identify the virus as itself. The HBV genome cannot be eliminated from mouse hepatocytes because its use is limited to research purposes, antiviral drug screening and evaluation (137).

6.3. PS-induced liver fibrosis model

Serum as a heterologous antigen stimulates the immune response in experimental animals and stimulates the body to actively release cytokines to activate HSC, causing excessive deposition of ECM leading to liver fibrosis (138). The injection of porcine serum (PS) into

model animals stimulates the production of antibodies to form immune complexes (IC) to activate complement, and the IC formed by long-term antigenic stimulation is deposited in the vascular wall, causing metaplasia resulting in vasculitis and perivasculitis, leading to liver injury and the formation of extensive progressive chronic inflammation, so that repeated hepatocyte degeneration, necrosis and hyperplasia gradually develop into fibrosis-like changes (139). Rats were injected intraperitoneally with 0.5 ml of PS twice a week for 16–24 weeks to induce liver fibrosis (138, 140). The PS-induced liver fibrosis model in rats exhibited changes similar to those of human liver disease (141). However, the modeling time is long and the experimental animals are prone to death due to allergic reactions.

6.4. Concanavalin A

Concanavalin A, a phytoagglutinin from knife-beans, is a common inducer of immune-mediated liver injury. The mechanism of liver fibrosis induction by knife-bezoar protein is to stimulate T-cell mitosis, promote the release of cytokines (TGF-β, TNF-α, etc.), cause an inflammatory response, and further the development of hepatitis into liver fibrosis (142). Immune-related liver fibrosis can be detected in mice after intravenous injection of Concanavalin A (143, 144). Some studies were performed by injecting Concanavalin A (10 mg/kg/wk./i.v) for 4–8 weeks in mice to induce hepatic fibrosis (145, 146).

Concanavalin A is a T cell-dependent model that causes immune-mediated hepatitis in a pattern similar to that induced by viral infection and is an ideal tool to study T cell-dependent immune-mediated liver injury (147, 148). Concanavalin A-induced liver fibrosis mimics that caused by autoimmune hepatitis, acute viral hepatitis or drug-induced immune activation in human immune-mediated liver fibrosis (149).

7. Combine induction methods

Some researchers can combine various factors to create an ideal animal model with more stable and precise mechanisms according to their model needs, and this combination of multiple factor approaches to create an animal model of liver fibrosis is called a composite model (63). Currently, the more widely used compound models are chemical and chemical, chemical and dietary, and transgenic and dietary combined induction.

7.1. Chemical+chemical

Although the alcohol *ad libitum* feeding model can be used as a "stand-alone" model for mild alcoholic liver injury, more and more studies are combining it with other stressors to stimulate inflammation, fibrosis or HCC in the liver, and combined CCl_4 and ethanol modeling is the most used model for chemical and chemically induced liver fibrosis (Figure 2). Brol et al. (150) treated mice with CCl_4 plus ethanol (16%) for 7 weeks to induce mice that exhibited strong inflammation with significant liver fibrosis and moderate steatosis, a pattern mostly similar to the relationship between fibrosis, proliferation and inflammation in human alcoholic liver disease, providing a model for further basic research and drug trials. Some researchers induced liver fibrosis by administering ethanol and CCl_4 together for 5–8 weeks, and liver sections showed typical pathological features, including marked steatosis, portal inflammation and necrosis, marked collagen deposition, hepatocellular fibrosis, and hepatocyte sparing (151, 152). Some researchers have also administered CCl_4 intraperitoneally twice a week for the first 6 weeks, and then administered ethanol continuously to mice through a gastric feeding tube for 3 weeks, and saw a significant increase in liver injury, showing a clear "chicken wire" pattern of hepatic steatosis or steatohepatitis and liver fibrosis (153). It is evident that a reasonable dosing schedule, whether given simultaneously or at different times, can induce liver fibrosis.

7.2. Chemical+diet

To increase the severity of liver injury in a rodent NASH model, streptozotocin (STZ) (154), ethanol (155), CCl4 (8), and TAA (150) have been added to a modified diet. The STAM model is a model in which STZ is combined with HFD to induce liver fibrosis by administering a low dose of STZ to two-day-old neonatal C57BL/6 male mice given low doses of STZ and subsequently fed an HFC diet starting at 4 weeks of age. Mice developed hepatic steatosis and diabetes mellitus, reached steatohepatitis within 3 weeks, followed by cirrhosis within 8 weeks (i.e., approximately 12 weeks of age) and hepatocellular carcinoma within 16 weeks (154). Zhou et al. (155) developed a HFD plus binge drinking ethanol challenge model that mimics binge drinking and obesity in humans. Its data showed that alcohol abuse and HFD synergistically induced steatohepatitis and fibrosis (155, 156). HFD plus ethanol binge drinking characterized by neutrophilic liver infiltration resulted in significant upregulation of a range of genes associated with HSC activation and fibrogenesis compared to HFD feeding only. Current data

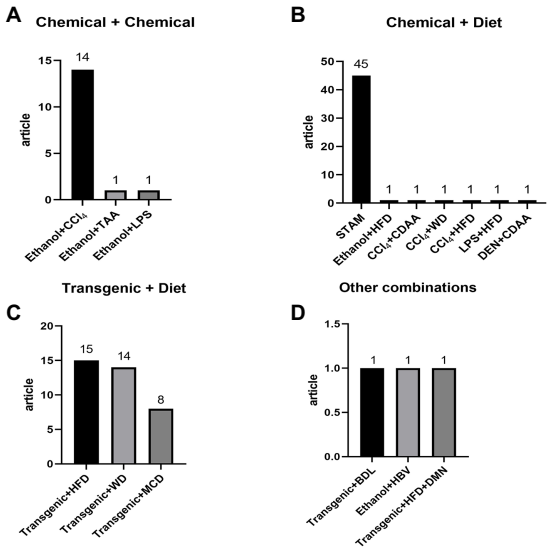

FIGURE 2
Detailed distribution of combine induction methods: **(A)** chemical+chemical: ethanol+CCl4 14, ethanol+TAA 1, ethanol+LPS 1; **(B)** chemical+diet: STAM 45, ethanol+HFD 1, CCl4+CDAA 1, CCl4+WD 1, CCl4+HFD 1, LPS+HFD 1, DEN+CDAA 1; **(C)** transgenic+diet: transgenic+HFD 15, transgenic+WD 14, transgenic+MCD 8; **(D)** other combinations: transgenic+BDL 1, ethanol+HBV, transgenic+HFD+DMN 1.

from an HFD plus binge ethanol-fed mouse model suggest that obesity and binge eating act synergistically to promote liver fibrosis, which is mediated in part through the interaction of neutrophils and HSC (155).

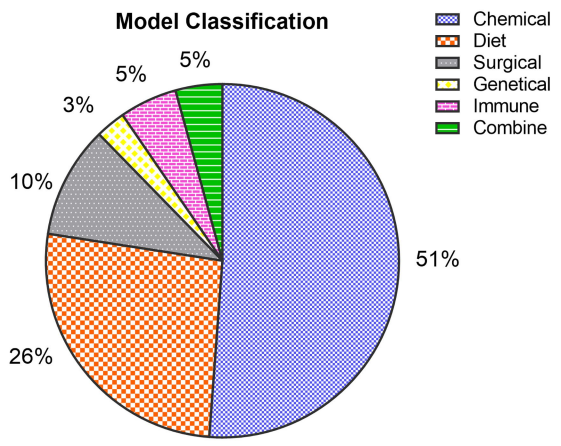

FIGURE 3
Classification of fibrosis models: Among the collected articles, 1,289 (51%) were chemically induced models, 661 (26%) were diet-induced models, 258 (10%) were surgically induced models, 69 (3%) were transgene-induced models, 133 (5%) were immune-induced models, and 108 (5%) were combine-induced animal models.

Tsuchida et al. (8) established a mouse model of NASH by weekly use of high-fat, high-fructose and high-cholesterol WD combined with low-dose intraperitoneal injection of CCl_4, which exhibited advanced fibrosis and rapid progression of HCC and mimicked the histological, immunological and transcriptomic features of human NASH. Related studies have shown that treatment of a mouse model of NASH with a combination of CCl_4 and WD for more than 12 weeks induced the most severe steatosis as well as significant liver fibrosis and moderate inflammation (150), demonstrating the histological and transcriptomic profile of human NASH (8). Co-administration of TAA with a FFD to C57BL/6 J mice for 8 weeks, a novel model that exhibited liver inflammation and fibrosis in just 8 weeks, could be used for rapid screening of novel anti-NAFLD and hepatic anti-fibrotic agents (157). As with chemical methods combined with chemical methods of modeling, simultaneous administration, or separate administration, can induce different degrees of liver fibrosis. It is necessary to screen the appropriate liver fibrosis model according to the purpose of one's study.

7.3. Transgenic+diet

The *ob* gene transcribes leptin, an adipocyte hormone involved in the regulation of food intake and insulin sensitivity.

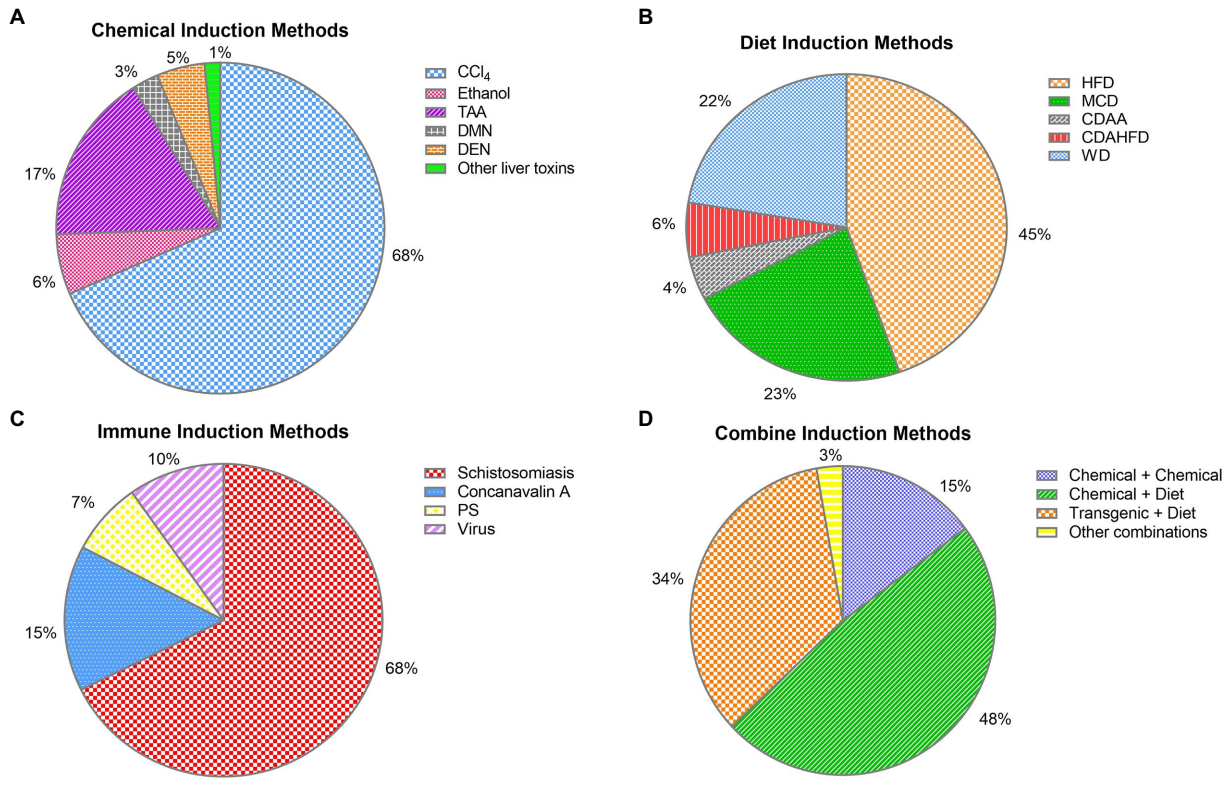

FIGURE 4
Specific distribution of liver fibrosis models: **(A)** chemical-induced liver fibrosis models were specifically distributed: CCl_4 883 (68%), TAA 215 (17%), ethanol 76 (6%), DEN 61 (5%), DMN 34 (3%), other liver toxins 20 (1%); **(B)** diet-induced liver fibrosis models were specifically distributed: HFD 295 (45%), WD 148 (22%), MCD 150 (23%), CDAHFD 38 (6%), CDAA 30 (4%); **(C)** immune-induced liver fibrosis models were specifically distributed: Schistosomiasis 90 (68%), concanavalin A 20 (15%), virus 13 (10%), and porcine serum 10 (7%). **(D)** combined-induced liver fibrosis was specifically distributed: chemical+chemical 16 (15%), chemistry+diet 52 (48%), transgenic+diet 37 (34%), and other combined methods 3 (3%).

Functional leptin production is defective in Lep^{ob}/Lep^{ob} (*ob/ob*) mice (68). The *ob/ob* mice are well known models of extreme obesity and insulin resistance (158). The $Lepr^{db}/Lepr^{db}$ (*db/db*) model has a metabolic phenotype similar to that of ob/ ob animals and exhibits leptin resistance caused by premature termination of leptin receptor transcription, a similar mutation exists in rats and has been described as $Lepr^{fa}/Lepr^{fa}$ (*fa/fa*), the *fa/fa* model exhibits a phenotype similar to that of *ob/ ob* and *db/ db* mice with spontaneous onset of severe obesity, insulin resistance and steatosis (22, 68). However, liver inflammation and fibrosis in genetically defective *ob/ob*, *db/db* mice, *fa/ fa* rats, or partially transgenic mice models are mild and can induce varying degrees of inflammation and liver fibrosis when combined with dietary measures (feeding MCD or HFD diet) (159). Kim et al. (160) fed *ob/ob* mice to a HFD for 20 weeks to establish an animal model of NASH with fibrosis. Treatment of *ob/ob* mice fed a long-term high-fat diet resulted in significant weight loss, adipose tissue hypertrophy and inflammation, hepatic steatosis, inflammation and fibrosis, and insulin resistance >1 year (161). MCD diet induces hepatic inflammation and fibrosis in $PPAR\alpha^{-/-}$ mice (162). This mouse model has been widely used to cause severe steatohepatitis and fibrosis, similar to human non-alcoholic steatohepatitis pathology (163). The pathogenesis involves the hepatic oxidative stress observed in human NASH (164). Chen et al. (165) gave adropin-deficient (adropin-KO) mice fed MCD diet for 4 weeks or WD diet for 16 weeks, adropin-KO mice exhibited more severe hepatic macrosteatosis, inflammation and ballooning with significantly higher NASA scores and increased areas of fibrosis with marked perisinusoidal fibrosis; fibrosis-related genes such as Col1a1, Acta2 and inflammation-related genes such as IL1b, IL6 and TNF were also induced in large numbers in Adropin-KO livers.

