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Unraveling the links between nutrients and metabolic dysfunction-associated liver disease: insights and implications

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Unraveling the links between nutrients and metabolic dysfunction-associated liver disease: insights and implications

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Editorial: Unraveling the links between nutrients and metabolic dysfunction-associated liver disease: insights and implications

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Editorial on the Research Topic
Unraveling the links between nutrients and metabolic dysfunction-associated liver disease: insights and implications

Metabolic dysfunction-associated steatotic liver disease (MASLD) and metabolic dysfunction-associated steatohepatitis (MASH) are the most prevalent liver diseases worldwide. Fortunately, in recent years, our understanding of the physiopathology of both diseases has expanded significantly, particularly regarding the roles of diet, lifestyle, and metabolic regulation. This Research Topic of studies provides critical insights into these aspects, presenting multifaceted approaches encompassing dietary and lifestyle influences, metabolic connections, therapeutic agents, and diagnostic advances. These studies underscore the potential of integrated strategies in preventing and managing liver disease.

A key theme within this collection is the impact of dietary intake and specific nutrients on liver health. Wang et al. conducted a meta-analysis on nearly half a million participants, revealing a protective effect of fruit and vegetable consumption against MASLD. They showed that higher intake of fruits and vegetables correlated with a reduced risk of MASLD, underscoring the importance of antioxidant-rich foods for liver health. The strength of this association varied by demographic factors, highlighting the necessity for population-specific dietary guidelines. The antioxidant theme continues in studies examining specific dietary components. Complementing these findings, Majeed et al. performed a clinical trial to evaluate the effects of a supplement containing garcinol, curcuminoids, and piperine in patients with mild to moderate MASH. This randomized, double-anonymized, placebo-controlled study suggests that this herbal combination may offer therapeutic benefits by targeting inflammation and oxidative stress. This supplementation approach could be a beneficial adjunct to conventional treatments, especially for patients in the early stages of liver disease. Using a different approach, Ravaut et al. examined the impact of the ketogenic diet on reversing steatosis. This study explains how low-carbohydrate, high-fat diets might influence liver status in diet-induced MALSD mice. Initial analysis in male

mice indicates that the ketogenic diet could support the reduction of hepatic steatosis, suggesting that carbohydrate restriction might help address metabolic liver disease. While promising, further research is needed to understand better the ketogenic diet's effects over longer durations and its potential for translation to human applications.

In a systematic review, Zhang X. et al. also investigated the Dietary Inflammatory Index (DII) and its association with MASLD. Analyzing data from two million participants, they found that higher DII scores, indicative of pro-inflammatory diets, are linked to a significantly elevated risk of MASLD. These findings highlight the importance of anti-inflammatory diets, emphasizing the role of dietary choices in reducing inflammation and, subsequently, MASLD risk. Complementing these findings, another study evaluated the oxidative balance score as a predictive measure for MASLD. This research emphasizes that dietary and lifestyle modifications aimed at improving oxidative balance may serve as valuable preventative measures for MASLD, further reinforcing the role of antioxidant-rich foods in liver health.

In studies addressing dietary biochemistry, Cai et al. explored the association between low serum folate levels and advanced liver disease stages in MASLD. They reveal that reduced levels of folate and 5-MTHF correlate with increased liver steatosis and fibrosis, pointing to the potential for folate supplementation as a modifiable factor in MASLD management. In addition, Xiang et al. investigated the relationship between blood chromium levels and hepatic steatosis, assessed via liver ultrasound transient elastography, using data from the National Health and Nutrition Examination Survey (2017-2020). The authors found a significant inverse association between blood chromium concentrations and the presence of hepatic steatosis, suggesting that higher chromium levels may be linked to a reduced risk of fatty liver. These findings support the potential of chromium as a modifiable factor in managing liver health, though further studies are needed to confirm causality.

In the area of therapeutic interventions to combat MASLD progression, Zhou et al. presented innovative research on peach gum polysaccharides (PGPs), investigating their antioxidant and hepatoprotective properties in models of alcohol-induced liver injury. Their findings suggest that PGPs can alleviate oxidative stress and inflammation while modulating amino acid metabolism, positioning PGPs as promising, plant-based candidates for treating liver disease with minimal toxicity. Further complementing these therapeutic insights is Ruan et al.'s review on metformin, a well-known diabetes medication that may also have promising applications for MASLD and hepatocellular carcinoma (HCC). The review highlights metformin's beneficial effects on lipid and glucose metabolism, AMPK activation, and gut microbiota modulation. Its anti-inflammatory properties suggest metformin could play a multifaceted role in managing liver disease, particularly in fibrosis prevention and HCC treatment. This review proposes that metformin, traditionally used in diabetes management, may have a broader impact on chronic liver disease due to its regulatory effects on metabolic and inflammatory pathways.

On the topic of lifestyle-based therapeutic strategies, Mambrini et al. advocated for a multidisciplinary approach in treating MASLD, focusing on personalized dietary and exercise interventions. Their review emphasizes the importance of improving insulin sensitivity and metabolic flexibility through customized lifestyle changes, highlighting the need for collaboration among dietitians, exercise specialists, and physicians. This comprehensive approach underscores the potential for tailored lifestyle modifications to impact patient outcomes and positively reduce disease progression in MASLD. Charlot et al. contributed further to our understanding of dietary approaches with their comparative study on hypercaloric low-carbohydrate, high-fat diets (LCHFD) vs. Western diets in mice. Their findings suggest that LCHFD may be protective against MASLD, despite equal caloric intake, indicating that carbohydrate reduction, rather than simple calorie restriction, could be a key factor in managing obesity-linked liver disease. This study encourages a re-evaluation of dietary guidelines, advocating for low-carbohydrate dietary patterns as an effective strategy for liver health.

Advances in diagnostic tools and nutritional assessment are also explored in this collection. He et al. examined malnutrition in patients with liver cirrhosis, comparing different assessment tools to address nutritional deficits more effectively. Their study suggests that the RFH-NPT screening tool is the most practical for malnutrition screening. At the same time, the GLIM criteria prove to be highly accurate for diagnosing malnutrition in cirrhotic patients. By highlighting the importance of early detection and intervention, this research underscores the need for improved nutritional assessment to enhance patient outcomes in advanced liver disease. Chen et al.'s mini-review synthesizes current knowledge on MASLD diagnosis and lifestyle interventions. They discuss the diagnostic value of combining serologic and imaging markers and emphasize the preventive role of diet and physical activity in MASLD management. This review calls for further research into MASLD pathogenesis and underscores the importance of individualized approaches, echoing the necessity of integrating diagnostic precision with personalized lifestyle interventions for optimal liver disease management.

Finally, in a systematic review, Zhang D. et al. explored the bidirectional relationship between *Helicobacter pylori* (*H. pylori*) infection and MASLD, underscoring their complex interplay with metabolic syndrome. Through a meta-analysis of 34 studies, the authors reveal that *H. pylori* infection increases the risk of MASLD and vice versa, establishing that these two conditions exacerbate each other. By linking *H. pylori* to metabolic factors that underlie MASLD and metabolic syndrome, this study opens new avenues for understanding the pathogenic overlap and invites further investigation into mechanisms by which bacterial infection might influence liver health.

In summary, this Research Topic brings together pioneering studies that reinforce the critical role of dietary, lifestyle, and therapeutic interventions in managing MASLD and related liver conditions. From antioxidant-rich foods and dietary patterns to personalized treatment approaches, these

articles present a robust framework for understanding the multifaceted pathways through which liver health can be supported. By integrating evidence from nutrition, pharmacology, and lifestyle medicine, this Research Topic advances a holistic perspective, inviting healthcare professionals and researchers to consider comprehensive strategies that encompass prevention and treatment in the fight against liver disease.

Author contributions

BR-M: Writing – original draft. CT-P: Writing – original draft. IT-V: Writing – original draft. MK: Writing – original draft, Writing – review & editing.