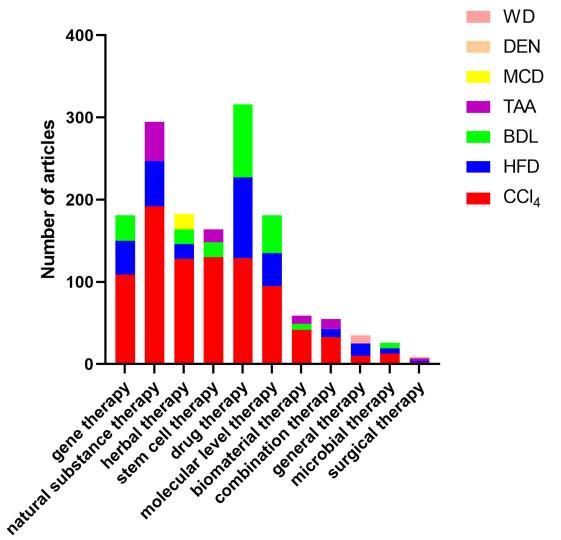

FIGURE 5
Top three animal models used in articles in the field of liver fibrosis treatment: Among the collected articles, the details of the top three animal models used in articles in each field of treatment are as follows: gene therapy: CCl_4 (109), HFD (41), BDL (31); natural substance therapy: CCl_4 (192), HFD (55), TAA (48); herbal therapy. CCl_4 (128), MCD (19), BDL (18), HFD (18); stem cell therapy: CCl_4 (130), BDL (18), TAA (16); drug therapy: CCl_4 (129), HFD (98), BDL (89); biomaterial therapy: CCl_4 (42), TAA (10), BDL (7); microbiological treatment: CCl_4 (13), BDL (7), HFD (6); surgical treatment: TAA (3), DEN (2), CCl_4 (2), HFD (2); general treatment: HFD (15), WD (10), CCl_4 (10); molecular level treatment: CCl_4 (95), BDL (46), HFD (40); combination therapy: CCl_4 (33), TAA (12), HFD (10).

Most of the animal models of compound liver fibrosis use 2 methods in combination, and 3 methods are used in combination (166), but rarely (Figure 2). With the development of society and the continuous improvement of living standards, modern human life has a certain complexity, which leads to the complex and variable factors of liver disease, and even a variety of compound factors together affect the formation and development of liver disease (167). To some extent, the compound model solves the problem that there is a gap between the single-factor animal model and the modern clinical patient's condition, and the compound animal model of liver fibrosis has a high modeling rate and a low morbidity and mortality rate of animals during the modeling period.

8. Discussions and prospect

It is now widely accepted that liver fibrosis is a reversible process and that early treatment can inhibit the progression of fibrosis or even reverse it, thus attracting a large number of researchers to study the therapeutic field of liver fibrosis. There are a hundred different treatment areas for liver fibrosis, including general therapy (exercise, dietary interventions), drug therapy, herbal therapy, stem cell therapy, gene therapy, natural substance therapy, biomaterial therapy, surgical therapy, molecular level therapy, microbial therapy, combination therapy, etc. Most of these fundamental articles for the treatment of liver fibrosis use animal experimental models for validation. Therefore, we conducted a PubMed search using the search term "liver fibrosis" "treatment" between 2017 and 2022 to collect articles on basic research in the field of liver fibrosis treatment, and a total of 2,518 articles used animal models of liver fibrosis.

The results from the collected data show that transgenic-induced liver fibrosis models are relatively less used, which may be attributed to the long development time and high price of this model (Figure 3). Chemical injury-induced liver fibrosis models are the most widely used (Figure 3), and these models use chemical drugs to enter hepatocytes to produce toxic metabolites that cause persistent liver injury and induce the formation of liver fibrosis. Among them, the CCl4-induced liver fibrosis animal model is similar to human liver fibrosis in some aspects of morphology and pathophysiology, and is the most used animal modeling method for liver fibrosis because of its short modeling time, low cost, and high reproducibility (Figures 4, 5). Animals in the CCl4-induced liver fibrosis model do not become obese or develop insulin resistance, which is very different from the pathophysiological features of patients with non-alcoholic fatty liver disease induced by metabolic disorders. The most common signs of fibrosis in NASH are mainly caused by excessive consumption of high-fat components, where patients absorb nutrients The HFD-induced liver fibrosis model overcomes the shortcomings of the MCD-induced liver fibrosis model, in which animals with increased body weight and peripheral insulin resistance develop and mimic the etiology of the disease by

replicating poor dietary habits, with phenotypic features similar to those of human nonalcoholic steatohepatitis. Fibrosis model, with a short modeling period, simple method and no need for exposure to toxic substances, is currently a common method for inducing cholestatic liver fibrosis models to study diseases related to biliary obstruction. As shown by our collected data, CCl4, HFD and BDL-induced liver fibrosis models, relative to other methods, are widely used in the basic field of liver fibrosis treatment (Figures 4, 5).

So far, researchers have successfully developed many models of liver fibrosis using different experimental animals and different methods. Each model has its disadvantages and advantages, and a reasonable method of model preparation needs to be selected according to the experimental purpose and requirements.

Author contributions

SW collected the data and wrote the manuscript. XW, WX, FL, ML, and KL revised the manuscript. YH and JW provided constructive comments on the review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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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EDITED BY
Ana Sandoval-Rodriguez,
University of Guadalajara, Mexico

REVIEWED BY
Jaime Arellanes-Robledo,
National Institute of Genomic Medicine (INMEGEN), Mexico
Yuzheng Zhuge,
Nanjing Drum Tower Hospital, China

*CORRESPONDENCE
Xiangyu Zhong
✉ zhongxiangyu@hrbmu.edu.cn
Yi Xu
✉ xuyihrb@pathology.hku.hk
Yunfu Cui
✉ yfcui7@163.com

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Liver fibrosis and MAFLD: the exploration of multi-drug combination therapy strategies

Qingfu Dong[1], Haolin Bao[1], Jiangang Wang[2], Wujiang Shi[1], Xinlei Zou[1], Jialin Sheng[1], Jianjun Gao[1], Canghai Guan[1], Haoming Xia[1], Jinglin Li[1], Pengcheng Kang[1], Yi Xu[1,3,4,5,6,7,8,9,10]*, Yunfu Cui[1]* and Xiangyu Zhong[1]*

[1]Department of Hepatopancreatobiliary Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China, [2]Department of General Surgery, Tangdu Hospital, Air Force Medical University, Xi'an, Shaanxi, China, [3]Key Laboratory of Basic Pharmacology of Ministry of Education, Zunyi Medical University, Zunyi, Guizhou, China, [4]Key Laboratory of Functional and Clinical Translational Medicine, Fujian Province University, Xiamen Medical College, Xiamen, Fujian, China, [5]Jiangsu Province Engineering Research Center of Tumor Targeted Nano Diagnostic and Therapeutic Materials, Yancheng Teachers University, Yancheng, Jiangsu, China, [6]Key Laboratory of Biomarkers and In Vitro Diagnosis Translation of Zhejiang Province, Hangzhou, Zhejiang, China, [7]Key Laboratory of Gastrointestinal Cancer, Ministry of Education, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian, China, [8]State Key Laboratory of Chemical Oncogenomics, Key Laboratory of Chemical Genomics, Peking University Shenzhen Graduate School, Shenzhen, Guangdong, China, [9]Department of Pathology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China, [10]Key Laboratory of Intelligent Pharmacy and Individualized Therapy of Huzhou, Department of Pharmacy, Changxing People's Hospital, Changxing, Zhejiang, China

In recent years, the prevalence of metabolic-associated fatty liver disease (MAFLD) has reached pandemic proportions as a leading cause of liver fibrosis worldwide. However, the stage of liver fibrosis is associated with an increased risk of severe liver-related and cardiovascular events and is the strongest predictor of mortality in MAFLD patients. More and more people believe that MAFLD is a multifactorial disease with multiple pathways are involved in promoting the progression of liver fibrosis. Numerous drug targets and drugs have been explored for various anti-fibrosis pathways. The treatment of single medicines is brutal to obtain satisfactory results, so the strategies of multi-drug combination therapies have attracted increasing attention. In this review, we discuss the mechanism of MAFLD-related liver fibrosis and its regression, summarize the current intervention and treatment methods for this disease, and focus on the analysis of drug combination strategies for MAFLD and its subsequent liver fibrosis in recent years to explore safer and more effective multi-drug combination therapy strategies.

KEYWORDS

liver fibrosis, metabolic-associated fatty liver disease (MAFLD), non-alcoholic steatohepatitis (NASH), cirrhosis, drug combination therapy

Introduction

With the prevalence and development of the disease, non-alcoholic fatty liver disease (NAFLD) has become the most general etiology of chronic liver disease worldwide. As one of the most common indications for liver transplantation (1, 2), it affects about 25% of the world's population, and its prevalence continues to increase (3). Increasing number of studies have reported that metabolic dysfunction, including obesity, type 2 diabetes mellitus (T2DM), hypertension, and metabolic syndrome, is closely associated with the complex pathological mechanism of NAFLD (4). To better integrate the present understanding of the heterogeneity of NAFLD patients, reflect the pathogenesis more accurately, realize stratified management of patients, and accelerate the translation of new treatments, in 2020, an expert

group proposed the new nomenclature "metabolic-associated fatty liver disease (MAFLD)" (5), which is now globally multi-stakeholder-agreed (6). From metabolic overload to durative hepatocyte injury, MAFLD will eventually lead to liver fibrosis, cirrhosis, and even HCC. In addition, the illness is closely related to various extrahepatic diseases, such as chronic kidney disease and cardiovascular complications (7, 8). Multiple factors, including race, age, sex, hormonal status, metabolic rate, diet, alcohol consumption, cigarette smoking, genetic predisposition, and microbiota, may influence the heterogeneity of disease progression and clinical manifestations in MAFLD (5). Consequently, efficacious treatments must consider various complex factors and may require personalized multi-drug combination therapies. This review introduces the mechanism of MAFLD-associated liver fibrosis progression and regression, discusses the role of lifestyle intervention, bariatric metabolic surgery, liver transplantation, and drug therapy, and focuses on analyzing drug combination therapy related to MAFLD and liver fibrosis in recent years. It aims to explore more effective multi-drug combination strategies for treating MAFLD and its related liver fibrosis and reducing the disease burden.

MAFLD-related liver fibrosis and its regression mechanism

During the past two decades, the incidence of HBV and HCV-related liver fibrosis and liver cancer has declined due to vaccination and new effective antiviral treatments. However, as the prevalence of MAFLD has reached pandemic levels, the incidence of MAFLD-related liver fibrosis is increasing (9–11). At the same time, studies have shown that the progression of liver fibrosis is significantly related to an increased chance of hepatocellular carcinoma (HCC); MAFLD has risen as one of the major causes of HCC (12); in addition, the increased risk of severe liver-related and cardiovascular events in MAFLD patients is closely related to the fibrotic stage (13) and is the strongest predictor of mortality in MAFLD patients (14), so it is crucial to understand the mechanism of MAFLD-related liver fibrosis (Figure 1) and explore effective antifibrotic therapeutic strategies. From metabolic disorders of fatty acids and carbohydrates to persistent liver injury, ultimately leading to liver fibrosis and cirrhosis, the pathogenesis of MAFLD-associated fibrosis relates to many complicated drivers and diverse mechanisms, such as high-concentration hepatic free fatty acid (FFA)-induced mitochondrial dysfunction, oxidative stress, endoplasmic reticulum (ER) stress and inflammation, subsequent hepatocyte apoptosis, and extracellular matrix (ECM) formation, which also involves the interaction of immunity and genetic and epigenetic regulations (15, 16). To date, the increase in hepatic FFA concentration is still considered the most critical stage in the development of MAFLD and activating of hepatic stellate cells (HSCs) is the key pathogenic event for the development of liver fibrosis (17). FFA comes from three sources (18): 15% comes from dietary fat absorbed in the gut, and bile acids play a crucial role in lipid absorption (19); 25% comes from *de novo* lipogenesis (DNL) of new fat synthesis, in which liver cells generate new fatty acids by converting excess carbohydrates (especially fructose), and acetyl-CoA carboxylase (ACC) is a crucial enzyme in the regulation of DNL, catalyzing the acetyl-CoA converse into malonyl-CoA; and 60% of fatty acids come from the non-esterified fatty acid pool or lipolysis of triglyceride (TG) in adipose tissue. In the hepatocytes, fatty acids' two significant fates are mitochondrial β-oxidation and re-esterification to form TG. A part of TG can be converted to very low-density lipoprotein (VLDL) and transported into the blood. Another part of TG is stored in lipid droplets, which undergo regulated lipolysis and release fatty acids into FFA pools (15). It has been suggested that FFA and its metabolites may represent the lipotoxic agents responsible for the development of MAFLD (20). When fatty acids are redundant, or their processing is impaired, they may serve as substrates to generate lipotoxic lipids that stimulate the ER stress, oxidative stress, and inflammasome activation, and release danger-associated molecular patterns, which lead to liver cell damage and induce diverse modes of cell death, including apoptosis and necrosis (15). Damaged hepatocytes can activate HSCs via paracrine signals. For example, lipotoxic hepatocytes can mediate the activation and proliferation of HSCs by producing exosomes, such as exosomal miR-27a and exosomal miR-1297 (21, 22). IL11 from lipotoxic hepatocytes stimulates HSCs to myofibroblast transformation in a paracrine manner (23). Lipotoxic-related reactive oxygen species (ROS) production in hepatocytes is a critical factor in the activation of HSCs in fibrosis (24, 25). The mitochondrial dysfunction, production of ROS, ER stress, and sterile hepatocyte death conduce to the pro-inflammatory environment of the liver, contributing to the pro-inflammatory environment. Bacterial translocation due to intestinal barrier dysfunction can induce an inflammatory response (26). Neutrophils remove apoptotic liver cells and produce various cytokines to participate in the occurrence of liver fibrosis. The transforming growth factor-β (TGF-β) from Kupffer cells is the most influential profibrotic factor (27). It should be noted that during MAFLD, immune mechanisms link the metabolic injury to inflammation and fibrosis; susceptibility to inflammatory liver states is also closely related to genetic and epigenetic backgrounds (16, 28, 29). During the injury–repair response, activated HSCs migrate to the injury site. They secrete ECM, accumulating and eventually forming fibrous scars and regenerative nodules that replace the damaged normal tissue, resulting in portal hypertension and cirrhosis. From an asymptomatic to a symptomatic phase (decompensated cirrhosis), associated complications often lead to hospitalization, poor quality of life, and higher mortality (30).

Metabolic-associated fatty liver disease-related liver fibrosis regression has been verified in many animal experiments and clinical practice (31), and serial liver biopsy has proved that bariatric metabolic surgery can effectively promote the regression of liver fibrosis in patients with non-alcoholic steatohepatitis (NASH) (32). After the etiology of chronic injury is eliminated, liver fibrosis stops progressing or even regresses, which is related to many mechanisms. Eliminating the etiology of chronic liver injury is the vital goal of antifibrotic therapy. However, not all causes of chronic liver injury can be effectively removed, especially for MAFLD-related liver fibrosis. In addition, direct anti-fibrosis or reverse fibrosis therapeutics are more hopeful strategies for patients with severe liver cirrhosis. Therefore, we need to deeply understand the mechanism of liver fibrosis regression to explore effective therapeutic targets. The mechanisms of liver fibrosis reversibility include HSCs' inactivation and apoptosis and the fibrous scar's

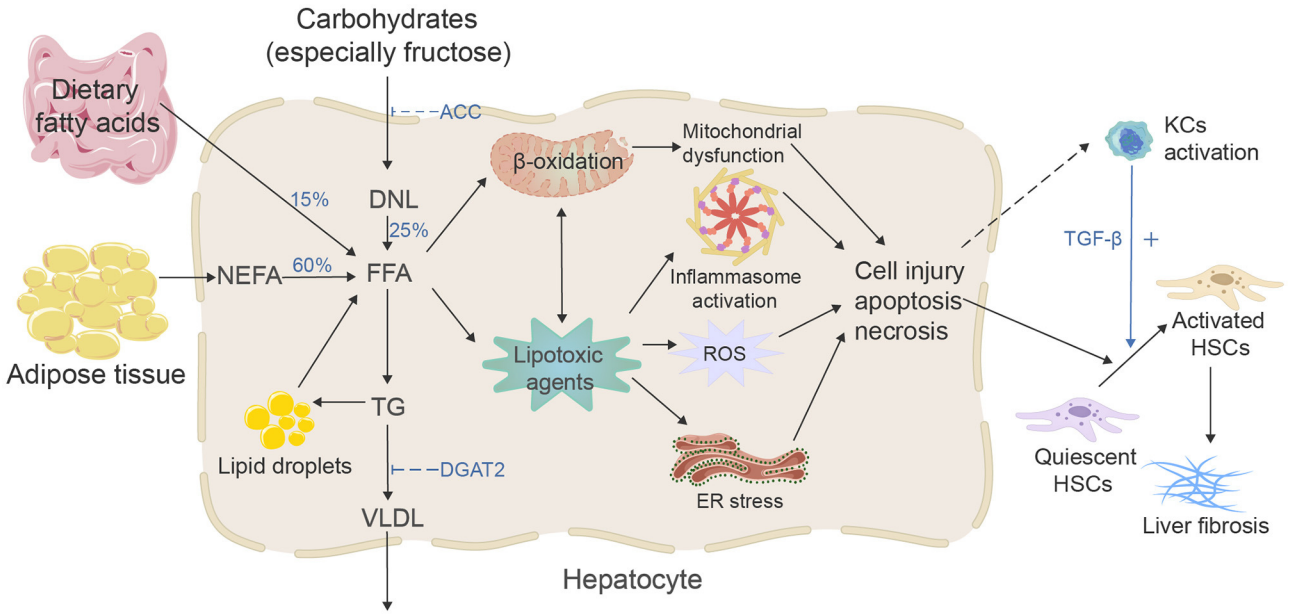

FIGURE 1
Mechanisms of MAFLD-related liver fibrosis. Free fatty acids (FFAs) and their metabolites may represent the lipotoxic agents that induced the development of MAFLD, and the increasing concentration of FFA is considered the most critical stage. There are three primary sources and two fates of hepatic FFA. Excessive FFA and their metabolites will cause mitochondrial dysfunction, stimulate ER stress, oxidative stress, and inflammasome activation, then activate the HSCs and eventually lead to liver fibrosis.

resorption (33). First, reducing activated HSCs is an essential target of antifibrotic therapy. On the one hand, we can reduce the number of HSCs by promoting senescence and apoptosis. Antiretroviral drugs against HIV can enhance the proliferation of hepatocytes and the apoptosis of HSCs (34). In addition, studies have shown that TNF-α activates a nuclear factor-κB-dependent gene program to promote HSCs survival and differentiation (35), which provides a possible target for us to explore the senescence and apoptosis of HSCs and inhibit the NF-kB pathway that may inhibit liver fibrosis by inducing apoptosis of HSCs. On the other hand, HSCs activation can be inhibited or reversed to fight liver fibrosis. Some studies have shown that peroxisome proliferator-activated receptor gamma (PPARγ) is a crucial mediator of HSCs activation and phenotypic changes and can affect the state of HSCs in the quiescent phase. The addition of PPARγ agonists *in vitro* and *in vivo* can reduce the activation of HSCs and promote the degradation of the ECM (36). Moreover, reducing collagen production, enhancing ECM degradation, and changing ECM's spatial conformation and matrix stiffness are also exploration targets for antifibrotic therapy (37, 38). For example, a study has shown that lysyl oxidase-like 2 (LOXL2) monoclonal antibodies can alleviate liver fibrosis and promote fibrosis reversal in mice (39).

Interventions/treatment measures for MAFLD and its related liver fibrosis

Lifestyle intervention

The development of MAFLD is closely related to lifestyle factors, especially excess caloric intake paired with insufficient physical exercise (40). Current studies suggest that dietary intervention improves MAFLD with or without physical activity, and training also reduces hepatic steatosis with or without dietary intervention (41). The 2018 ASSLD Practice Guidelines state that a combination of a low-calorie diet and moderate-intensity exercise may lead to sustained weight loss over time, with a 3–5% weight loss improving steatosis and a 7–10% weight loss improving fibrosis (42). However, relevant clinical trials found that most patients could not achieve the level of weight loss that can improve liver fibrosis (43). It is difficult to achieve the goal of enhancing fibrosis through lifestyle intervention alone. A recent prospective cohort study found that healthy lifestyles positively correlate with all-cause mortality in MAFLD patients (44). The COVID-19 pandemic has affected people's lifestyles seriously, and new MAFLD diagnoses have increased during the pandemic. A retrospective study including 973 participants found that before the pandemic (2018–2019), the independent lifestyle predictor of MAFLD was regular late-night eating, while in the epidemic (2019–2020), it was higher daily alcohol intake (45). The research in mice showed that respiratory exposure to silica nanoparticles induces hepatotoxicity, resulting in inflammatory infiltration, and even causes the deposition of collagen (46). Another cross-sectional study conducted in China proves that the rising sickness rate of MAFLD in the real world is significantly related to long-term exposure to ambient PM1, PM2.5, PM10, and NO2, particularly those who are men, alcohol drinkers, cigarettes smokers, high-fat diet consumers, and central obesity (47). Lifestyle changes make MAFLD more and more common. There is no doubt that healthy lifestyles can help prevent the occurrence of MAFLD, and timely change in unhealthy lifestyles is very important for MAFLD patients.

However, as a preventive strategy that can be extended to the whole population, lifestyle interventions alone have yet to control the prevalence of MAFLD and the development of MAFD-related liver fibrosis, so we must actively explore other preventive and therapeutic measures.

Bariatric metabolic surgery

Bariatric metabolic surgery is now recommended as an effectual treatment for clinically severe obesity and its interrelated comorbidities and has generally been accepted by patients in recent years (48). In a bariatric metabolic surgery center in France, Guillaume Lassailly conducted a long-term follow-up on 180 severely obese patients who were biopsy-confirmed with NASH and underwent bariatric metabolic surgery; they found that 84% of the participants had regression of NASH in liver samples after 5 years, indicating that the fibrosis of the liver was reduced from the first to the fifth year (32). Although it is generally believed that MAFLD is closely related to obesity, there is growing evidence proving that not all overweight individuals have MAFLD. Approximately 40% of MAFLD patients are classified as non-obese (49, 50). Does bariatric metabolic surgery have a therapeutic effect on low BMI MAFLD patients? Adrian T Billeter researched the curative effect of Roux-en-Y gastric bypass (RYGB) in advanced MAFLD; 20 patients participated in this prospective trial and underwent RYGB surgery; liver biopsy was performed during the operation and followed up 3 years later. The results showed that after 3 years of RYGB treatment, MAFLD completely disappeared in all patients, and fibrosis was also improved, 55% of the patients stopped insulin therapy, glycosylated hemoglobin decreased significantly, new lipogenesis decreased, β-oxidation was enhanced, and finally, the secretion of gastrointestinal hormones and adipokines was favorably altered (51). The aforementioned results suggest that bariatric metabolic surgery positively affects MAFLD regardless of obesity, but there are still certain risks in these surgical treatments for MAFLD. For example, the mortality rate of patients with decompensated liver cirrhosis after bariatric metabolic surgery is as high as 16.3% (52). Therefore, bariatric metabolic surgery is excluded as the first-line treatment of MAFLD. It is believed that with the continuous improvement of relevant clinical research, clinicians can more accurately evaluate the pros and cons of bariatric metabolic surgery for MAFLD, better grasp the indications for surgical treatment, and make more patients benefit from it.

Liver transplantation

Because of the severe scarcity of liver resources, the high cost of liver transplantation (LT), and a series of problems, such as immune rejection after transplantation, LT is just considered for advanced MAFLD patients with severe complications in most cases. However, it cannot be ignored that MAFLD is the fastest-rising indication for LT in Western countries. Its interrelated end-stage liver disease and HCC have grown to be LT's common indications worldwide (2). Severe cirrhosis, liver failure, and severe portal hypertension caused by advanced liver fibrosis usually require LT. Some patients with non-resettable HCC also need LT for better treatment (53, 54). Although the survival rate of liver transplant recipients with MAFLD is similar to that of liver transplant recipients with other etiologies, liver transplant recipients still seem prone to relapse MAFLD due to the persistence of diseases such as metabolic syndrome (55). This proportion is as high as 78–88%, usually relapsing within the first 5 years after LT. However, ∼11–14% may develop cirrhosis within 5 years after LT (56). As the number of liver transplant recipients continues to increase, their quality of life continues to improve, their survival time continues to increase, and increased attention has been paid to the occurrence of MAFLD after transplantation. In addition, due to the shortage of liver resources and the prevalence of MAFLD, some donor livers with steatosis also need to be used in LT.

Pharmacological treatment

Pharmacological treatment is very attractive to MAFLD and liver fibrosis, not only due to its convenience but also because various mechanisms of disease progression can be targeted. At present, many drugs are actively developed for the therapy of MAFLD and its related liver fibrosis, which are mainly divided into the following categories according to the main mechanism (18, 57): The first category is agents acting on lipid syntheses and fat accumulation, such as glucagon-like peptide 1 (GLP-1) agonists, ACC inhibitors, Fanitol X receptor (FXR) agonists, and PPAR-α/δ agonists. The second category is drugs that act on cellular stress and apoptosis, including vitamin E and caspase inhibitors. The third category is drugs that play roles in the immune and inflammatory response, such as C-C chemokine receptor type 2 and type 5 antagonists. The fourth category is drugs that directly target the fiber formation process, such as LOXL2 monoclonal antibodies. In addition, new studies have also found that anti-angiogenic drugs can improve liver fibrosis, such as recombinant vascular endothelial growth factor (rVEGF) and bevacizumab (58). These drugs have some efficacy in the therapy of MAFLD and its associated liver fibrosis. However, few pharmacological treatments reached satisfactory endpoints assessed by liver biopsy or with negligible side effects in clinical trials (18). It is essential to accelerate the discovery of new pharmacotherapeutics and explore better multi-drug combination therapies.

In summary (Table 1), measures such as lifestyle intervention have failed to effectively control the increasing prevalence of MAFLD and the progression of liver fibrosis. Bariatric metabolic surgery is still not suitable as the first-line treatment for MAFLD. LT, an option method to save lives for patients with MAFLD-related non-resettable HCC or end-stage liver diseases, is not a good solution for decreasing the burden of MAFLD and its associated liver fibrosis. In addition, the advance of MAFLD-related liver fibrosis involves numerous complicating factors, and the impact of single-drug therapy is very limited. Effective pharmaceutical therapies may need to consider multiple mechanisms, such as metabolic disorders, inflammation, immunity, and fibrosis. Combination pharmaceutical therapies may be an inevitable choice to achieve adequate control of MAFLD and its related liver fibrosis in the future.

Multi-drug combination therapies

In the following, we will analyze some pharmaceutical combination therapies for MAFLD and liver fibrosis in recent years (Table 2).

Combination therapy based on glucagon-like peptide 1 receptor agonists

Glucagon-like peptide 1 (GLP-1), a pleiotropic peptide hormone secreted by intestinal L cells (59), controls insulin hormone secretion, intestinal motility, and body weight. GLP-1 receptor agonists, developed for treating T2DM and obesity recently, have demonstrated a favorable benefit and decreased the occurrence of cardiovascular-related adverse events in T2DM patients. The current analysis considers all people with T2DM, and people with a liver fat content of >5% are deemed to have MAFLD (60). MAFLD increases cardiovascular morbidity and mortality (18). In recent years, the potential role of the combination therapy of GLP-1 receptor agonists and sodium–glucose cotransporter-2 (SGLT-2) inhibitors in treating MAFLD has attracted increased attention. Numerous clinical trials link this combination treatment to reductions in intrahepatic triglyceride accumulation and liver fibrosis, even though none of the GLP-1 or SGLT-2 receptors are expressed in the liver (61). Therefore, GLP-1 receptor agonists are a potentially valuable element of combination therapy to address different complementary pathways in MAFLD therapy (62). For example, in a 24-week exploratory phase 2 trial, the GLP-1 receptor agonist semaglutide alleviates cilofexor and firsocostat-induced hypertriglyceridemia, resulting in more significant reductions in liver enzymes, liver fat, and non-invasive imaging assessed liver fibrosis (NCT03987074) (63). In conclusion, GLP-1 receptor agonists are very suitable as the primary drugs for the combination therapy of MAFLD characterized by metabolic disorders.

TABLE 1 Comparison of different therapeutics for MAFLD.

Therapeutics	Superiorities	Shortcomings
Lifestyle intervention	Applicable to the whole population Reduces all-cause mortality	Difficult to persist Failed to control the prevalence of MAFLD effectively
Bariatric metabolic surgery	Surgery is becoming less invasive Gets rid of taking medicines every day	Risk of post-operative complications Lack of adequate clinical research Is excluded as the first-line treatment of MAFLD
Liver transplantation	A life-saving method	Expensive Lack of liver resources Immune rejection, MAFLD relapsing
Pharmacological therapies	Convenient Affordable Various mechanisms of disease progression can be targeted	No specific drugs Drug side effects The impact of single-drug therapy is minimal

Combined use of acetyl-CoA carboxylase inhibitors

Acetyl-CoA carboxylase (ACC) is a critical enzyme in *de novo* lipogenesis (DNL), catalyzing the rate-limiting step in converting acetyl-CoA to malonyl-CoA, regulating the fatty acids' mitochondrial β-oxidation and playing a vital role in the accumulation of TG in hepatocytes. Animal studies have confirmed that inhibiting ACC in rat models can reduce liver fibrosis (18).

TABLE 2 Some combination treatments for MAFLD and liver fibrosis in recent years.

Agents	Primary mechanism	Patients	Pros and cons of combined therapy	NCT number (and Phase)
Cilofexor + firsocostat	FXR agonist ACC inhibitor	392 patients with bridging fibrosis or compensated cirrhosis (F3–F4)	Combined therapy has better anti-fibrosis potential but still induces hypertriglyceridemia	NCT03449446 (Phase 2b)
Cilofexor + firsocostat + semaglutide	FXR agonist ACC inhibitor GLP-1 receptor agonist	Patients with NASH	Cilofexor and firsocostat-induced hypertriglyceridemia is alleviated by semaglutide	NCT03987074 (phase 2)
Cilofexor + firsocostat + fenofibrate	FXR agonist ACC inhibitor PPARα agonist	Patients with NASH with elevated TG (≥150 and <500 mg/dL)	Fenofibrate was safe and effectively mitigated increases in TG associated with ACC inhibitor	NCT02781584
PF-05221304 + PF-06865571	ACC inhibitor DGAT2 inhibitor	Adults with NAFLD	ACC inhibitor-mediated serum TG elevation was mitigated	NCT03776175 (phase 2a)
OCA + atorvastatin	FXR agonist HMGR inhibitors	84 participants with NASH	Atorvastatin attenuates OCA-induced LDL-C elevation	NCT02633956 (Phase 2)
Pioglitazone +tofogliflozin	PPARγ agonist SGLT-2 inhibitor	Patients with NAFLD with T2DM and a hepatic fat fraction of ≥10%	Therapeutic potential to prevent the progression of NASH to HCC	/
HXT + vitamin E	Natural compounds Antioxidant	Children with biopsy-proven NAFLD	Ameliorate steatosis and hypertriglyceridemia, reducing the fibrosis stage	NCT02842567

Currently used ACC inhibitors mainly include firsocostat (formerly GS-0976) and PF-05221304. These two acetyl-CoA carboxylase inhibitors affect serum TG. In a study of NASH patients, it was found that treating with GS-0976 20 mg per day for 12 weeks reduced liver steatosis, selective markers of liver fibrosis, and biochemistry but caused significant increases in serum TG levels in most patients (64); this asymptomatic hypertriglyceridemia can be partially resolved by fibrate (belonging to PPARα agonists). Lawitz EJ compared the curative effects of Vascepa or fenofibrate in mitigating triglyceride elevation in patients with NASH treated with cilofexor and firsocostat. NASH patients with elevated TG were randomly divided into two groups: one group treated with Vascepa 2 g twice a day for 2 weeks, and another with fenofibrate 145 mg once a day; both groups followed these with cilofexor 30 mg and firsocostat 20 mg once a day for 6 weeks, then safety, blood lipids, and liver biochemistry were monitored. After 6 weeks of combination therapy, fenofibrate has a better curative effect than Vascepa in reducing elevated TG in patients (NCT02781584) (65). Similarly, the ACC inhibitor PF-05221304 alone significantly reduced hepatic steatosis and induced an asymptomatic increase in serum TG levels; the latter may represent an adverse cardiometabolic profile limiting the long-term use of this class of drugs (66). Diacylglycerol acyltransferase 2 (DGAT2) is an enzyme that catalyzes the last step of TG synthesis. It plays a role in regulating VLDL production in rodents (67); PF-06865571 is an inhibitor of DGAT2 (68). In a Phase 2a pilot study combining PF-05221304 and PF-06865571, a significant attenuation of ACC inhibitor-mediated effects on serum TG was observed (NCT03776175) (69). We look forward to longer-period research including liver biopsies to further demonstrate the impact of co-administration of PF-05221304 and PF-06865571 on NASH regression and fibrosis in NASH patients. In summary, ACC inhibitors are currently attractive target drugs to restore the balance of hepatic fatty acid metabolism in patients with MAFLD. Combined use with drugs, such as FXR agonists, can reduce liver fibrosis, but attention still needs to be paid to the combination use of other medications that regulate lipogenesis to minimize the impact on blood lipids.