Conflict of interest

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Metformin: update on mechanisms of action on liver diseases

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Substantial attention has been paid to the various effects of metformin on liver diseases; the liver is the targeted organ where metformin exerts its antihyperglycemic properties. In non-alcoholic fatty liver disease (NAFLD), studies have shown that metformin affects the ATP/AMP ratio to activate AMPK, subsequently governing lipid metabolism. The latest research showed that low-dose metformin targets the lysosomal AMPK pathway to decrease hepatic triglyceride levels through the PEN2-ATP6AP1 axis in an AMP-independent manner. Metformin regulates caspase-3, eukaryotic initiation factor-2a (eIF2a), and insulin receptor substrate-1 (IRS-1) in palmitate-exposed HepG2 cells, alleviating endoplasmic reticulum (ER) stress. Recent observations highlighted the critical association with intestinal flora, as confirmed by the finding that metformin decreased the relative abundance of *Bacteroides fragilis* while increasing *Akkermansia muciniphila* and *Bifidobacterium bifidum*. The suppression of intestinal farnesoid X receptor (FXR) and the elevation of short-chain fatty acids resulted in the upregulation of tight junction protein and the alleviation of hepatic inflammation induced by lipopolysaccharide (LPS). Additionally, metformin delayed the progression of cirrhosis by regulating the activation and proliferation of hepatic stellate cells (HSCs) via the TGF-β1/Smad3 and succinate-GPR91 pathways. In hepatocellular carcinoma (HCC), metformin impeded the cell cycle and enhanced the curative effect of antitumor medications. Moreover, metformin protects against chemical-induced and drug-induced liver injury (DILI) against hepatotoxic drugs. These findings suggest that metformin may have pharmacological efficacy against liver diseases.

KEYWORDS

non-alcoholic liver disease, metformin, cirrhosis, AMPK pathway, drug-induced liver injury

1 Introduction

Metformin is a classical oral hypoglycemic agent derived from a leguminous caudex named *Galega officinalis* (1). Its antihyperglycemic properties were first discovered in 1918, and in 1957, Jean Sterne successfully used it to treat diabetes mellitus (2). Given its substantial safety and efficacy, metformin is indicated as a first-line medication for type 2 diabetes treatment (3).

The liver is the primary target organ of metformin, which inhibits hepatic glucose output through the AMPK signaling pathway in the following ways. (1) Metformin upregulates the small hetero-dimer partner through the AMPK signaling pathway, which interacts directly with the cAMP-response-element-binding factor (CREB) to block the recruitment of CREB to

CREB-regulated transcription co-activator 2 (CRTC2), downregulating the expression of gluconeogenic genes (4); (2) Through the AMPK signaling pathway, metformin upregulates the expression of the hepatic deacetylase sirtuin 1 (SIRT1), which deacetylates CRTC2 and promotes its ubiquitinated degradation, downregulating the expression of gluconeogenic genes (5). The lysosomal v-ATPase-regulator complex is a common activator for AMPK and mTORC1, acting as a switch between catabolism and anabolism (6). However, it is yet unknown how metformin activates AMPK (Figure 1).

Later, it was found that metformin activates AMPK through the lysosomal pathway and disrupts metabolic processes such as ATP synthesis through oxidative phosphorylation. Mechanistically, it acts on vacuolar H + -ATPase (v-ATPase) and promotes the translocation of AXIN/LKB1 onto the surface of lysosome to form complex with v-ATPase-regulator and then dissociates raptor and mTOR, leading to AMPK activation and turning off the activity of mTORC1, a master regulator for anabolic pathways (7).

Ma et al. synthesized a photoactive metformin probe to identify potential direct targets. By promoting intestinal GLP-1 secretion through the lysosomal AMPK pathway, low-dose metformin lowers blood glucose via PEN2 in a AMP-independent manner. However, even though metformin inhibits hepatic gluconeogenesis, researchers found that low-dose metformin did not behave in such a way, based on pyruvate tolerance tests and the quantification of gluconeogenic genes. Furthermore, metformin exerted two supplementary beneficial effects through the AMPK-determined PEN2-ATP6AP1 axis, specifically diminishing hepatic lipid accumulation in mice liver and prolonging the lifespan of *Caenorhabditis elegans*, in addition to this postprandial glucose reduction (8).

In recent years, several lines of evidence revealed various biological effects of metformin in addition to improving glucometabolic status, including anti-inflammation, antioxidation, antitumor, and anti-fibrosis; metformin holds considerable potential to prevent or treat non-alcoholic fatty liver disease (9), cirrhosis (10), hepatic malignancies (11), drug-induced or chemical-induced liver

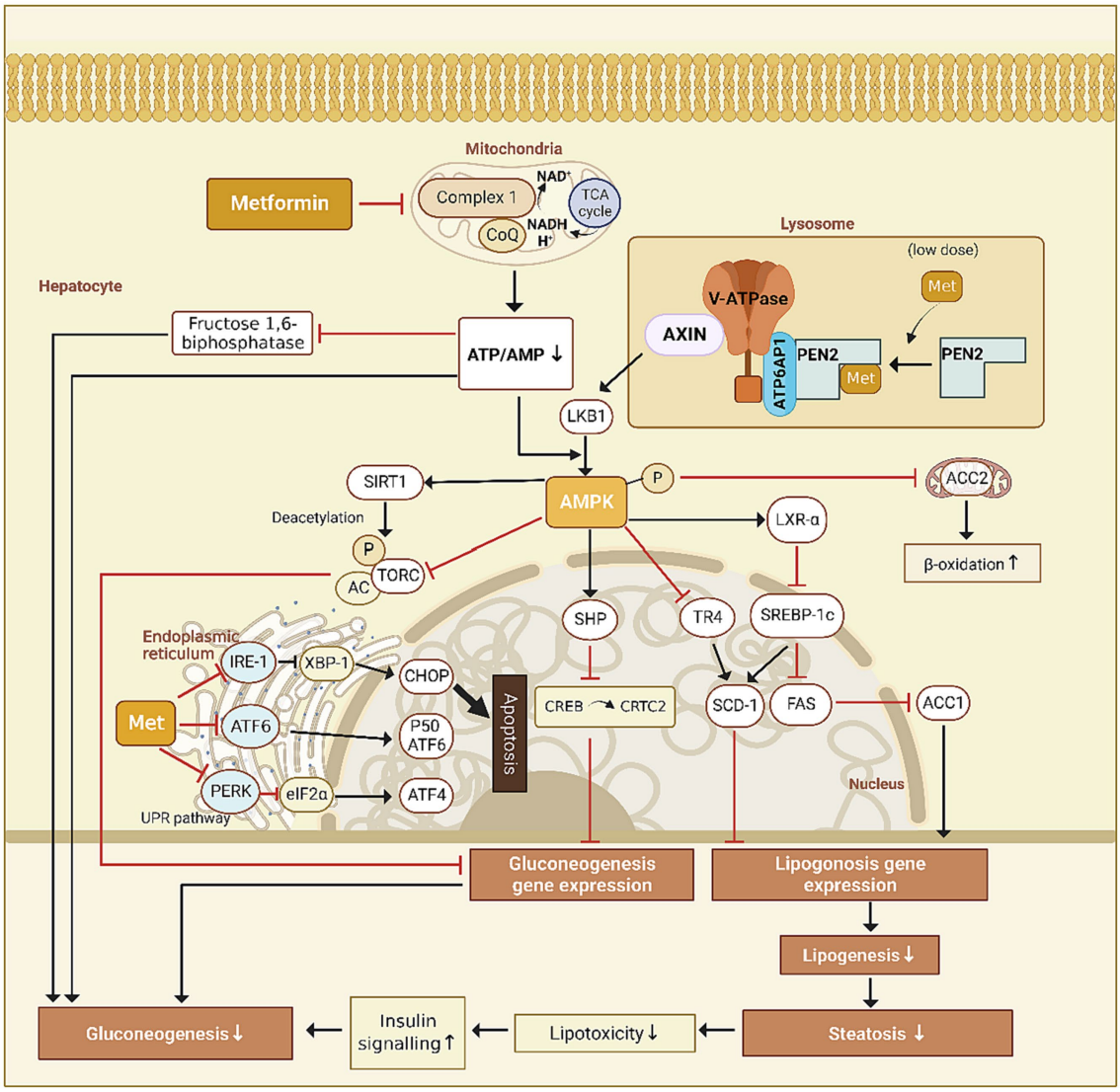

FIGURE 1
Proposed mechanisms of action for metformin.

injury (12), and other liver diseases. This article reviews its role and underlying mechanisms in liver disease treatment.