Combination of Fanitol X receptor agonists and other drugs

Fanitol X receptor (FXR) is a nuclear receptor abundantly expressed in the liver and intestinal epithelia, which is vital in the perception of bile acid signals. It regulates inflammatory pathways by reducing pro-inflammatory cytokines, inhibiting the activation of inflammasomes, and upregulating anti-inflammatory mediators (70). Studies have confirmed that activating the FXR in HSCs can reduce the HSCs' response to profibrotic signals such as TGFβ, thereby decreasing ECM formation and inhibiting the development of fibrosis (71). FXR agonists mainly include obeticholic acid (OCA) and cilofexor. One serious limitation of the OCA therapy is dyslipidemia (elevated low-density lipoprotein cholesterol), which may lead to a rising risk in NASH patients with atherosclerosis. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) plays a pivotal role in the biosynthesis of cholesterol; HMGR inhibitors inhibit cholesterol synthesis by atorvastatin (72). Clinical research about the combination of OCA and a statin found that atorvastatin attenuated OCA-induced low-density lipoprotein cholesterol (LDL-C) elevation in patients with NASH after 16 weeks of treatment (NCT02633956) (73). Cilofexor can significantly reduce liver steatosis, biochemical markers, and serum bile acid levels. Studies have shown that it has better anti-fibrosis profit when combined with ACC inhibitor firsocostat but still faces the problem of hypertriglyceridemia (NCT03449446) (53). This issue limits the application of this pharmacological combination therapy strategy. Therefore, it is necessary to explore more precise and effective drug targets; otherwise, multi-drug combination therapies are needed. As previously mentioned, adding fenofibrate or semaglutide relieves elevated TG induced by the combination therapy of cilofexor and firsocostat (63, 65). In addition, the FXR receptor agonist tropifexor and the C-C chemokine receptor type 2 and type 5 antagonist cenicriviroc target steatosis, inflammation, and fibrosis pathways involved in MAFLD. FXR agonists, which restore bile acid metabolism and suppress inflammation, are essential to future combination therapy for MAFLD.

Peroxisome proliferator-activated receptor modulators in combination with other drugs

Peroxisome proliferator-activated receptors (PPARs) are a class of nuclear hormone superfamily receptors widely involved in regulating inflammatory responses and metabolic homeostasis. Some agonists targeting PPAR combined with other different classes of drugs have a complementary effect in treating liver fibrosis, such as fenofibrate mentioned above (65, 74). In addition, pemafibrate is a selective PPARα modulator, and clinically relevant doses of pemafibrate were demonstrated to effectively and safely lower serum TG in mice (75). A study containing 118 patients evaluated the therapeutical effect of pemafibrate for MAFLD patients and showed that pemafibrate reduced liver stiffness but had no effect in reducing liver fat content (76). Pemafibrate may be a hopeful drug for treating MAFLD combined with medicines to lower hepatic fat. Another trial, including 70 participants with ultrasound-confirmed MAFLD, showed that the combination of ezetimibe plus rosuvastatin lowered hepatic fat (77). Further studies are needed to determine whether the combined use of pemafibrate, ezetimibe, and rosuvastatin can achieve more clinical benefits. In addition, a mouse model study showed that, at the two-time points of onset of NASH progression and HCC survival, combined treatment with pemafibrate and tofogliflozin (an SGLT-2 inhibitor) not only significantly relieved hyperglycemia and hypertriglyceridemia but also reduced ballooning of hepatocytes, reduced expression of ER stress-related genes level (such as Ire1a, Grp78, Xbp1, and Phlda3), and significantly improved the survival rate and decreased the tumors' numbers in the liver. It suggests that PPARα modulator and SGLT-2 inhibitor combined treatment has the potential to inhibit the progression of NASH to HCC (78). Pioglitazone belongs to the first-generation thiazolidinediones, which is a PPARγ agonist, and it was proved that pioglitazone could improve liver fibrosis scores in non-diabetic patients with

NASH (79). In patients with T2DM and MAFLD, 32 suitable patients were treated with pioglitazone and tofogliflozin; compared with every single-drug therapy group, combination therapy gained additional improvement in HbA1C. Weight gain mediated by pioglitazone was reduced with the concomitant use of tofogliflozin (80). In conclusion, agonists of PPAR are widely involved in regulating metabolic homeostasis and inflammatory response, have therapeutic potential in preventing the progression of MAFLD to HCC, and have a prominent position in combination therapy.

Combination therapy of natural compounds

Up to now, there is no specific clinically useful therapy for MAFLD, thus some people try to screen and study natural products or synthetic compounds to find efficacious drugs for the treatment of MAFLD, such as the natural sesquiterpene ketone (Nok) (81), hydroxytyrosol (HXT), and vitamin E. Both HXT and vitamin E have good antioxidant properties (82), and oxidative stress is an influential factor that induces HSCs to activate and leads to liver fibrosis (83). HSCs can be activated by tumor growth factor TGF-β, leading to a significant increase in proliferation rate (84). Nadia Panera used this cell as an *in vitro* model to conduct experiments, indicating that the use of HXT and vitamin E alone or in combination treatment resulted in a marked decrease in this TGF-β-dependent pro-proliferative effect. The combined therapy of HXT + vitamin E more effectively inhibited the impact of TGF-β on HSCs. HXT + vitamin E significantly reduced the pattern of liver fibrosis observed in a mouse model which was fed a carbon tetrachloride plus Western diet (82). In addition, in children with biopsy-proven MAFLD, a 4-month-old short-term HXT + vitamin E treatment responds to DNA damage recovery by increasing circulating IL-10 levels, ultimately ameliorating steatosis and hypertriglyceridemia, reducing the fibrosis stage in children with MAFLD, and this beneficial effect is extended over time (NCT02842567) (85). Screening and research on natural products or synthetic compounds to treat MAFLD will help explore new antifibrotic therapeutic targets, which may provide new elements for pharmaceutical combination therapies.

Combined use of different endothelial cell modulators

Liver fibrosis is due to the excessive formation of extracellular matrix, often accompanied by neovascularization and changes in vascular structure, ultimately causing organ injury and failure (86, 87). In recent years, angiogenesis inhibitors such as bevacizumab have made great progress in the treatment of tumors (88). Simultaneously, people are also actively exploring the application of angiogenesis modulators in treating liver fibrosis. The microvessels in the liver contain portal veins, hepatic sinusoids, and central vessels, and different vessels play different roles in the development of liver fibrosis (58). Therefore, achieving effective anti-fibrosis through targeted vascular therapy may require a combination of varying angiogenesis modulators. Leukocyte cell-derived chemotaxin 2 (LECT2), a newly discovered hepatic factor, is significantly increased in MAFLD patients (89). Ec-specific receptor Tie1 is necessary for the maturation of blood vessels. Meng Xu found that direct binding of LECT2 to Tie1 can inhibit portal vein angiogenesis, induce hepatic sinusoidal capillarization, and promote liver fibrosis. On the other hand, adeno-associated virus vector serotype 9 carrying LECT2 short hairpin RNA (AAV9-LECT2-shRNA) can target mouse LECT2 to inhibit LECT2/Tie1 signaling, thereby inducing portal angiogenesis, suppressing hepatic sinusoidal capillarization, and alleviating liver fibrosis (90). Yuan Lin and Meng Xu further explored the effect of AAV9-LECT2-shRNA combined with rVEGF or bevacizumab in the targeted therapy of liver fibrosis in mice. The shortcomings of bevacizumab and rVEGF in regulating different microvessels in the treatment of liver fibrosis are made up for by AAV9-LECT2 shRNA, the combination of varying angiogenesis modulators further improves the therapeutic effect on liver fibrosis, and the side effects of bevacizumab combination therapy are relatively less (58). In comparison, vascular endothelial cell regulators are aimed at the changes of angiogenesis in the development of liver fibrosis, directly anti-fibrosis, and in combination with other drugs targeting metabolic disorders, inflammation, and other mechanisms; theoretically speaking, the complementary advantages are apparent, and it is a direction worth exploring.

Conclusion and perspectives

As MAFLD has become the primary cause of liver fibrosis and one of the most common indications for LT worldwide, the global health problems caused by the MAFLD pandemic cannot be ignored. In the face of a considerable disease burden, lifestyle interventions have failed to control the prevalence of MAFLD effectively, and bariatric metabolic surgery is unsuitable as a first-line treatment. It is important to explore safe and effective drug treatment options. The occurrence of MAFLD and its liver fibrosis progression involves many complex factors and mechanisms, such as metabolic disorders, inflammation, immunity, and ECM formation. Some new drugs with multiple mechanisms of action have been discovered, such as FXR agonists that can regulate bile acid metabolism and inflammatory response and PPAR agonists that target metabolic disorders and inflammation simultaneously, but it is still challenging to achieve satisfactory results when these drugs are used alone. Hence, a strategy for combining different types of drugs is necessary. In recent years, appropriate drug combination therapy has mainly focused on driving factors such as metabolic disorders, inflammation, and oxidative stress. In the future, it is believed that there will be more explorations of multi-drug combination therapy strategies targeting different pro-fibrosis pathways and fibrosis regression mechanisms.

Author contributions

QD and HB wrote the original draft and further revised it. JW and WS organized and created tables. XZo and JS drew the figure. YX, YC, and XZh were responsible for project administration,

revising, and approving the manuscript. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The 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.

Publisher's note

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EDITED BY
Ana Sandoval-Rodriguez,
University of Guadalajara, Mexico

REVIEWED BY
Ming Yang,
University of Missouri, United States,
Valentina Cossiga,
University of Naples Federico II, Italy

*CORRESPONDENCE
Lyndsey F. Meyer
✉ Lyndsey.meyer@pfizer.com

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A continuous-time Markov chain model of fibrosis progression in NAFLD and NASH

Lyndsey F. Meyer*, Cynthia J. Musante and Richard Allen

Pfizer Worldwide Research Development and Medical, Cambridge, MA, United States

The specific pathways, timescales, and dynamics driving the progression of fibrosis in NAFLD and NASH are not yet fully understood. Hence, a mechanistic model of the pathogenesis and treatment of fibrosis in NASH will necessarily have significant uncertainties. The rate of fibrosis progression and the heterogeneity of pathogenesis across patients are not thoroughly quantified. To address this problem, we have developed a continuous-time Markov chain model that is able to capture the heterogeneity of fibrosis progression observed in the clinic. We estimated the average time of disease progression through various stages of fibrosis using seven published clinical studies involving paired liver biopsies. Sensitivity analysis revealed therapeutic intervention at stage F1 or stage F2 results in greatest potential improvement in the average fibrosis scores for a typical patient cohort distribution. These results were in good agreement with a retrospective analysis of placebo-controlled pioglitazone clinical trials for the treatment of NAFLD and NASH. This model provides support for determining patient populations, duration, and potential successful endpoints for clinical trial design in the area of NAFLD and NASH.

KEYWORDS

NAFLD, NASH, fibrosis progression, Markov chain model, liver biopsy, fibrosis score

1. Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) is the most common liver disease in the United States. In approximately 20% of the affected population, NAFLD progresses to nonalcoholic steatohepatitis (NASH) the hallmarks of which are inflammation, hepatocellular ballooning, and subsequent worsening fibrosis (1). Left untreated, NASH can ultimately progress to cirrhosis of the liver and hepatocellular carcinoma. NAFLD and NASH are the root cause of ~30% of all liver transplants in the United States (2). Currently, there are no FDA approved treatments for NAFLD or NASH resulting in a substantial unmet medical need.

It is hypothesized that liver injury is initiated following excess hepatic lipid accumulation leading to oxidative stress and inflammation (3). However, the complex dynamical relationships between the key biological pathways and processes leading to fibrosis are not well understood. Hepatocyte stress, ballooning, and death contribute to the recruitment of macrophages (4). Macrophages potentiate collagen deposition *via* hepatic stellate cell activation through cytokines, such as transforming growth factor-beta (TGFβ) and platelet-derived growth factor (PDGF) (5). A particular challenge in understanding the pathogenesis is that some patients exhibit rapid disease progression while others progress more slowly, and some portion of the observed population may exhibit stable disease or improve (6).

Currently, liver biopsy is considered the "gold standard" for the clinical diagnosis of NAFLD and NASH (7). Trained pathologists analyze biopsy samples and score fibrosis progression according to the criteria specified by Brunt et al. in Table 1 (8, 9). The invasive nature of biopsy and the histological assessment of fibrosis each contribute to significant challenges in evaluating the time course of disease progression. Due to the risks involved with obtaining a liver biopsy, it is often difficult to obtain multiple biopsies from a single patient and in cases where a patient's fibrosis score is in stage 4 it may be unethical to resample. Additionally, there are several sources of variability associated with histological assessment, including variability from the area of the liver sampled, as well as the expertise of the pathologist and the discrete nature of the fibrosis score (7). In addition, the scoring can be subjective, for example, even an experienced pathologist may score the same histological sample differently on the same day.

The objective of this work is to develop a computational model capable of capturing the time-course of fibrosis progression and the associated variability in order to provide insights into successful clinical trial design. Given the discrete scoring system and variability of the observed data, we chose to develop a continuous-time Markov chain (CTMC) model. Examples from the literature highlight the application of CTMC models to describe disease progression, such as renal function and hepatitis C (10, 11). In a CTMC model, there are defined states and transition rates, which determine the time and the next state. In our case, the discrete states correspond to fibrosis stage. Markov chain models are probabilistic and time independent. The critical property is that the model is memoryless; that is, the probability of changing states in a given interval is fixed. Therefore, the model requires no assumptions about the previous state of the "agent" (in this case, a patient) and the probability of disease progression or improvement is the same within a given time interval. Here, we describe model development and an application of the model to quantify pioglitazone effects on fibrosis progression as a proof of principle, as well as a power analysis to aid in clinical trial design. Pioglitazone is hypothesized to improve lipid metabolism and insulin sensitivity *via* PPARγ agonism and has been studied in clinical trials as a potential treatment for NAFLD (12).

2. Methods

2.1. Model development

Given the challenges associated with quantifying the specific mechanisms governing fibrosis progression as well the discrete nature of fibrosis clinical assessment, we chose to employ a continuous-time Markov chain model shown in Figure 1. There are five potential states of the model representing each stage of fibrosis. Each subject scored at F0 was designated as a progressor or non-progressor depending on the probability of progression parameter (p_0) estimated by the model. Progressors move through the various stages of fibrosis with a probability of progression or regression that is independent of how long the subject was in that stage of fibrosis. These characteristics allow us to capture the heterogeneity of clinical cohorts, as well as variability due to different biopsy sampling regions, and histological scoring variations between studies.

To simulate the model, we used a next-reaction, τ leaping algorithm from Thanh et al. in which the reaction rate, τ, is assumed to be a constant. The firing time is then drawn from an exponential distribution where r is a random number from 0 to 1 (13).

$$\tau = \frac{1}{\lambda}\ln\left(\frac{1}{r}\right) \quad (1)$$

At each time point, the algorithm determines the τ for both the forward and reverse reactions and chooses the state associated with the shortest reaction time. Parameters (p_1–p_8) governing transition between states (Figure 1) represent the average reaction rate; hence, the reciprocal of a parameter is the average time to disease progression or improvement event. The timescale of the model was chosen to be months based on the frequency of sampling and duration of clinical studies. Model development and parameter estimation was carried out in MATLAB (v. R2019b, Mathworks, Natick, MA, United States). We employed a genetic algorithm with a maximum population of 200 and maximum number of generations up to 100 to identify parameter estimates. In order to compare the final model to the data, we simulated the initial distribution 100 times and averaged the results. Parameters were fit with a sum of squared error objective function weighted by the number of patients at each time point. Simulation time is on the order of minutes for a single trial simulation. Complete MATLAB (v.2019b, Mathworks, Natick, MA, United States) model code is available at https://github.com/pfizer-opensource/CTMC-NAFLD-fibrosis.

TABLE 1 Fibrosis scoring definitions.

Stage	Fibrosis
F0	None
F1	Zone 3 Perisinusoidal
F2	F1 + Periportal
F3	Bridging
F4	Cirrhosis

Fibrosis scoring definitions summarized from Kleiner and Brunt et al. (8).

2.2. Disease progression data fittings

A literature search identified published clinical studies in which patients had biopsy proven NAFLD or NASH. The studies chosen for further analysis were those reporting both the initial number of patients in each stage of fibrosis as well as the final distribution for each initial stage. Studies were included for model fitting if patients underwent paired liver biopsy; the time between biopsies was clearly defined; and the fibrosis scoring was done according to Kleiner or Brunt scoring. As a result, data for fitting was acquired for 6, 24, 36, 48, 60, 72, 96, and 156 months for a total of 218 patients. Data were sourced from Harrison et al., Ratzui et al., Wong et al., Hui et al., Chan et al., Evans et al., and Ekstedt et al. (14–20). Patient demographics varied slightly between studies but most included patients with an average age of 47 years, BMI greater than 25 kg/m^2, and a mixture of patients with and without diabetes (14–20). All studies excluded patients with excessive alcohol consumption in the last 2 years as well as patients testing positive for hepatitis B surface antigen or anti-hepatitis C virus antibody; patients with secondary causes of hepatic

steatosis were also excluded. Data were fit simultaneously using the methods described above.

2.3. Sensitivity analysis

Next, we performed a sensitivity analysis to identify which parameters have the most influence on the average change in fibrosis score. In this scenario, we chose to use an initial distribution from a clinical trial of pioglitazone (21). We then simulated the outcome of the trial 500 times and quantified the average change in fibrosis score resulting from a change to a single parameter from 0.001 to 100-fold. We conducted the simulation for each parameter and plotted the change in average fibrosis score +/− the standard deviation vs. the parameter fold-change corrected by the placebo change in fibrosis score. The most sensitive parameters altered the change in average fibrosis score the most.

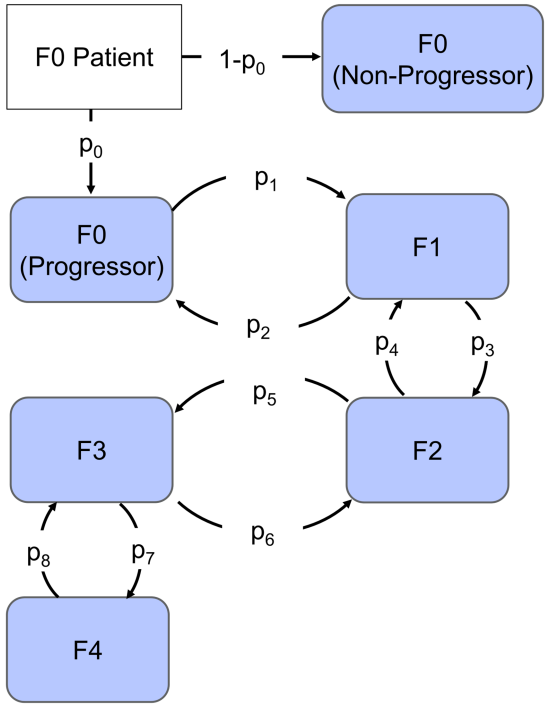

FIGURE 1
Continuous-time Markov chain model. Each compartment represents the stage of Fibrosis from F0 to F4. The arrows represent rates of progression (p1, p3, p5, and p7) or regression (p2, p4, p6, and p8), the fraction that progresses is indicated by parameter p0 and non-progressors are represented by the remaining fraction.

2.4. Pioglitazone intervention

Following the sensitivity analysis, we assessed how an intervention such as pioglitazone impacts both the forward and reverse model parameters and compared the results with the sensitivity analysis. We again performed a literature search to identify published clinical data that included a placebo and pioglitazone treatment group with paired liver biopsies. Three studies met this inclusion criteria, including Cusi et al., Belfort et al., and Aithal et al. (21–23). The doses ranged in each study from 30 to 45 mg/day of pioglitazone. The clinical trial endpoints were assessed at 6, 12, and 18 months for Belfort et al., Aithal et al., and Cusi et al. data, respectively. Data reported by each of these studies did not track individual patient starting and end fibrosis stages; only the initial distribution and final distribution were reported for each group as shown in Table 2.

The impact of pioglitazone on parameter estimates was estimated in a three-step process. First, we assumed each study exhibits a separate placebo effect and this placebo effect only impacts the forward disease progression. To capture the distribution of patients in the placebo group of each study, the parameters were fixed to the observational disease progression fitted parameters, however, the forward parameters were allowed to vary by a scale-factor (α1 α3 α5 α7). Each study has its own set of α-parameters to account for study specific differences in patient response to the trial, such as adherence to the recommended diet and exercise regimens. The second step was to similarly estimate the scale-factor change from observational fitted parameters for both the forward and reverse parameters for treatment with pioglitazone. Under the assumption that the same set of coefficients would capture all the pioglitazone data, we used the Cusi et al. data set to fit these parameters because it had the longest study end time. We then fixed the Cusi et al. parameters with their respective α-parameters and estimated a second set of scale-factors (β1–β8). Lastly, we attempted to validate the pioglitazone parameters by simulating the outcome of the Belfort et al. and Aithal et al. studies (22, 23). A flowchart describing the parameter fitting and validation sequence is shown in Figure 2.

2.5. Clinical trial design

Finally, we simulated the model to demonstrate how it may be useful in clinical trial design. One of the many challenges in designing a clinical trial for the treatment of NAFLD and NASH is determining the number of patients required to reach clinical significance given large interpatient and intrapatient variability in fibrosis score. The CTMC model is well-posed to address this problem since the stochastic nature of the model is agnostic to the sources of variability. The first assessment performed was to determine how the

TABLE 2 Pioglitazone fibrosis data extracted from Appendix Table 1 in Cusi et al. (13).

Stage	Placebo	18 months	Pioglitazone	18 months
F0	20	18	15	22
F1	22	16	22	13
F2	4	3	6	2
F3	5	5	7	3

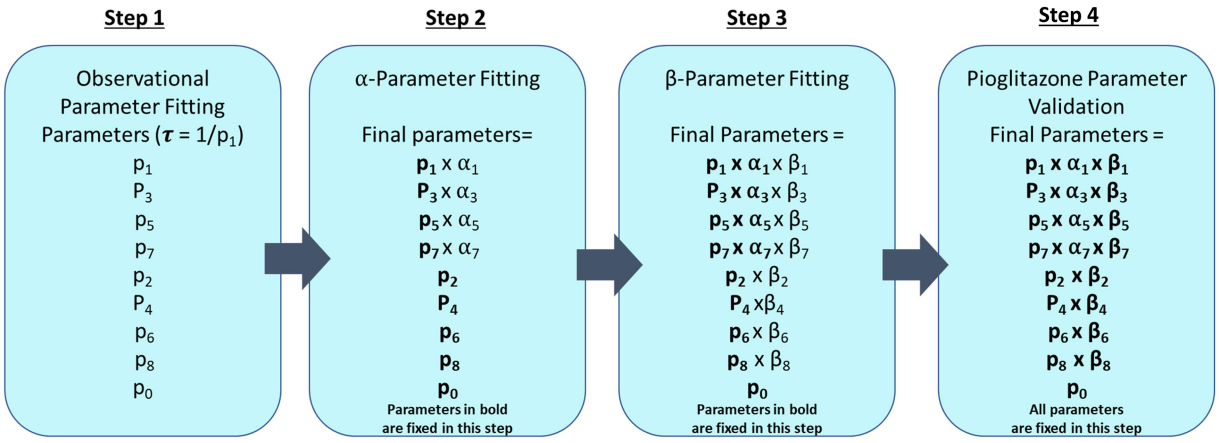

FIGURE 2
Parameter fitting flowchart. This diagram describes each step that was taken to fit CTMC model parameters where fixed parameters are in bold type. The first step fits all forward (p1, p3, p5, and p7) and reverse parameters (p2, p4, p6, and p8) and progression fraction (p0) to observational data from paired liver biopsy studies Harrison et al., Ratzui et al., Wong et al., Hui et al., Chan et al., Evans et al., and Ekstedt et al. (14–20). Observational parameters are then fixed for subsequent steps. In step 2, a scale-factor (α) is fit for the corresponding forward parameters to describe the placebo effect in Cusi et al., Belfort et al., and Aithal et al. (21–23) trials. Step 3 fits the pioglitazone effect with an additional scale factor (β) on both forward (p1, p3, p5, and p7) and reverse parameters (p2, p4, p6, and p8) for just the Cusi et al. (21) trial. Step 4 fixes all previously estimated parameters to predict the outcome of the pioglitazone arm in Belfort et al. and Aithal et al. (22, 23).

placebo response may change given different distributions of the patient population at each stage of fibrosis. Each stage of fibrosis was simulated with 100 patients for a duration of 12 months to determine the percentage of that population whose scores would improve or worsen over time.

The second assessment was to use information from model simulations to identify the minimum number of patients necessary to power a clinical trial with a drug effect similar to that of pioglitazone. To do this we simulated a minimum of 90 different initial distributions for a given number of virtual patients. The patient distribution was randomly selected according to the proportions of the initial distributions in the Cusi et al. (21) data set. For each set of data, a virtual clinical trial was simulated 200 times to generate statistics for the average change in fibrosis score, and standard deviation for that given data set. We then calculated the power for each initial distribution using a two-sample *t*-test. The number of virtual patients in each trial was varied from 5 to 125 by increments of 5.

3. Results

3.1. Observational data fitting

Results of the model fitting are shown in Figure 3. Observed data are presented side by side with model fittings. The model fittings performed reasonably well to capture the observed data. Upon visual inspection, 85% of the fitted data is within two standard deviations of the observed data.

3.2. Sensitivity analysis

Sensitivity analysis showed the average fibrosis score was most sensitive to changes in parameters p1, p2, p3, and p4 as shown in Figure 4. The most sensitive parameter was shown to be the reverse parameter from F1 to F0, p2, a 10-fold increase results in a 0.5-point decrease in the average fibrosis score. Parameters p5, p6, p7, and p8 have less influence on the average fibrosis score since there are fewer patients represented in F3 and F4, which is typical of the published clinical trial cohorts.

3.3. Pioglitazone intervention

Final parameter estimates for α and β coefficients are shown in Table 3. Placebo effects (α-parameters) on fibrosis progression ranged from less than 2- to 4-fold compared to observational disease progression parameter estimates. Pioglitazone effects ranged from 2- to 30-fold for Cusi et al. data whereas some β-parameter estimates were from 25-fold to upward of 40-fold for data from Belfort et al. and Aithal et al. β-parameter estimates suggest that pioglitazone not only slows disease progression but reverses fibrosis in the liver as indicated by a faster transition to lower fibrosis scores. Figure 5 shows the model simulations compared to observed data.

3.4. Clinical trial design

The percent of patient improvement depends on the initial stage of fibrosis. The model estimates that within 1 year, 40% of patients categorized with stage 1 fibrosis will improve by one stage, whereas only 20% of patients in stage 2 will improve by 1 stage or more. These results suggest that the expected placebo effect will be dependent on the initial distribution of the patient population as shown in Figure 6.

The power analysis shown in Figure 7 suggests a sample size of 65 patients in each cohort of a clinical trial would be sufficient to detect a difference in the average fibrosis score of 0.5 between control and treatment groups with 80% power. These results assume that the drug

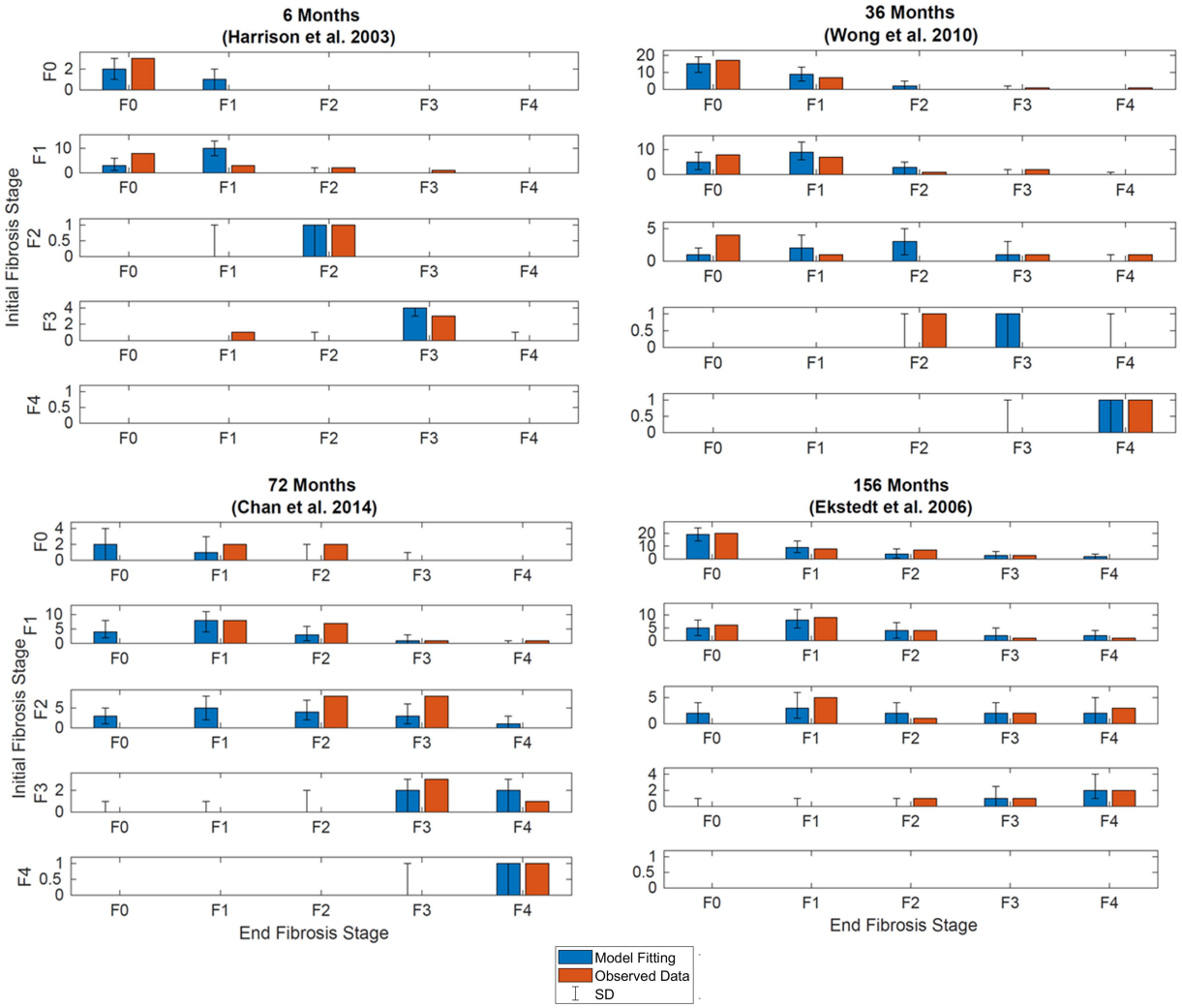

FIGURE 3
Simultaneous model fitting of observational data. Observed (orange bars) and model (blue bars). **(A)** Model fitting at 6 months. Compared to data digitized from Harrison et al. (20, p.2488). **(B)** Model fitting at 36 months. Compared to data extracted from Wong et al. (14, p.972). **(C)** Model fitting at 72 months. Compared to data extracted from Chan et al. (19, p.550). **(D)** Model fitting at 56 months. Compared to data extracted from Ekstedt et al. (17, p.871). Initial stages of fibrosis are indicated on the *y*-axis, the final stages of fibrosis for patients in the respective starting stages are shown on the *x*-axis. Error bars illustrate the standard deviation after simulating the model with the same initial distribution 500 times.

effect is similar to pioglitazone and the patient population inclusion criteria are similar to that of Cusi et al.