2 NAFLD

NAFLD is a clinical-pathological syndrome not directly linked to alcohol or other etiologies. It is characterized by excessive lipid accumulation in hepatocytes, one of the most common types of chronic liver disease worldwide (13, 14). NAFLD includes the benign NAFLD and the more severe non-alcoholic steatohepatitis (NASH), which carries a higher risk of progression to severe liver disease (15, 16). Lipotoxicity theory explains the progression of NAFLD (17). According to the theory, large amounts of free fatty acids (FFAs) ectopically accumulate in the liver, which causes impaired hepatocytes' ability to output FFAs into triglycerides (TGs) and subsequent toxic effects. This phenomenon only increases insulin resistance (18) and also promotes disease progression (17). Metformin can relieve NAFLD by reducing lipotoxicity in the following ways.

Recently, a consensus has been reached to replace the term NAFLD with metabolic dysfunction–associated steatotic liver disease (MASLD) following a modified Delphi process (19). MASLD is defined as the presence of hepatic steatosis and at least one of five cardiometabolic risk factors. This change in terminology aims to increase disease awareness, reduce stigma, and accelerate the development of drugs and biomarkers for the benefit of patients. However, the proposed nomenclature concept has limitations in classifying subtypes of NAFLD due to insufficient data. It is important to highlight that metabolic syndrome and its components are strongly associated with NAFLD/MASLD (20). Therefore, metformin, a drug targeting various factors related to metabolic syndrome, such as fasting plasma glucose levels and waist circumference, may also alleviate NAFLD in the following ways.

2.1 Metformin regulates lipids metabolism by activation of AMPK

Several studies dealt with the downstream signaling pathway of AMPK. Nevertheless, none identified a specific target for AMPK activation by metformin before Ma et al. found that lysosomal PEN2 is recruited to ATP6AP1 subunit upon binding to metformin to regulate v-ATPase, which provides the impetus for the translocation of AXIN and liver kinase B1 (LKB1) to the lysosomal surface to activate AMPK, enabling low concentrations of metformin to utilize the AMP-independent AMPK activation pathway (8).

Metformin activates LKB1 to induce AMPK phosphorylation and inhibits acetyl-CoA carboxylase (ACC), which has several complex effects. Fullerton et al. demonstrated that metformin treatment alters hepatic lipid homeostasis and increases insulin sensitivity by suppressing ACC (21). 103 In fasting, Metformin increases AMPK activity, which increases the activity of LXR-α. LXR-α then suppresses sterol regulatory element binding protein-1c (SREBP-1c, a master regulator of liver lipogenesis gene programme), which in turn suppresses enzymes of lipogenesis [ACC1, fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1)], thereby inhibiting lipogenesis (22). AMPK can also directly inhibit ACC2, decrease malonyl Co-A, and activate carnitine palmitoyl transferase 1 (CPT1), and thereby enhancing β-oxidation. In addition, ACC1 (cytosolic) is highly expressed in lipogenic tissues, such as liver and adipose tissue, while ACC2 (mitochondrial membrane) is majorly expressed in oxidative tissues, such as cardiac and skeletal muscle, which are consistent with the functions of ACC1 in lipogenesis and ACC2 in regulating fatty acid β-oxidation (23).

Activated by metformin, AMPK phosphorylates nuclear receptor TR4 at Ser351 to inhibit transcriptional activation of TR4, repressing the expression of TR4-mediated gene for stearyl coenzyme A dehydrogenase-1 (24). It also phosphorylates SREBP-1c at Ser372, which inhibits proteolytic maturation, nuclear translocation of SREBP-1c and the expression of downstream fatty acid synthase gene, thus increasing fatty acid β-oxidation and reducing *de novo* synthesis of TG (25) to prevent hepatocyte steatosis by reducing the accumulation of TG in HepG2 cells (26).

Metformin-induced NAFLD remission could be attributed to the cooperative roles of Kuffer cells (KCs) and hepatocytes, which are mediated by the presence of tristetraprolin (TTP), an mRNA-binding protein. Metformin activates TTP in hepatocytes and KCs through the AMPK/Sirt1 pathway. TTP inhibited TNF-α production in KCs, resulting in a subsequent reduction in hepatocyte necroptosis. Metformin-induced TTP activation inhibited mTORC1 via destabilization of Rheb, which promotes transcription factor-EB nuclear translocation to promote hepatocyte lipophagy, treating obesity-related NAFLD (27).

2.2 Metformin alleviates endoplasmic reticulum (ER) stress

ER stress refers to a homeostatic imbalance within the ER caused by external stimulus or changes in the intracellular environment, which further leads to the accumulation of unfolded or misfolded proteins in the ER. It can cause cytotoxic effects and unfolded protein response (UPR) (28). Although proper UPR acts as a protective agent against misfolding, enhancing the ER folding capacity, and degrades misfolded proteins, excessive or persistent UPR can trigger cellular insults, such as the execution of apoptosis, which accelerates NAFLD progression (29). Cell culture and animal studies found that metformin may act to reduce ER stress. Indeed, metformin attenuated palmitate-induced UPR events in RINm5F rat insulinoma cells (30) and HepG2 cells (31), and alleviated hepatic ER stress of Male albino Wistar rats induced by a high-calorie diet (29). The UPR is initiated through three sensors: the protein kinase RNA-like ER kinase (PERK), the inositol requiring enzyme-1 (IRE-1), and the activating transcription factor-6 (ATF-6). Accumulated unfolded proteins bind to immunoglobulin-binding proteins, activating PERK, IRE-1, as well as transcription factor-6 and phosphorylating eIF2a. In response to activation of IRE-1, active ATF6 translocates to the Golgi apparatus, enters the nucleus, and triggers apoptosis through C/EBP homologous protein (CHOP) (29). As reported by Kim et al., metformin significantly suppresses caspase-3 and eIF2a phosphorylation, blocking the induction of the ER stress markers in palmitate-exposed HepG2 cells. The expression of spliced XBP-1 mRNA and cleaved ATF6 was at low levels, suggesting that metformin regulates ATF6 processing, inhibits selected IRE-1 activities, and regulates the expression of pro-apoptotic CHOP (31). Metformin also reversed the palmitate-induced serine phosphorylation of IRS-1, thus attenuating

ER stress to inhibit hepatocellular apoptosis resulting from lipotoxicity (30).However, it should be noted that a study by Geng et al. showed that while metformin inhibited palmitate-induced eIF2α phosphorylation in HepG2 cells, it did not downregulate the expression of CHOP, Gadd34, or GRP78, which are the downstream target genes of p-eIF2α (32). This finding suggests that the dephosphorylation of *p*-eIF2α does not lead to deactivation of the downstream UPR signaling pathway, meaning that metformin is not directly related to lower ER stress. The precise process is debated.

2.3 Metformin restores mitochondrial function

Lipotoxicity can potentially harm the mitochondrial respiratory chain by accelerating the tricarboxylic acid cycle flux in NAFLD (33, 34). Geng et al. discovered that metformin inhibits the mitochondrial respiratory chain complex to prevent the electron transport from NADH to coenzyme Q, which somewhat lowers mitochondrial basal and maximal respiration and mildly limits ATP synthesis. In response to metformin treatment, primary rat hepatocytes exposed to palmitate experienced a recovery of mitochondrial respiration. Further study showed that palmitate decreased mitochondrial membrane potential and increased cell reactive oxygen species (ROS) by detecting mitochondrial membrane potential. At the same time, metformin restored the membrane potential, reducing mitochondrial proton leak and ROS generation by inducing the expression of superoxide dismutase 2. Thus, the protective effects of metformin on palmitate-induced cell death could result from partially inhibiting mitochondrial complex I, which would restore mitochondrial function and demonstrate the necessity of mitochondrial complex I in retarding or stopping the progression of NAFLD (32). Before this study, the concentrations of metformin used in reports investigating the mechanism of action of metformin varied widely, often at much higher than the blood concentration of the therapeutic dose of metformin. Consequently, research on the mechanism of action of metformin has produced contradictory findings. Emphasis should be given to the importance of proper inhibition of mitochondrial complex I since excessive or insufficient metformin cannot have its intended therapeutic effect. Studies will be needed to determine the appropriate metformin dose for use in NAFLD therapy.

2.4 Metformin downregulates non-coding RNA associated with NAFLD

In recent years, the critical role of non-coding RNA such as microRNA, lncRNA, and circRNA has become recognized in NAFLD (35–37). Dysregulation of non-coding RNA disrupts the gene regulatory network, leading to metabolic syndrome and related diseases. MicroRNAs are small non-coding RNAs involved in post-transcriptional gene expression regulation by binding to the 3′-untranslated region of target mRNAs and inhibiting their expression (38). A broad spectrum of NAFLD has aberrantly enhanced miR-34a expression in humans and mice (39). By coordinating the regulation of lipid metabolism, mRNAs regulate NAFLD development and progression. Silencing miR-34a led to an initially increased expression of hepatic peroxisome proliferation-activated receptor-α (PPARα), SIRT1, then PPARα and SIRT1 activated the AMPK pathway. PPARα and pAMPKα 1 increased fat oxidation and improve the steatosis finally (40). *In vitro* experiments conducted by Xu et al.showed that the ablation of hepatocyte miR-34a resulted in the inhibition of intestinal lipid absorption and hepatic TG synthesis, as well as a decrease in inflammation, ROS production, apoptosis, and an induction of hepatic fatty acid oxidation. Furthermore, the *in vivo* experiments showed that miR-34a inhibitors reduced hepatic levels of TG and FFAs. These findings were further supported by a reduction in hepatic expression of genes associated with bile acid synthesis (CYP7A1 and CYP8B1) and fatty acid synthesis (SREBP-1c, ACC1, and FAS), while genes involved in fatty acid oxidation (PPARα, CPT1, CPT2, and PDK4) were upregulated, which may account for the mechanism by which inhibition of miR-34a expression protects against the development of steatohepatitis (41). Others reported that miR-34a was upregulated in MCD-fed mice, and following treatment with metformin, this effect attenuated miRNA expression (42). However, no further studies were conducted. Thus, the study mentioned above may provide insight into the potential mechanism by which metformin halts the advancement of NAFLD.

2.5 Metformin reshapes the gut microbiota and intestinal barrier function

The occurrence and development of NAFLD are associated with impaired intestinal flora metabolism and intestinal permeability. Several lines of evidence suggest that metformin improves intestinal microflora composition and intestinal barrier dysfunction (43–45), suggesting that this pathway might be essential for metformin against NALFD. The FXR, a bile acid-activated nuclear receptor prominently expressed in the liver and intestine, regulates the expression of genes involved in cholesterol and bile acid homeostasis, hepatic lipogenesis, and inflammation, in addition to maintaining the intestinal barrier integrity, preventing bacterial translocation and preserving the gut microbiota eubiosis. Based on previous studies, the imbalance of FXR is one of the critical mechanisms in the development of NAFLD (46). Metformin treatment decreased the abundance of species of *Bacteroides fragilis* and its bile salt hydrolase activity in the intestines of individuals with T2D, increasing the levels of the bile acid glycoursodeoxycholic acid to inhibit intestinal FXR signaling (47). Another study showed that metformin is associated with higher relative abundance of mucin-degrading *Akkermansia muciniphila* and *Bifidobacterium bifidum* (43, 48, 49) Both strains protected against NAFLD by activating hepatic FXR, suppressing intestinal FXR expression, modulating the gut microbiota, and improving intestinal mucosal permeability (50). The administration of *A. muciniphila* restored metabolic disturbances in diet-induced obese mice. These imbalances include heightened adiposity and metabolic endotoxemia. Damage to the intestinal barrier is an early core event and an essential mechanism for NAFLD development. Li et al. (51) discovered that *A. muciniphila* given by daily oral gavage in high-fat diet-fed mice partially restored the thickness of the mucin layer and upregulated epithelial tight junction proteins, specifically zona occludens 1 and occludin. These alterations ultimately lead to a reduction in the influx of pro-inflammatory LPS into the systemic circulation, resulting in a subsequent decrease in the expression of hepatic inflammatory

markers. Furthermore, *A. muciniphila* raised the level of gut endocannabinoids, pivotal in controlling inflammation, maintaining intestinal barrier integrity, and encouraging the release of gut peptides from intestinal L-cells. Experimental evidence from *in vivo* and *in vitro* studies substantiated the endocannabinoid system's role in regulating the gut-intestinal barrier via a mechanism dependent on the CB1 receptor (52). A cross-sectional metagenomic study conducted across three countries revealed a positive correlation between the utilization of metformin and the presence of short-chain fatty acid-producing bacteria, including *Bifidobacterium bifidum*, *Butyrivibrio*, *Megasphaera*, and an operational taxonomic unit of *Prevotella* (48, 53). Acetate promoted by carbohydrate transporter in bifidobacterial mediates anti-apoptotic and anti-inflammatory responses in the host colonic epithelium, protecting the host against lethal infection (54). Cani et al. (55) demonstrated that manipulating gut microbiota in mice favoring the *Bifidobacterium* spp. significantly improves gut permeability and obesity-induced hepatic inflammatory phenotype in a glucagon-like peptide-2-dependent way. These findings suggest that microbes at least partially mediate the positive effects of metformin on host metabolism. The findings further illustrate the diversified modes of metformin in NAFLD treatment with direct effects on the liver and indirect actions via extrahepatic pathways.

3 Hepatic fibrosis and cirrhosis

Hepatic cirrhosis is a diffuse liver injury characterized by degeneration and necrosis of hepatocytes, replacement of normal liver tissue by fibrotic tissues and nodules, and progressive loss of liver function (56). Hepatic fibrosis is an essential pathological process in which liver lesions develop into cirrhosis (57), characterized by excessive accumulation of connective tissue and abnormal formation of fibrous septum, the primary component of which is type I collagen produced by activated HSCs (58). HSC activation is the classical mechanism for promoting liver fibrosis (59). In response to injury and inflammatory factors, HSCs transform into highly proliferative and migratory myofibroblasts, upregulate the expression of α-SMA, a sign of HSC activation, and produce a large amount of collagen and other extracellular matrix (ECM), leading to fibrosis (60, 61). Metformin improves outcomes by treating NAFLD/NASH to delay the progression to cirrhosis and inhibits hepatic fibrosis (62). Mounting evidence has indicated that it can control the activation and proliferation of HSC through various signal pathways.

3.1 Metformin activates AMPK to inhibit the platelet-derived growth factor (PDGF) pathway

The PDGF signaling pathway is one of the most characteristic pathways in HSC activation. PDGF-BB, a subtype of the PDGF family, is induced by the most potent stimulator of HSC growth and intracellular signal transduction. PDGF induces the activation of extracellular signal-regulated kinase and Akt/mTOR pathways, which are serine/threonine protein kinases that are critical in cell growth, differentiation, proliferation, migration, and survival. Metformin activated AMPK to regulate PDGF-BB-induced phenotypic changes in HSC activation (63, 64). Adachi et al. demonstrated that metformin inhibited PDGF-induced phosphorylation of AKT, FoxO1 and mTOR/p70S6K (two downstream targets of the phosphatidylinositol 3-kinase/AKT pathway), resulting in the inhibition of HSC proliferation and migration, the reduction of extracellular matrix secretion consisting of α-SMA, type I collagen and fibronectin, which leads to inhibition of fibrosis (64, 65).