4. Discussion

The progression of fibrosis in NAFLD and NASH is not well characterized, and surrogate plasma biomarkers have remained elusive. One approach to enhance our understanding of disease progression is to estimate the average number of stages patients' progress in a year from paired liver biopsy studies as illustrated by Singh et al. In this systematic review, the authors estimated the fibrosis progression rate differentiating between NAFLD patients and NASH patients. They found the average progression rate from stage 0 to stage 1 was 0.07 years for patients with NAFLD and 0.14 years for patients with NASH (24). One shortcoming of this estimation method, however, is that it cannot account for the heterogeneity of disease progression observed in the population. In addition, the authors faced challenges estimating the fibrosis progression rate for stages 2–3 and 3–4 citing that the negative lower limit of the confidence intervals suggest there could be net regression of fibrosis stage. These observations motivated us to develop a modeling approach in order to quantify fibrosis progression, regression, and its associated variability.

We chose to employ a continuous-time Markov chain model as clinical data collected from histological assessment of NAFLD and NASH lends itself readily to Markov chain modeling. A CTMC model can estimate both rates of progression as well as rates of regression at each stage in fibrosis. It is necessary to include the regression mechanism as the data in literature suggests fibrosis improves in patients advised on diet and exercise, as well as patients treated with pioglitazone (25). The disadvantage of this modeling approach is that it requires sufficient data to estimate each parameter and there is limited data for patients in the later stages of fibrosis.

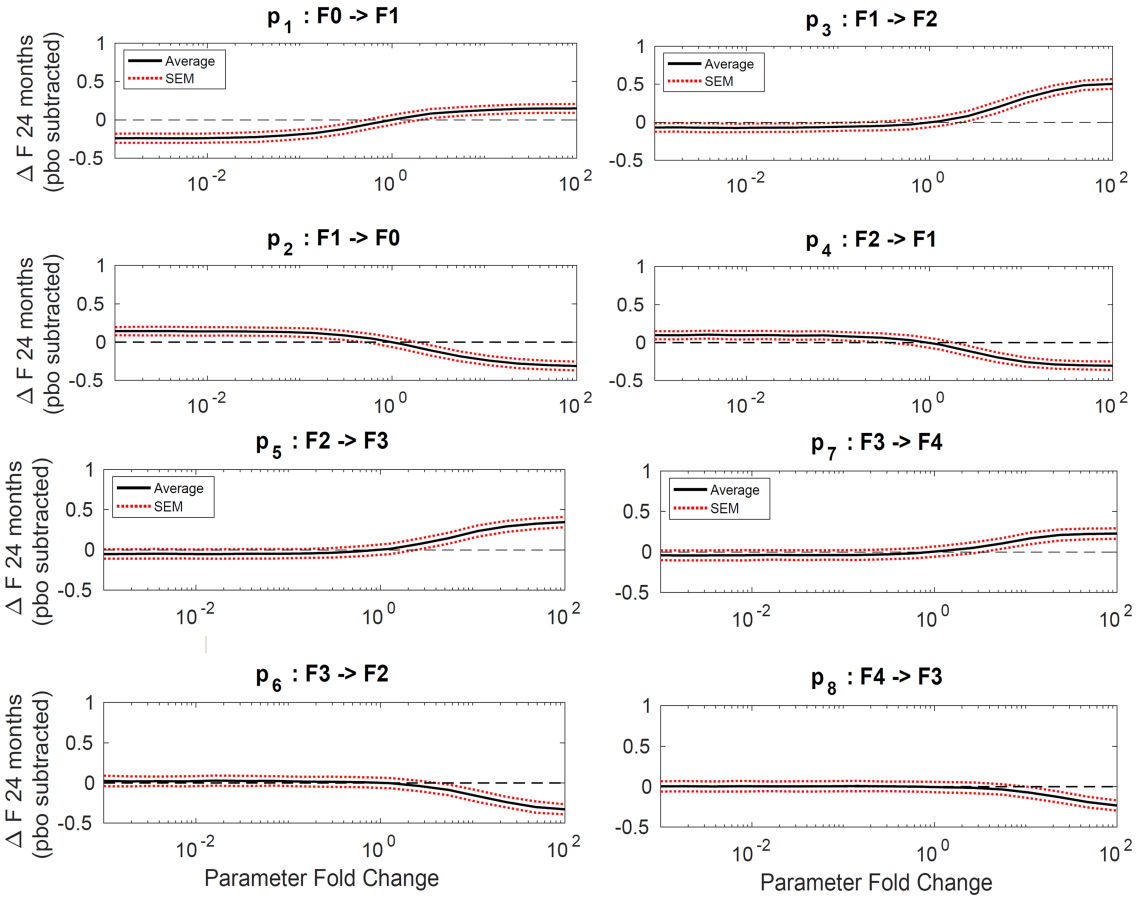

FIGURE 4
Sensitivity analysis. Plots of average change in fibrosis score vs. fold change in a single parameter while all others remain constant. Progressor fraction was fixed to the fitted value. Solid lines represent the average change in fibrosis score and the dashed lines are the standard error of the mean.

In our approach, we collected data from several studies reporting the fibrosis scores from paired liver biopsy studies; many of the studies overlap with the analysis performed by Singh et al. These studies consisted of patients with biopsy confirmed NAFLD or NASH and included patients with diabetes, metabolic syndrome, hypertension, reduced insulin sensitivity, and obese and non-obese patients. All patients in the studies were advised on diet and exercise but were otherwise not specifically treated for NAFLD or NASH. Using all the available information, we were able to observe reasonable fits between the model and observed data as shown in Figure 3.

The fraction of patients progressing from stage 0 was estimated to be 64%. This agrees with the raw data showing that 58% of patients that started in F0 at all time points ended in F0 as well as the Ekstedt et al. data which shows 53% of patients starting in F0 remained in F0 at 13 year follow up (17). Further work is necessary to elucidate potential biomarkers to differentiate progressors from non-progressors. The rate of progression from F0 to F1 for a progressive population was estimated to be 5.7 months or 0.47 years, which is similar to the estimation presented by Singh et al. for NASH patients (0.14 years) (24). The advantage to using a modeling approach is that we can now make predictions about a given initial distribution and leverage the stochastic property of the model to incorporate variability.

Sensitivity analysis indicates the maximum decrease in average fibrosis score by altering a single parameter is 0.5 stages. However, this does not suggest that any putative therapy designed to have a larger impact on the disease would necessarily have to exhibit poly-pharmacology. In this framework, we have made no mechanistic interpretation of the parameters and in fact the same biological process could be captured in multiple parameters (for example, collagen deposition might be encapsulated in all forward parameters). We found the parameters that have the most impact over the average fibrosis score are p_1, p_2, p_3, and p_4. Since the majority of patients in the initial distribution are in stages F0 and F1, the influence of changing p_5, for example, does not significantly alter the average fibrosis score. One caveat to the sensitivity analysis is also that the simulation duration was 24 months. Parameter estimates for stages F2, F3, and F4 are estimated to have average reaction times from 86 up to 116 months as shown in Table 3. This may exclude the impact of changing a parameter 100-fold like for parameters p_5 and p_6, which are 97 and 208 months, respectively, for a population with more patients in stages F3 and F4.

Next, we investigated the effects of placebo and pioglitazone from a clinical trial as a proof of principle for the CTMC model. Parameter estimates to capture the placebo effect suggested the recommended diet and exercise regimen in the placebo group slows disease progression. Weight loss was also observed in the placebo

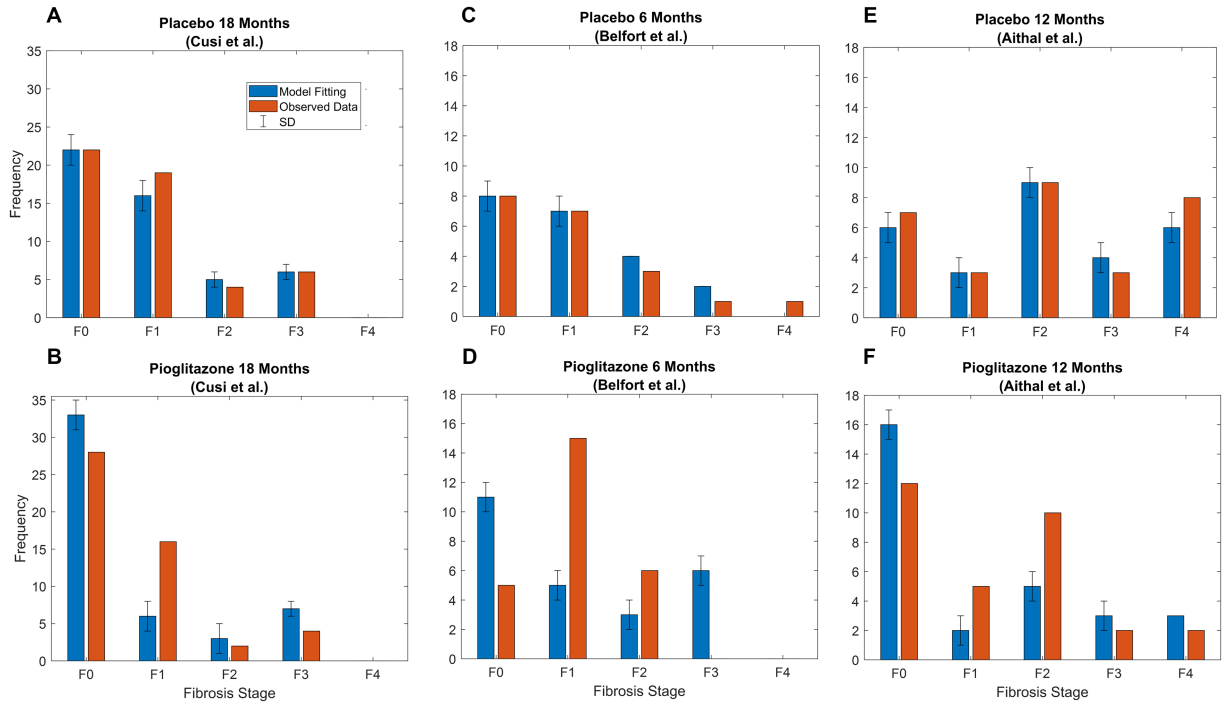

FIGURE 5
Pioglitazone simulation results. Observed (orange bars) and predicted (blue bars) data ±SD. **(A)** Fitting of Cusi et al. data after 18 months [Appendix (21)] with just α parameters, which are the fold changes for forward parameters. **(B)** Fitting for β parameters representing fold change for pioglitazone effect in the Cusi et al. study. **(C)** Fitted placebo α parameters for Belfort et al. **(D)** Fixed parameters representing the placebo effect and pioglitazone effect for Belfort et al. data extracted from p.2305 (22) after treatment. **(E)** Fitted placebo α parameters for Aithal et al. **(F)** Fixed parameters representing the placebo effect and pioglitazone effect for Aithal et al. data extracted from p.1179 (23) after treatment.

TABLE 3 Final parameter estimates.

Parameters	Fitted parameters		Placebo (α)		Pioglitazone (αβ)	
	Forward	Backward	Forward	Backward	Forward	Backward
	τ (months)	τ (months)	τ (months)	τ (months)	τ (months)	τ (months)
F0 < -> F1	5.7	10	9.3	10	139	3.8
F1 < -> F2	86	51	68	51	1,750	7.6
F2 < -> F3	97	208	52	208	1,570	171
F3 < -> F4	116	526	374	526	374,000	2,140
Progressor fraction	0.64	Estimated	0.64	Fixed	0.64	Fixed

τ_n Reaction Time (months) $1/p_n$.

groups of Aithal et al. (22) and Belfort et al. (23) studies. Pioglitazone, however, has notable effects on both slowing disease progression and improving the fibrosis score, as suggested by alterations in both the forward and reverse parameters; i.e., the data could not be captured without accounting for the reversal mechanism corresponding to a decrease in fibrosis score. These results also agree with the sensitivity analysis; we found p_1 reaction time increased by 10-fold, and p_4 reaction time decreased by 6-fold resulting in a decrease of 0.5 for the average fibrosis score with pioglitazone. Future work is necessary to validate the parameter estimates for the impact of pioglitazone. Cusi et al. reported only initial and final distributions for the placebo and treated groups—if the data had been reported categorically for each stage, such as Ekstedt et al. (17), then our estimates might be more robust. Perhaps due to this limitation, estimating individual placebo effects and simulating the results for data presented in Belfort et al. and Aithal et al. did not capture the data, as indicated in Figures 5D,F. Perhaps more crucially, the patient populations recruited for each trial were different; for example, the Aithal et al. trial excluded patients with type 2 diabetes. Additionally, the sample size for each study was relatively low, with only 20 or 30 patients. This approach applied to richer larger datasets, with individual level data, may be able to elucidate treatment effects with more clarity.

Model simulations were performed to gain insight on disease progression variability to inform clinical trial design. Based on our findings from parameter fitting, sensitivity analysis revealed the

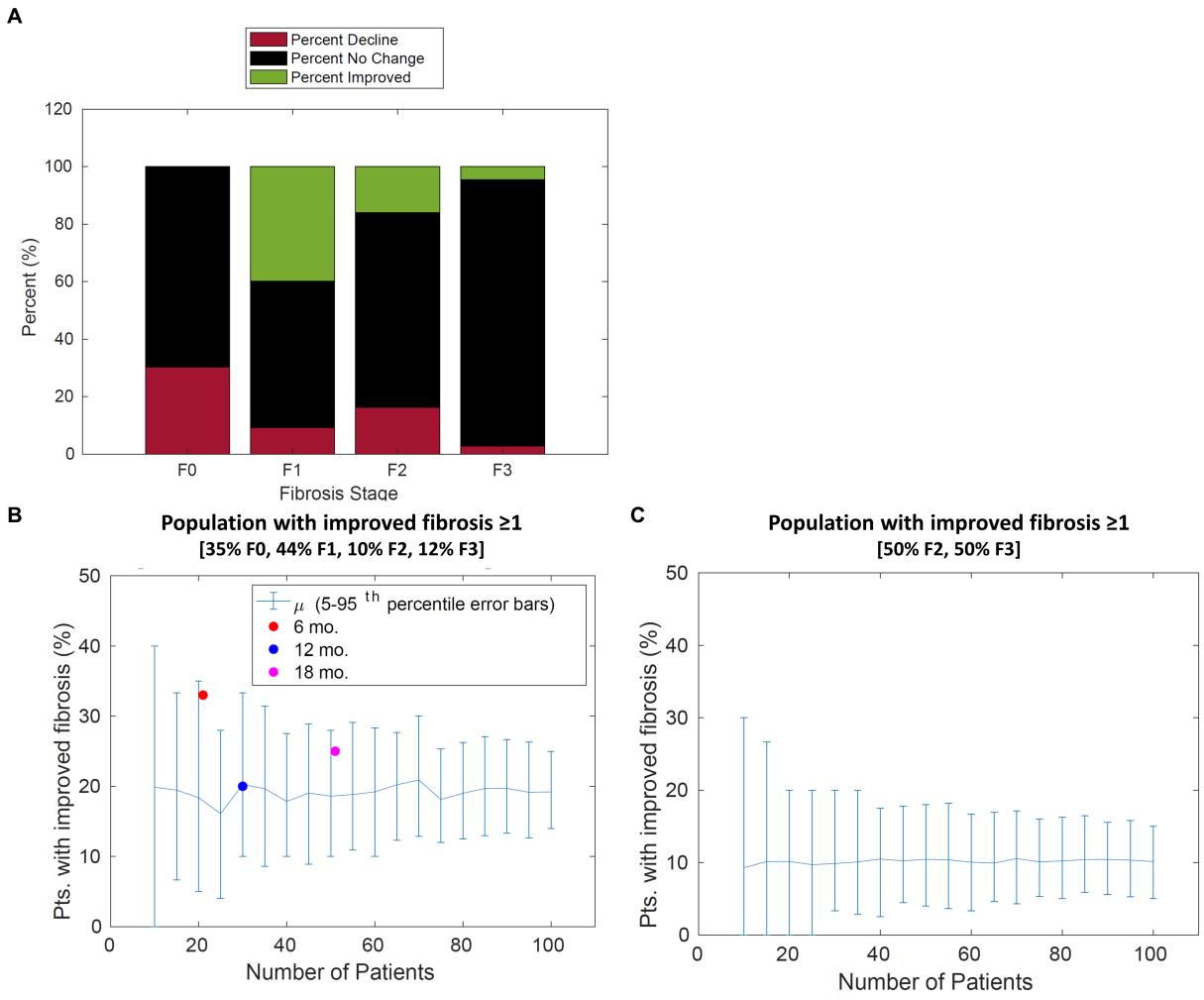

FIGURE 6
Population change in fibrosis score over 12 months. **(A)** For each stage of fibrosis, the average predicted percent of the population that improved (green bars), declined (red bars), or stayed the same (black bars). **(B)** The predicted percentage of patients with improvement in fibrosis score of 1 or more vs. the number of patients in the study for a typical patient cohort. The percentage of patients improved from Cusi et al. extracted from [Appendix, (21)], Belfort et al. extracted from p. 2305, and Aithal et al. extracted from p. 1179 (21–23) is overlaid with model simulations. **(C)** The predicted percentage of patients with improvement in fibrosis score of 1 or more vs. the number of patients in the study for a cohort with 50% F2 and 50% F3 patients. Error bars represent the 90th percentile.

expected placebo response (i.e., the percentage of patients improving) may depend on the initial distribution. Simulations predict the placebo response will be greater if a larger number of patients in stage F1 are included due to the shorter transition time. The placebo response ranges from 20% of patients improve by one stage or more for a typical distribution (35% F0, 44% F1, 10% F2, 12% F3, and 0% F4) compared to 13% for a distribution containing 50% F2 and 50% F3 patients (Figures 6B,C).

Finally, we simulated the outcome of a clinical trial multiple times for a range of patient sample sizes to calculate the average power of a study, with a random initial distribution. We found a sample size of 65 patients in each group would be sufficient to power a study with 80% power to detect a difference of 0.5 in average fibrosis score with a significance of 0.05. The clinical trial design, however, will depend on the drug effect and a clearly defined patient population.

5. Conclusion

The CTMC modeling approach enabled us to estimate forward and reverse parameters for fibrosis in NAFLD and NASH. We found that intervening at earlier stages of fibrosis is more likely to improve the average fibrosis score. This finding is confirmed in clinical trials of pioglitazone (21–23). In addition, model fitting suggests pioglitazone plays a role in reversing fibrosis progression, though, these results may be caveated by small sampling size, biopsy sampling variability, and pathologist variability. Based on our analysis, a study powered at 80% to detect a −0.5 change in average fibrosis score, as observed by Cusi et al., would require a larger sample size to reduce the risk of a type 1 error. The modeling work presented here is well-suited for better understanding the placebo response for a clinical trial. We found the CTMC model reproduces the trends in the data and broadly recapitulates the variability associated with fibrosis score.

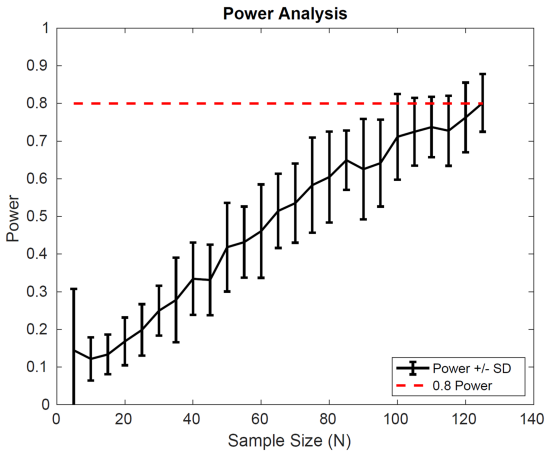

FIGURE 7
Simulated power Analysis. Power of a clinical trial vs. the number of patients in each cohort of the trial. The endpoint for this analysis was a 0.5 difference in the average fibrosis score between control and treatment groups with 80% power. Power was calculated based on two-sample *t*-test of simulated outputs.

Future applications of this model could include bridging a quantitative systems pharmacology (QSP) model to the CTMC model. A QSP model would facilitate connecting mechanistic drivers of disease progression to clinically measured fibrosis score. To combine the CTMC model with a more mechanistic model, it may be necessary to incorporate variability in the reaction rates. This can be accomplished by back-calculating the integrals of the reaction rates (13) in order to couple disease progression to a more mechanistic model of underlying disease pathogenesis. As more data from ongoing clinical studies is published, the model will become more powerful at predicting fibrosis progression for NAFLD/NASH patients.

Data availability statement

The data used in this study are available in PubMed Central with the following accession numbers: PMID 14638353, PMID 10833486, PMID 20581244, PMID 15709991, PMID 25060399, PMID 12195000, PMID 17006923, PMID 27322798, PMID 17135584, PMID 18718471.

Author contributions

RA contributed to conception and design of the study. LM and RA executed the analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that this study received funding from Pfizer, Inc. The funder had the following involvement in the study: study design, collection, analysis, interpretation of data, the writing of this article, and the decision to submit it for publication.

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EDITED BY
Ana Sandoval-Rodriguez,
University of Guadalajara, Mexico

REVIEWED BY
Ab Qayoom Naik,
Barkatullah University, India

*CORRESPONDENCE
Consolato M. Sergi
✉ csergi@cheo.on.ca

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MASLD and aspartame: are new studies in the horizon?

Consolato M. Sergi[1,2]*

[1]Department of Laboratory Medicine, University of Alberta, Edmonton, AB, Canada, [2]Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, ON, Canada

Fatty liver disease has been on the rise in the past few decades, and there is no hope that it will stop. The terminology change that has been recently proposed may not be sufficient to advocate for a reduction of steatogenic foods and a change in lifestyle. A course change may be supported by the recent labeling of aspartame sweetener as a possible carcinogenic compound by the International Association for Research on Cancer (IARC), an agency of the World Health Organization (WHO). Aspartame sweeteners and other edulcorating molecular compounds besides colorings may trigger liver cancer other than fatty liver disease, despite limited data supporting it. An essential bias in human cohort studies is indeed the exclusion of all confounding factors, which may be barely impossible for human studies. In this perspective, we suggest that the activation of the NOD-like receptor-enclosing protein 3 (NLRP3) inflammasome and the stimulation of the tumor suppression gene *TP53* may be critical in the progression from fatty liver to liver inflammation and liver cancer. Aspartame reduces a transcriptional coactivator, precisely the peroxisomal proliferator-initiated receptor-γ (gamma) coactivator 1-α (alpha) (or PGC1α). This coactivator upregulates mitochondrial bioformation, oxidative phosphorylation, respiratory capacity, and fatty acid β-oxidation. Aspartame acts in this way, probably through the activation of *TP53*. These events have been accountable for the variations in the lipid outline in serum and total lipid storage as well as for the impairment of gluconeogenesis in the liver, as supported by the downregulation of the gluconeogenic enzymes in experimental animals, and may be relevant in humans as well.

KEYWORDS

fatty liver, NLRP3, cancer, aspartame, NOD-like receptor-enclosing protein 3

Introduction

Undoubtedly, one of the epidemics we are facing right now is the spread of fatty liver disease in our technological society. Most countries have individuals of all ages affected with fatty liver disease (1–3). Most recently, fatty liver disease has increased in professional and lay circles because of its morbidities associated with life style changes. There has been an outcry that the most contemporary COVID-19 pandemic restrictions may have had a role in the current rate of fatty liver due to the decrease in physical activity and an increase of both high-fructose corn syrup and adulterated food and beverages during lockdowns and school closures (4). However, it is scapegoating, and probably this trend was already significant before the COVID-19 pandemic. On the other hand, there is no doubt that the decrease in physical activity and diffuse online learning may have been responsible for hijacking and incrementing this trend, making entire cohorts of youth and children overweight and even obese.

NAFLD vs. MASLD

Liver disease is an ongoing epidemic affecting multiple countries worldwide and different ethnic groups of individuals. Most recently, a global consensus has been reached among various panels of experts, including hepatology researchers and hepatology clinicians, who changed the acronym non-alcoholic fatty liver disease (NAFLD) into metabolic dysfunction-associated steatotic hepatic disease (MASLD) from the intermediate term of original metabolic dysfunction-linked fatty liver disease (MAFLD) (5, 6). The new terminology will probably dominate in the next few years once more publications emerge. Still, we do not need to absquatulate terms for new terms when rediscovering Greek or Latin books. The new terminology recalls the Greek etymology of fat. The panelists determined fatty was considered stigmatizing and opted for a return to the Greek term "steatotic," which arises from "steatos" or fatty (the Greek term "στέατος" is the genitive singular of στέαρ or hard fat). The terminology of steatotic liver disease will cover MASLD and MetALD (metabolic alcoholic liver disease) simultaneously. MetALD incorporates alcoholic liver disease in individuals who consume more than 210 g/week in men and more than 140 g/week in women. The term non-alcoholic steatohepatitis or NASH will be replaced by MASH, which is metabolic dysfunction associated with steatohepatitis.

MASLD individuals commonly harbor a cardiac metabolic risk factor, such as type 2 diabetes mellitus, which is not trivial and affects several organs and systems in the human organism. The comorbidities do not need to be neglected. Individuals without metabolic parameters and an unknown etiology will be classified as patients with cryptogenic steatotic liver disease (SLD). In MetALD, there is precisely a continuum in which the liver disease can be appreciated to be either ALD or MASLD leading. The new nomenclature incorporates the agreement that even moderate alcohol consumption may alter the natural progression of fatty liver disease. We hope that randomized clinical trials will soon be carried out to identify the minimal alcohol consumption that can affect metabolic dysfunction in specific individuals or ethnically different individuals. The effort involved 236 panelists. They were from 56 individual countries and took part in several rounds of online service. They used a Delphi process. Professionals and patient advocates participated in building up the new nomenclature and change well, relying on the classic "supermajority of opinion." A few key international medical societies participated in building the new nomenclature, including the Latin American, North American, and European Associations for the Study of the Liver (ASLD, EASL, and LASL).

Clinical aspects

Non-alcoholic steatosis of the liver is a problematic issue because it can evolve into inflammation (NASH) and cancer (Figure 1). Steatosis is associated with metabolic syndrome in about 2/3 of cases. The increase in delivery of free fatty acids to the hepatic organ originates from inappropriate dietary fat intake, large consumption of soft drinks, increased oxidative stress in the hepatocytes, and insulin resistance. The delivery of free fatty acid to the liver and the increase of triglyceride buildup in this organ give the "fatty" change of the hepatocyte, showing histologically a multivacuolar to univacuolar transformation of the hepatocytic phenotype, which looks like a space in the hepatocyte displacing the nucleus at the periphery. NAFLD is a significant health issue, with 1/4 to 1/3 of the worldwide adult population affected (7). NAFLD can subsequently progress to NASH. There is a 20–50% risk of progression to liver fibrosis, a 30% risk of developing liver cirrhosis, and a 5% risk of developing liver neoplasm, namely, hepatocellular carcinoma (HCC) (8, 9). The presence of hyperlipidemia, hypertension, non-hypertensive cardiovascular diseases, and type 2 diabetes mellitus, in addition to NAFLD, characterizes the metabolic syndrome, which is currently harboring the name change of MASLD. It has been a matter of controversy if healthy metabolic syndrome truly exists, and several publications came out in the most recent years supporting the theory that, indeed, some individuals who are obese may also be healthy. Resveratrol is a stilbenoid (hydroxylated derivative of stilbene), i.e., a variety of phenol that occurs in nature. It is a phytoalexin. Resveratrol is produced by several plants in reaction to injury. There are multiple sources of resveratrol in food. The most salient food varieties, including resveratrol, are the skins of grapes, raspberries, mulberries, blueberries, and peanuts. Recently, a methodical search and meta-analysis was piloted to determine if cardiometabolic risk factors may be curbed by resveratrol targeting individuals with metabolic syndrome (Met-S) and individuals who are obese/healthy (O/H) (10). The first group was harboring MetS, well-defined as a gathering of obesity at the abdomen location, dyslipidemia, hyperglycemia, and

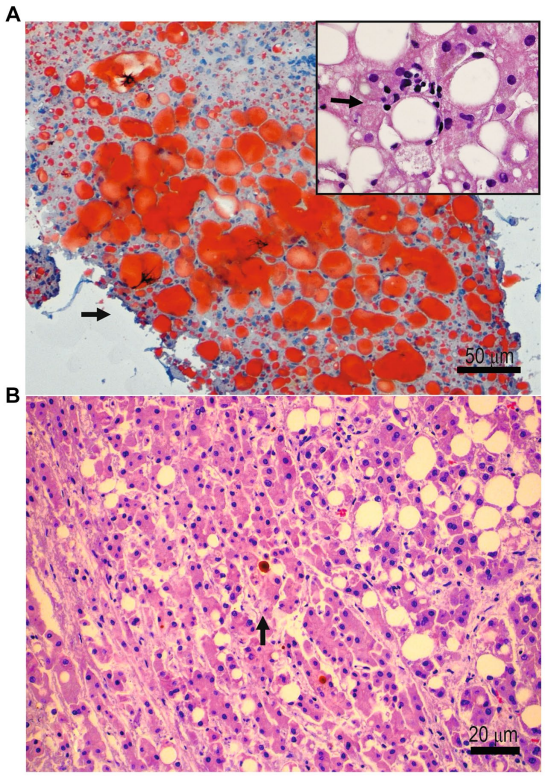

FIGURE 1
(A) Liver histopathology showing oil-red O-stained vacuoles in a patient affected by non-alcoholic fatty liver disease. Inset: a focus on inflammation in a patient with non-alcoholic steatohepatitis. **(B)** Liver histopathology showing neoplastic growth of cells with features of hepatocellular carcinoma in a patient with long-standing non-alcoholic fatty liver disease.

hypertension in a single subject. In contrast, the second group was composed of "obese/healthy" people, i.e., healthy subjects with or without demonstrating a clinical view of obesity. Data from randomized clinical trials (RCT) studies (total: 17) comprising 651 participants were mined for analysis. Generally, resveratrol had a substantial effect on HOMA-IR, i.e., the Homeostatic Model Assessment-Insulin Resistance (HOMA-IR). It resulted in a mean difference of −0.520665 ($p = 0.001$). In Met-S, RES pointedly reduced low-density lipoprotein cholesterol (LDL-C), and total cholesterol (T-Chol) other than glucose, as discovered by the mean difference of −0.924 ($p = 0.040$), −1.246 ($p = 0.022$), and −1.069 ($p = 0.043$), respectively. Despite some perceived heterogeneity in world people, the supplementation of resveratrol improved cardiometabolic health and clearly decreased some risk factors (HOMA-IR, LDL-C, and T-Chol) allied with cardiovascular disease (10).