3.2 Metformin inhibits the TGF-β1/Smad3 pathway

Another mechanism of HSC activation is the cooperation of TGF-β1-related signaling pathways and other signals, including ROS, PDGF, and connective tissue growth factors (66). Smad3 is the primary downstream target. In human and rat HSC lines, activated AMPK Inhibited TGF-β-Induced fibrotic responses of HSCs by regulating transcription co-activator P300 without altering TGF-β-induced Smad3 phosphorylation, nuclear localization, and Smad3 promoter binding activity. This regulation manifested as increasing proteasomal degradation to induce p300 downregulation but not attenuating p300 expression (67). Fan. et al. studied CCl4-exposed mice and found that metformin reduced the expression of TGF-β1 and inhibited the TGF-β1/Smad3 pathway, thereby inhibiting the expression of fibroblast genes and fibroblast markers such as α-SMA and E-cadherin directly mediated by Smad3 (68–70). Xiao et al. (71) established a model of myocardial fibrosis. They found that metformin can directly bind to TGF-β1 through an AMPK-independent pathway, reducing its binding probability to TGF-β1 type II receptor rather than binding force and leading to the reduction of downstream signal transduction, which might explain how metformin inhibits TGF-β1 signaling.

3.3 Metformin inhibits the succinate-GPR91 pathway

Succinate is an intermediate product of the tricarboxylic acid cycle, which can be converted into fumaric acid by succinate dehydrogenase. In addition to its critical role in energy metabolism, it acts as an extracellular signaling molecule that binds to and activates its specific G-protein-coupled receptor (GPR91), a receptor in HSCs whose role in hepatic fibrosis remains unclear. In an *in vitro* and *in vivo* study, Li. et al. found that HSCs cultured and treated directly with succinate or succinate dehydrogenase inhibitors increased the expression of GPR91 and the expression of α-SMA, TGF-β, and type I collagen, which are markers of the fibrotic response. These findings suggested that succinate accumulation and GPR91 overexpression are pathological features of hepatic fibrosis (72). Nguyen et al. (10) found that in mice fed with a methionine-choline-deficient diet, the expression of α-SMA and GPR91 is enhanced, while metformin administration reduced overexpression in the liver, ameliorating steatohepatitis and liver fibrosis in mice. In addition, metformin significantly reduced the enhanced HSC activation, proliferation, and migration processes induced by succinate and inhibited the expression of inflammatory cytokines (e.g., IL-6 and TGF-β1). These findings suggest that metformin acts as an antifibrotic agent by inhibiting the succinate-GPR91 pathway.

4 HCC

Hepatoma is a common malignant digestive system tumor with significant morbidity and mortality. HCC, which makes up 70–90% of the pathological types of primary liver cancer, is now the second most common cause of cancer death worldwide. Epidemiological evidence suggests that metformin can reduce the risk of cancer in diabetic patients (73), while HCC is one of the cancer types with the most significant reduction in incidence after the application of metformin (74). Here, we discuss how metformin exerts its underlying anti-cancer effects through the following pathways.

4.1 Metformin inhibits the growth of liver cancer cells

Activation of the mTOR signaling pathway, a branch downstream of AMPK, plays an essential role in the occurrence and progression of cancer, primarily hepatocellular carcinoma. The AMPK activation by metformin enhances the binding of DEPTOR and mTOR by increasing DEPTOR production and inhibiting proteasomal degradation, thereby interfering with the downstream target p70-S6 kinase and ribosomal protein S6 and inhibiting the proliferation of hepatocellular carcinoma cells (75, 76). Metformin-activated AMPK also reduces the degradation of intracellular caspase-3 protein by inhibiting the proteasome, inducing apoptosis in tumor cells (77, 78).

4.2 Metformin interferes with energy metabolism of liver cancer cells

The impact of metformin on tumor energy metabolism may be what causes it to have antitumor properties. Increased glycolysis is a characteristic of many tumors (79, 80). Glycolysis provides tumor cells with the energy they need for basal activities, and its metabolites can be employed to synthesize biomacromolecules and NADPH necessary for developing and invading cancerous cells. A study found that a hypoglycemia-metformin combination restrains tumor growth by activating glycogen synthase kinase 3β (GSK3β) downstream of PP2A. Mechanically, metformin inhibits CIP2A, a PP2A suppressor. At the same time, hypoglycemia upregulates B56δ, the PP2A regulatory subunit, resulting in the formation of a PP2A-B56δ active complex with a high affinity toward GSK3β to specifically activate the PP2A-GSK3β axis, leading to a decline in the pro-survival protein MCL-1, thereby interfering with glycolysis in hepatocellular carcinoma cells (81). Due to the rapid growth of tumor tissue, local hypoxia is ubiquitous in tissues, including liver cancer cells. Composed of α and β subunits, the heterodimeric hypoxia-inducible factor 1 (HIF-1), a critical transcription factor that regulates the response of tumor cells to hypoxia, can cause the transcription of a variety of genes to make it possible for the tumor cells to tolerate oxygen environment (82). HIF-1α is upregulated in many malignant tumors and reflects the tendency of tumor metastasis and poor prognosis (83). Metformin inhibits mitochondrial respiration by inhibiting respiratory chain complex I and reduces oxygen consumption to improve hypoxia (84), thereby promoting the ubiquitination of HIF-1α and the degradation of HIF-1α by the proteasome. The stability of HIF-1α and the translocation of the α subunit to the nucleus to bind to HIF-1β is reduced, resulting in the decrease of its ability to activate the cancer-promoting target genes glucose transporter 1 and carbonic anhydrase IX, exerting the role of inhibiting liver cancer progression (85–87). The effect of metformin on promoting degradation of HIF-1α can also inhibit the allosteric activator of phosphofructokinase-1 6-phosphofructose-2-kinase/fructose-2, 6-bisphosphatase 3 (PFKFB3), downregulating the expression of PFK1 mediated by PFKFB3 to inhibit glycolysis and cancer cell proliferation (88).

4.3 Epigenetic modification

MiRNAs play critical roles in liver cancer (89). Among these RNAs, miR-21 is oncogenic for HCC. Liu et al. (90) found that miR-21 is highly upregulated in liver cancer cells; the reduced expression of miR-21 inhibited the proliferation and migration of liver cancer cells through the phosphate and tension homology and triggered G2/M cell cycle arrest in cancer cells. Miyoshi et al. used metformin to treat liver cancer cells *in vivo* and *in vitro* and found that metformin inhibits human HCC cell proliferation and arrests the G1 cell cycle by suppressing the cell cycle-related molecules via alteration of miRNAs. Fifty-one differentially expressed miRNAs were identified, among which the let-7 family (let-7a, let-7b, and let-7e) was upregulated in metformin-treated liver cancer cells (91). The human let-7 family contains 13 members and is a class of tumor suppressor miRNAs (92). The upregulated miRNAs in these studies included miRNAs overexpressed in HCC (e.g., miR-21). The investigators speculated that this phenomenon might be related to altered survival responses in malignant cells. Additional investigations are required to determine whether each miRNA reflects the cause or effect of metformin treatment.

4.4 Metformin with cytotoxic therapy

Several studies explored the possibility of combining metformin with cytotoxic therapy for liver cancer; several lines of evidence suggest that metformin enhances the anti-cancer effect of these agents. WP631 is a structural analog of doxorubicin that exerts anti-cancer effects by inducing cell apoptosis. Its molecular mechanism includes activating NF-κB and increasing Bax, p53, and caspases-3, 8, and 9. Sliwinska et al. found that metformin can accelerate the death of cancer cells induced by WP631 and reduce the dose of WP631, which may be related to a significant increase in the level of NF-κB in liver cancer cells (93). These authors also discovered that metformin enhanced the anti-hepatoma effect of tubulin depolymerization inhibitor epothilone A by enhancing its pro-apoptotic effect and increasing levels of NF-κB (94).