Sweeteners and fatty liver disease

A dramatic change occurred in the past few decades in dietary habits worldwide with the food industry's introduction of sweeteners such as sucrose and fructose. Regular soft and fruit drinks are significant sources of high-fructose corn syrup or sugar. They increased from 3.9% of the total energy consumption in 1977 to 9.2% in 2001 (11). Thus, there is a 135% increment in just two decades. In addition to this change in dietary habits, soft drinks have increased their presence in the market. They are contained in many diet styles and have raised some health concerns. Diet soft drinks encompass aspartame sweetener (Figure 2) and often contain caramel coloring. These compounds stimulate advanced glycation end products. Aspartame sweeteners and caramel coloring are diffused in soft drinks, potentially increasing insulin resistance and inflammation (12). The increase in soft drink intake has been linked to NAFLD, a non-dependent metabolic syndrome. NAFLD patients have been seen consuming five times the quantity of carbohydrates from soft drinks compared to healthy individuals. The increase of more than one "soft" drink *per diem* has been associated with a high rate of metabolic syndrome. Some other individuals consume less than one "soft" drink daily (13). In June 2023, an assembly of 25 scientists and researchers from twelve countries met in Lyon, France, at the International Agency for Research on Cancer (IARC). They target evaluating the carcinogenesis of aspartame, among other chemicals (14). The aspartame sweetener was categorized as a compound possibly carcinogenic to humans. This classification was based on some, but limited, evidence for neoplasms in humans. The panel of 25 scientists also indicated that there was some, but limited, evidence for carcinogenicity in some experimental animals. Finally, there was little evidence of "mechanistic" data for carcinogenicity. To the best of our knowledge, the NutriNet-Sante study is leading. It is the only more extensive cohort human study that prospectively evaluated the exposure to aspartame from totally dietary sources. This cardinal investigation reported that the link with aspartame increases the mammary gland cancer risk (obesity-related) and the cumulative cancer risk. Still, they did not examine in detail the link between aspartame and liver cancer risk. On the other side, the working group recognized four prospective human cohorts that evaluated the link between artificially sweetened "soft" drinks and liver cancer risk. In three investigations, a positive association was determined between artificially sweetened drink intake and mortality due to cancer. Old studies were perused in detail and controlled for potential confounding variables. Nevertheless, the authors could not entirely rule out some confounders, and the evidence of HCC following aspartame sweetener "soft" drinks was considered to be limited.

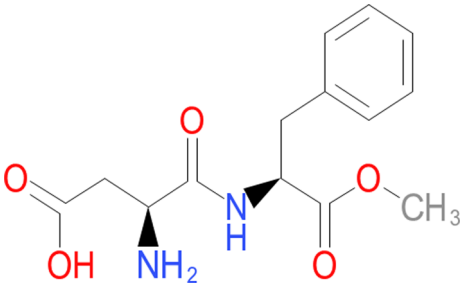

FIGURE 2
Chemical formula of aspartame.

In experimental animals, there was a split view in interpreting data for limited or sufficient evidence for carcinogenicity. Indeed, there were concerns regarding the diagnosis of lymphomas and related combinations and the likely impossible exclusion of the inflammation. However, the data from the study on animals indicated that the molecule of aspartame (methyl L-α-aspartyl-L-phenylalaninate) had clear-cut carcinogenic activity. The majority of the scientific working group pondered that the signal for oncogenesis should be considered limited concerning the appropriateness of the design and adequate reporting of the litter effect, which may have led to some false positive results. A minority of the scientific working group indicated that such concerns were indeed considered but assessed as not consistently critical. This subgroup suggested that the evidence for cancer and experimental animals was "sufficient." An examination of the key characteristics of carcinogenicity identified that the mechanistic signal for aspartame being carcinogenic was present but still limited, despite some prominent and outstanding positive findings about genotoxicity in several investigations. These studies indicated that alterations in insulin sensitivity may have played a serious time factor. Overall, insulin sensitivity can be an important carcinogenicity factor (14).

In a most recent experimental animal study from Spain, Finamor et al. found that prolonged (long-term) aspartame administration leads ineluctably to hepatic fibrosis (15). It can elicit inflammasome activation and, subsequently, an impairment of gluconeogenesis in the liver, at least in experimental animals. They studied male Swiss mice kept in temperature- and humidity-controlled quarters. The animals were fed standard rodent chow and tap water. Aspartame produced hepatic tissue damage and a remarkable increase in transaminase levels, prompting liver fibrosis and upregulating pro-fibrotic markers, including transforming growth factor β1, collagen type 1A1, and α smooth muscle actin (SMA). Interestingly, aspartame clearly reduced the activation of nuclear factor erythroid 2-related factor 2 (Nrf2). This compound also decreased the enzymatic activity of antioxidants and caused an increase in lipid peroxidation. These events elicited the activation of the NOD-like receptor-containing protein 3 (NLRP3) inflammasome and the induction of the tumor suppression gene *TP53*. They also found that aspartame reduced the peroxisomal proliferator-activated receptor γ coactivator 1A, probably through the

activation of P53. These events have been accountable for the modifications in the profile of lipids in serum and total lipid accumulation, as well as a deficiency of gluconeogenesis in the liver, as supported by the downregulation of gluconeogenic enzymes.

NLRP3 inflammasome and cancer

NLRP3 inflammasome initiation is linked with the onset of liver cancer, particularly HCC (16). New clinical trials should target NLRP3 inflammasome activation and compounds that may be able to act on it (17). The initiation of the NLRP3 inflammasome requires two steps. First, the priming signal stimulates the nuclear factor kappa-B (NF-κB) pathway. It permits the transcription of NLRP3 and other genes. All these gene products encode some pro-inflammatory molecules (e.g., cytokines or interleukins pro-IL-1β and pro-IL-18). This initial step is generated by binding molecules of microorganisms or endogenously generated (pathogen/damage or endogenous molecular patterns, also abbreviated as PAMP and DAMP) to their receptors, labeled PRRs or "pattern recognition receptors". The proper stimulation or second signal prompts the suitable gathering of the inflammasome (18). NLRP3 animal models may be critical to study the pathogenesis of diseases, and an animal model based on NLRP3 has been considered to be critical in studying COVID-19 properly (19). We are familiar with a rodent model because of our previous studies on inflammatory bowel disease (IBD), an idiopathic gastrointestinal disease with an inflammatory character characterized by chronicity (20, 21). *Citrobacter rodentium*, a non-invasive Gram-negative microorganism, is a natural mouse bacterium in these animal models. This microbe is generally used to explore enteric infections as well as microorganism-promoted inflammation, resembling enterohemorrhagic *Escherichia coli* infection and IBD in humans (20, 21). Mice lacking the *Nlrp3* gene (*Nlrp3*$^{-/-}$) are critical for these studies. They are more susceptible to induced experimental IBD. *Nlrp3*$^{-/-}$ macrophages did not respond to pathogen-associated microorganismal patterns. Formerly, we established that compensation of IL-1b in rodents (mice) missing the NLRP3 inflammasome might stimulate the clearance of *C. rodentium* by encouraging the recruitment of inflammatory macrophages early during the infection. Conversely, IL-1b overcompensation may be detrimental in wild-type animals (20). There is considerable data regarding the protagonist role of the NRLP3 inflammasome in HCC. Wei et al. (22) demonstrated that E2 supports NLRP3 inflammasome-caspase-1-dependent pyroptosis. Such an event is triggered by the inhibition of autophagy following the suppression of the AMPK/mTOR pathway in HCC cells (22). Zhang et al. (23) showed that AIF prevents the growth and metastasis of HCC cells by prompting NLRP3 inflammasome-mediated pyroptosis via the inhibition of autophagy in HCC cell lines (HepG2, Huh7, Bel7402, and SMMC 7721). Wei et al. (24) showed that E2 impedes HCC growth through the promotion of the NLRP3 inflammasome via activating the ERβ/MAPK/ERK pathway in human primary HCC samples and human HCC cell lines, including HepG2 cell lines, among others. Fan et al. (25) demonstrated that luteoloside defeats the proliferation and metastasis of HCC cells by inhibiting the NLRP3 inflammasome via decreasing the intracellular ROS (reactive oxygen species) in human neoplastic cell lines of the liver (Hep3B and SNU-449) and in an HCC animal model. Wan et al. (26) showed that miR-223-3p quashes the NLRP3 inflammasome. In this study, there is an induction of apoptosis. Finally, there is the inhibition of the proliferation of HCC cells in the Hep 3B2.1–7 cell line (26). ANI (Anisodamine or ANI) is an alkaloid extracted from Anisodus) has been suggested to promote apoptosis by suppressing the NLRP3 inflammasome in the HepG2 hepatoma cell line and HCC rodent model (27). Finally, the NLRP3 shortage in HCC augments the cytotoxicity of NK cells to HCC via the interaction of NKG2D-MIC-A. It eventually promotes the immunosurveillance of NK cells in the NK cell line NK-92, in human HCC cells, and a HCC rodent animal model (28). SHD stimulates the NLRP3 inflammasome through the promotion of the release of ROS and the suppression of the NF-κB pathway. It inhibits the growth and migration of neoplastic liver cells *in vitro* and *in vivo* (Hep3B and HepG2, among others) and in an HCC model of mice (29).

NLRP3 inflammasome and natural compounds

Different therapeutic strategies targeting events upstream of NLRP3 inflammasome cascade activation or downstream have been evaluated for managing patients with COVID-19 (30). These treatments may be repositioned in the context of managing MASLD. Chemarin et al. enumerate colchicine, emricasan, and dapansutrile as inhibitors of NLRP3 inflammasome activation and canakinumab, anakinra, disulfiram, and dimethyl fumarate as inhibitors of the NLRP inflammasome-promoted inflammatory cascade. However, numerous natural products and chemicals can potentially target NLRP3 inflammasome cascade activation and subsequent inflammatory compounds. Some other NLRP3 inflammasome inhibitors using natural compounds are conceivably more important to highlight here. They include isoandrographolide, which targets NOD-like receptors (NLRs) and has cell differentiation-inducing and hepatoprotective properties. This molecule inhibits activation of the NLRP3 inflammasome and suppresses pneumoconiosis (silicosis) in mice. Marveloside A is one of the major biologically active components of mulberry (*Morus alba L.*). It targets TNF-α receptors and tyrosinase. Marveloside A decreases the expression of IL-1β, IL-6, and TNF-α. It has been identified that this molecule inhibits the activation of NLRP3, caspase-1, and NF-κB and the phosphorylation of ERK, JNK, and p38 and shows anti-(necro) inflammatory and anti-apoptotic effects. Muscone is probably the most important active monomer in traditional Chinese medicine. Muscon inhibits NLRP3 inflammasome activation and the upgrade of NF-κB. Muscone targets IL-6 receptors, NF-κB, NOD-like receptors (NLRs), and TNF-α receptors. Muscon markedly decreases inflammatory cytokines (IL-1β, IL-6, and TNF-α). It ultimately improves cardiac function and survival. Licochalcone B targets amyloid-β, apoptosis, and NOD-like receptors (NLRs). Licochalcone B is a compound extracted from the root of the plant *Lycopersicon esculentum*. Licochalcone B prevents amyloid-β self-aggregation, disassembles preformed Aβ42 fibers, and diminishes metal-induced Aβ42 aggregation by chelating metal ions. In addition, Licochalcone B inhibits phosphorylation of NF-κB p65 in the LPS pathway. Licochalcone B inhibits the proliferation of pulmonary carcinoma cells and induces their apoptosis. Licochalcone B inhibits the NLRP3 inflammasome by blocking the NEK7-NLRP3 interaction. Ruscogenin is an important steroidal sapogenin from *Ophiopogon japonicus* that targets NOD-like receptors. By inhibiting TXNIP/NLRP3 inflammasome activation and the MAPK pathway, Ruscogenin suppresses blood–brain barrier dysfunction caused by cerebral

ischemia and exhibits marked anti-inflammatory and anti-thrombotic activity. Arglabin targets NOD-like receptors (NLRs), farnesyltransferases, and autophagy. Arglabin is also a natural product. This molecule arises from *Artemisia glabella* and is an NLRP3 inflammasome inhibitor. Arglabin exhibits anti-inflammatory and antitumor activity. The anti-neoplastic activity of algravine ensues via inhibition of farnesyltransferase, leading to activation of the RAS protogene. 4′-Methoxy resveratrol is a polyphenol. It derives from the *Dipterocarpaceae* family and has remarkable antifungal, antiandrogenic, and anti-inflammatory properties (10). 4′-Methoxy resveratrol alleviates AGE-promoted inflammation by inhibiting the RAGE-mediated MAPK/NF-κB signaling pathway and NLRP3 inflammasome activation. Soya saponin II is a saponin with substantial antiviral activity. Soya saponin II inhibits the replication of several viruses. They include herpes simplex virus 1, cytomegalovirus, influenza virus, and human immunodeficiency virus 1. Soya saponin II inhibits YB-1 phosphorylation and NLRP3 inflammasome priming. It may protect rodents from LPS/GalN-induced acute or fulminant liver failure. Picroside II is an iridoid molecular compound extracted from *Picrorhiza*. It exhibits anti-inflammatory and anti-apoptotic cellular activity. Picroside II assuages the inflammatory response in sepsis. It improves immune function by inhibiting the NLRP3 inflammasome and NF-κB pathway activation. Picroside II is an antioxidant molecular compound. It shows marked neuroprotective effects. Such effects are determined by lowering the assembly of ROS. It may be crucial in the emergency department and neurology because it protects the blood–brain barrier after a cerebral ischemia–reperfusion injury. Picroside II has pharmacological activities such as antioxidant, anti-inflammatory, and antiviral effects, in addition to immunomodulatory activities. It targets NF-κB, ROS, apoptosis, and the influenza virus.

Conclusion

Research studies on aspartame consequences have one major factor that may affect the true impact of the investigations, i.e., dose and duration. It is extremely important to emphasize that studies in a vacuum, i.e., excluding other potential confounders, are difficult to realize, at least in humans. However, the evidence from animal studies may be comparable to that identified for other chemical compounds examined at the IARC in previous monographs.

Extensive consumption of soft drinks is prominent in determining an increase in obesity and cardio-metabolic risk factors in children, adolescents, and adults. Artificially sweetened soft drinks have been modeled as a healthier alternative to other carbonated and non-carbonated soft drinks. The evidence is that there is no protection against non-alcoholic fatty liver disease (NAFLD) using edulcorated soft drinks. We are extremely concerned about the use of sweeteners, including aspartame. It seems that aspartame, saccharine, acesulfame-K, sucralose, and neotame do not determine birth defects, but the evidence of behavioral or neurological effects in children, such as attention deficit and hyperactivity disorders, seems to be controversial. We truly hope that further research with randomized clinical trials may be set up to establish or finally exclude a potential causal relationship. In the meantime, we may suggest that diet drinks, sugar-free chewing gums, gelatins, toothpaste, and medications such as cough drops should not be part of the diet of any child, and parents should pay attention to the composition and ingredients of food given to children. In the time being, dose and duration may affect the onset of pediatric diseases. Thus, in terms of safety, aspartame should probably be banned completely, at least in pediatrics.

In addition to fatty changes in the liver and inflammation, there is a substantial risk of developing cancer. Since well-designed studies that address specific, practical, psychological, and public health issues are substantially lacking, further research is incredibly necessary. On the other hand, there is enough current theoretical conjecture to support the use of molecular compounds targeting the NLRP3 inflammasome for MASLD individuals, and randomized clinical trials are urgently warranted.

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 author.

Author contributions

CS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author declares 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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