5 Drug-induced and chemical-induced liver injury (DILI)

DILI refers to many forms of liver injury during medication exposure, resulting from metabolite hepatotoxicity or poor drug tolerance in specific patient populations. Given that the incidence is increasing yearly in China and Western countries because of substance abuse (95). DILI is the most common cause of acute liver failure (96). Chemical hepatotoxicants cause chemical-induced liver injury and are similar to DILI in mechanism. Hence, these two diseases will be discussed together.

5.1 Metformin ameliorates APAP-induced liver injury

In the Western world, APAP overdose is the most common cause of DILI and acute liver failure (97). Excessive APAP is converted to N-acetylbenzoquinone imine (NAPQI) by hepatic cytochrome P450 2E1. It depletes reduced glutathione in mitochondria, and the remaining NAPQI then reacts to form covalent links with cellular biological macromolecules, especially proteins, resulting in mitochondrial damage and necrotic cell death. This process increases ROS production, triggering JNK phosphorylation and sustained JNK activation, which contributes to liver cell death (98). Kim et al. (12) found that metformin protects against APAP overdose-evoked hepatotoxicity via growth arrest and DNA damage 45β (GADD45β)-dependent JNK regulation. Metformin can increase the expression of growth arrest and GADD45β to inhibit the phosphorylation of mitogen-activated protein kinase kinase 4, inhibiting JNK phosphorylation to protect hepatocytes from oxidative damage. However, another study indicated that metformin does not inhibit JNK activation or mitochondrial JNK translocation; metformin significantly reduced APAP protein adducts in mitochondria. Additionally, metformin inhibits mitochondrial respiratory chain complex I, which lowers proton leak and ROS generation in hepatocyte mitochondria, thereby reducing hepatocyte apoptosis to treat DILI (99).

5.2 Metformin ameliorates DILI induced by other chemicals

Metformin protects against chemical-induced liver injury. D-galactosamine increases the level of LPS and TNF-α in hepatocytes, causing acute liver injury. Metformin can reduce inflammatory indicators such as myeloperoxidase and malondialdehyde by activating the classic AMPK signaling pathway to inhibit apoptosis induced by LPS and TNF-α, thereby alleviating liver injury (100, 101). Moreover, arsenic trioxide, a chemotherapy agent used to treat promyelocytic leukemia, causes liver damage by generating ROS, while metformin can inhibit mitochondrial respiratory chain complex I and increase NAD+/NADH ratio, protecting against ATO damage. In addition, metformin can prevent carbon tetrachloride-induced hepatotoxicity, possibly associated with the increase of glutathione in hepatocyte mitochondria (102).

6 Conclusion

Given recent advances in understanding metformin's role in activating AMPK, attenuating lipotoxicity, optimizing gut microbiome, and regulating HSCs and liver cancer cells, the drug has potential therapeutic applications in several liver diseases. However, the actual clinical application of metformin for various *in vivo* disorders needs to be approached with caution due to limited relevant clinical research, and the correlation of dose must be considered when designing new metformin-based therapies. More research in these fields will advance our knowledge of metformin as a novel treatment for several liver diseases.

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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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A scientifically validated combination of garcinol, curcuminoids, and piperine for mild to moderate nonalcoholic steatohepatitis patients—results from a randomized, double-blind, placebo-controlled study

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Background: Garcinol is a naturally occurring compound from the fruit rind of the *Garcinia indica*, with antioxidant, anti-inflammatory, and anticancer properties. Curcuminoids are the active molecule from the rhizome of *Curcuma longa*, studied extensively for its health benefits as an anti-inflammatory and antioxidant activities. Non-alcoholic steatohepatitis (NASH) is the progressive form of nonalcoholic steatohepatitis characterized by liver fat and inflammation.

Objective: To evaluate the clinical efficacy and safety of Garcinol, Curcuminoids and piperine (GCP) combination in patients with mild to moderate NASH in a randomized, double-blind, placebo-controlled study.

Methods: The patients received one tablet (450 mg) of GCP containing garcinol-50 mg, curcuminoids –250 mg and piperine 5 mg or a placebo (450 mg of microcrystalline cellulose) twice daily for 90 days. Changes in circulating aspartate aminotransferase (AST), alanine transaminase (ALT) levels, liver stiffness measurement (LSM), and controlled attenuation parameter (CAP) using Fibroscan were compared from baseline to day 90. Anthropometric parameters, serum levels of lipids, Interleukin (IL-6), hsCRP, and adiponectin were estimated. Safety was evaluated by laboratory parameters and by monitoring adverse events.

Results: Seventy-two patients were randomized and 63 (GCP = 32, Placebo = 31) completed the study. The mean age of the patients was 48.3 $\pm$ 8.7 years (36 males and 27 females). The mean reduction in AST (U/L) was 9.53 in GCP and 3.16 in placebo ($p < 0.001$) and that of ALT (U/L) was 13.47 in GCP and 7.43 in Placebo ($p = 0.002$). The liver stiffness and CAP scores showed a better reduction in GCP (0.56 kPa and 12.38 db/m) compared to placebo (0.064 kPa and 10.42 db/m) $p < 0.05$. Consequently, the noninvasive Fibroscan-AST (FAST) score reduction was also found to be significant in GCP compared to placebo. Additionally, body weight, lipid levels, hsCRP, and IL-6 in serum decreased, while adiponectin levels increased in GCP-supplemented participants compared to placebo. The combination of garcinol and curcuminoids was well tolerated with no significant

changes in hematological and clinical laboratory parameters during the 90-day supplementation.

Conclusion: Our results suggest that GCP could be a possible supplement for the management of NASH.

Clinical trial registration: https://clinicaltrials.gov/, identifier CTRI/2019/11/022147.

KEYWORDS

nonalcoholic steatohepatitis, curcuminoids, garcinol, piperine, Fibroscan, liver stiffness, FAST score

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease caused by the accumulation of excess liver fat in the absence of excessive alcohol consumption or any other liver injury. The rise in obesity and metabolic syndrome across the globe has increased the prevalence and severity of the disease, rendering it the most common indication for liver transplantation (1). About 10–20% of individuals with NAFLD progress to nonalcoholic steatohepatitis (NASH), an inflammatory progressive disease with a higher risk of liver cirrhosis and hepatocellular carcinoma (2).

The most alarming fact about NAFLD is its asymptomatic nature which may remain unnoticed for years (3). The incidence of NAFD is exponentially increasing, with a global prevalence of 30.05% in adults and an alarming increase in pediatric population (4, 5).

The prevalence of NAFLD is 75% in overweight people and 90% in obese individuals, suggesting obesity (6) to be a major risk factor. The disease is regarded as the hepatic manifestation of metabolic syndrome and is found to be associated with type-2 diabetes, high body-mass index, insulin resistance, polycystic ovarian syndrome, hyperlipidemia, and old age. Although drug discovery for NAFLD is a priority, no drug has been approved by the United States Food and Drug Administration or the European Medicines Agency till today (7, 8).

The most effective treatment for fatty liver and NASH is lifestyle changes like exercise, caloric restriction, and nutrition. Vitamin E, pioglitazone, and new diabetic medications are used to treat NASH and its progressive conditions. Natural extracts and herbal products offer an alternate treatment for NAFLD. Curcuminoids refer to the three natural molecules: Curcumin (75–81%), Demethoxycurcumin (15–19%), and Bisdemethoxycurcumin (2.2–6.5%), present in the Curcumin C3 complex, the commercial extract from *Curcuma longa* rhizomes (turmeric). Garcinol is a polyisoprenylated benzophenone, extracted from the fruit rinds of *Garcinia indica* (Figure 1).

Curcuminoids were suggested to be a promising treatment for NAFLD in a meta-analysis of randomized clinical trials with a significant reduction in ALT, AST, and NAFLD severity (9, 10). Curcumin C3 complex at a dose of 500 mg with 5 mg piperine was reported to reduce NAFLD severity and decreased the serum levels of tumor necrosis factor, and monocyte chemoattractant protein in NAFLD patients (11). In another trial, Curcumin C3 complex at the same dose was reported to reduce ALT, AST, and lipid levels (12). Garcinol has been evaluated as an effective supplement for weight loss but has not been studied for NAFLD (13, 14). We have earlier reported that the combination of curcuminoids and garcinol was significantly more effective than the individual treatment in controlling the progression of liver steatosis to inflammation and fibrosis in the Stelic animal model (STAM™) model of NASH (15). In the present study, we explored the clinical efficacy of the combination of garcinol and curcuminoids with piperine in patients with mild to moderate NASH without liver cirrhosis. We used piperine as a bioenhancer in the present study, as the bioavailability of curcumin is known to increase in the presence of piperine (16). Bioenhancers are chemical entities, which promote and augment the bioavailability of natural molecules and drugs without showing any significant effect on their own. Piperine has been shown to increase the bioavailability of several natural products and drugs including curcumin (16–21). The dose of 250 mg of curcuminoids and 50 mg of 20% Garcinol, was derived from the preclinical study reported earlier (15).

2 Materials and methods

2.1 Materials

The test material was a combination of 20% garcinol from *Garcinia indica* (Livinol®—50 mg), 95% curcuminoids from *Curcuma longa* (Curcumin C3 Complex®—250 mg), and 95% piperine from *Piper nigrum* (BioPerine®—5 mg) as a bioavailability enhancer, referred to as GCP. This blend was formulated into a tablet (450 mg). The placebo tablet was formulated using microcrystalline cellulose (450 mg). The product was provided by Sami-Sabinsa Group Limited (LivLonga®). The details of the formulation are given in Supplementary methods section.

Curcumin C3 Complex was extracted from the dried rhizomes of *Curcuma longa*. In nature, the three curcuminoids exist together with Curcumin being the most abundant. The Curcumin C3 Complex used in the present study is composed of curcumin (75–81%), demethoxycurcumin (15–19%), and bisdemethoxycurcumin (2.2–6.5%). The product was standardized to contain not less than 95% w/w curcuminoids, with curcumin (80.22%), demethoxycurcumin (17.1%), and bisdemethoxycurcumin (2.68%) as per US pharmacopeial specifications.

Garcinol was extracted from the dried fruit rind of *Garcinia indica,* concentrated, and crystallized to get the pale-yellow needle-like crystals of garcinol with 75–80% purity. This 75–80% w/w of garcinol was diluted using microcrystalline cellulose to get 20% garcinol, containing around 5–7% of plant material, contributing to its stability at room temperature. Piperine was extracted from black pepper fruits (*Piper nigrum*) and

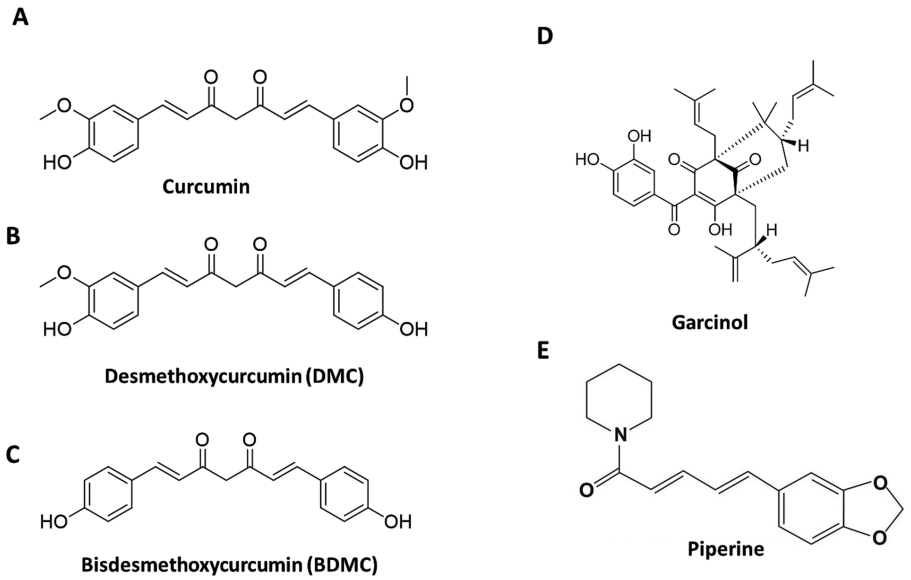

FIGURE 1
Structure of the active phytochemicals in the formulation. The structures of the Cucuminoids **(A–C)**, Garcinol **(D)**, and Piperine **(E)** present in the formulation.

standardized to contain 95% piperine. The HPLC chromatograms of Garcinol, Curcumin C3 Complex and Piperine are included in Supplementary Figures S1–S3.

GCP was formulated with garcinol (50 mg), curcuminoids (250 mg), and piperine (5 mg).

2.2 Analysis of GCP

Garcinol was again estimated by HPLC using a C18 ODS column, 4.6 mm (diameter) × 250 mm (length) with particle size 5 µ. The mobile phase consisted of 100% acetonitrile at a flow rate of 0.7 mL/min. A Photodiode array detector was used to detect garcinol at 240 nm. The additional early eluting peaks seen in the chromatogram are due to curcuminoids and piperine (Figure 2A).

Curcuminoids and Piperine were analyzed by HPLC using a C18 ODS column, 4.6 mm (diameter) × 250 mm (length) with particle size 5 µ. The mobile phase consisted of tetrahydrofuran with 0.1% w/v citric acid solution in the ratio 40:60 at a flow rate of 1 mL/min. A Photodiode array detector was used to detect curcuminoids at 420 nm (Figure 2B) and piperine at 341 nm (Figure 2C). and piperine at 341 nm using a UV detector Additional late eluting peaks seen in the chromatogram of piperine are due to curcuminoids.

2.3 Study design and ethics

A randomized, double-blind, placebo-controlled study was conducted from October 2020 to November 2021 in India. The study was designed at four centers; however, executed only at two sites, JNM Hospital, Kalyani, West Bengal, and Santosh Hospital, Bangalore, as the other two centers could not recruit any patients. All study documents were reviewed and approved by the institutional ethics committee of MS Ramiah Medical College and Hospital as per the study protocol number CPL/79/G&C_NASH/JUN/19 on 24 December, Dr. Satyprakash's Center for Digestive and liver diseases, 11 October 2019, College of Medicine and JNM Hospital, 22 June 2020 and Santosh Hospital, 21 January 2021. The study was conducted following Good Clinical Practice as required by the International Conference on Harmonization. The trial was registered prospectively with the Clinical Trial Registry of India (CTRI) with the registration number CTRI/2019/11/022147.

2.4 Sample size

The sample size was calculated for an alpha error of 0.05 and power at 80% based on the proportion of subjects with an effective response at the end of the treatment period. The details of the calculations are given in Supplementary material section.

2.5 Study population

2.5.1 Inclusion criteria

The study included male and female participants (30–65 years) with mild to moderate (≤ stage 2) nonalcoholic steatohepatitis (NASH) with or without fibrosis as diagnosed by elevated ALT and Ultrasonography. Normal echotexture of the liver was graded as the absence of steatosis (score 0), a slight and diffuse increase of liver echogenicity with normal visualization of the diaphragm and portal vein as mild (score 1), a moderate increase of liver echogenicity with an impaired appearance of the diaphragm and portal vein as moderate (score 2), and as severe (score 3), when the liver echogenicity showed a marked increase with poor or no visualization of the portal vein wall, diaphragm, and posterior part of the right liver lobe (22). The participants had a body mass index (BMI) in the range of 25–35 kg/m^2 and had controlled diabetes, hyperlipidemia, or hypertension for at least 3 months before

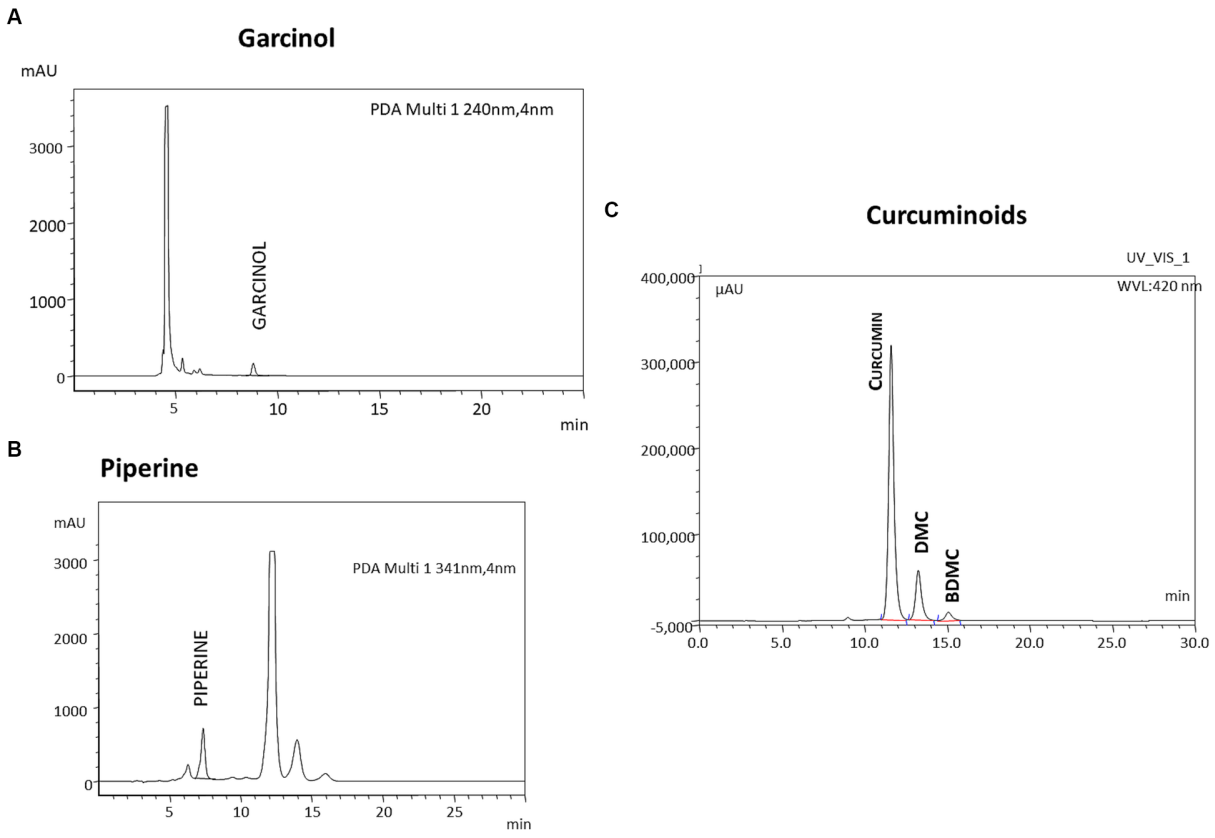

FIGURE 2
Representative HPLC profile of GCP. The formulation GCP was analyzed for the individual components using HPLC. **(A)** Garcinol is analyzed by reverse phase HPLC using Acetonitrile as mobile Phase and UV detector at 240 nm. **(C)** Curcuminoids (detected at 420 nm) and piperine (detected at 341 nm) were analyzed by reverse phase HPLC using Tetrahydrofuran and 0.1% Citric acid in the ratio of 40:60 (v: v) as **(B)** mobile phase.

screening. Other inclusion criteria were agreeing to use a barrier method of contraception during the study period for female patients and agreeing to adhere to assessments and visit schedules. Informed consent to participate in the study was signed by all the participants.

2.5.2 Exclusion criteria

Individuals with liver enzymes three times higher than the upper limit of the normal, fatty liver caused by viral hepatitis, autoimmune hepatitis, Wilson disease, any drugs, or alcoholism, as defined by the inability to control drinking due to both physical & emotional dependence on alcohol, were excluded from the study. Patients with biliary tract disease, diabetes (HbA1c >7.2%), dyslipidemia, endocrine diseases, and clinical suspicion of advanced liver disease, cirrhosis, or malignancy were also excluded from the study. Other criteria for exclusion were a history of renal, pulmonary, epileptic, hematologic, cardiovascular, neurological, or psychiatric illness, immunodeficiency diseases, positive HIV test results, and participation in any other clinical trial within the last 3 months.

2.5.3 Randomization

Subjects were randomized using a predetermined block randomization schedule generated using a computer-based randomization software (SAS 9.3), prepared by a statistician, independent of the sponsoring organization, and not involved in the conduct or reporting of the study. The randomization codes were kept strictly confidential and were accessible only to authorized persons on an emergency basis as per the standard operating procedures until the time of unblinding.

2.5.4 Intervention

The study participants were instructed to consume active (GCP-450 mg tablet) or a matching placebo containing microcrystalline cellulose (450 mg tablet) twice a day after breakfast and dinner for 90 days. Compliance was assessed by recording the number of tablets dispensed to and administered by the subject and returned at each visit in the case record form. All the patients enrolled in the study were asked to initiate lifestyle changes (a healthy diet with cardio/exercise for 5 days a week for at least 30 min) along with placebo or GCP supplements.

2.6 Outcome

The primary efficacy endpoint was the mean changes in AST, and ALT enzymes from the screening visit to day 90 and the changes in liver fat content as measured by Fibroscan with CAP from the baseline visit to day 90. The secondary endpoints included changes in liver

parameters (total bilirubin, alkaline phosphatase), lipid Profile (LDL, VLDL, HDL, TC, and TG), glycemic Profile (fasting glucose, HbA1c), biomarkers (IL-6, hs-CRP, adiponectin), anthropometric parameters (BMI, abdominal circumference) from baseline to day90 and safety outcome by the incidence of adverse events.

2.6.1 Transient elastography

The transient elastography devices used in this study were Fibroscan 430 Mini Plus (Echosens, France) by trained nurses and physicians. Fibroscan analysis was performed after 3 h of fasting. Patients were placed in a supine position and measurements were done by scanning the liver lobe through an intercostal space. CAP is an average estimate of ultrasound attenuation at 3·5 MHz and is expressed in db/m. LSM by VCTE is an average estimate of stiffness (Young's modulus) at a shear wave frequency of 50 Hz and is expressed as kPa. Only examinations with at least 10 valid individual measurements were deemed reliable.

2.6.2 Biochemical analysis

Fasting blood samples (10 mL) were collected from each participant, via venipuncture with a Vacutainer system (Becton Dickinson Biosciences, NJ, United States) by trained laboratory staff (EDTA treated tubes for hematology and untreated tubes for biochemistry tests). Hematological indices were measured by Sysmex XN1000 (Sysmex Corporation, Kobe, Japan); and biochemical indices by Cobas 400 (Roche Diagnostics, Mannheim, Germany) per manufacturer's instructions. Biomarkers IL6 and adiponectin were measured using commercial ELISA kits as per manufacturer's instructions.

The FibroScan-AST (FAST) score is a noninvasive diagnostic score, which takes the LSM, CAP measurements by Fibroscan, and the AST values into consideration, and provides an efficient method to identify patients at risk of progressive NASH in clinical trials or treatments (23). The FAST scores were calculated from LSM, CAP, and AST values using the equation,

$$\begin{aligned}\mathrm{FAST} = \{&\exp(-1.65 + 1.07 \times \ln(\mathrm{LSM}) \\ &+ 2.66 \times 10^{-8} \times \mathrm{CAP}^3 - 63.3 \times \mathrm{AST}^{-1})\} \\ &/\{1 + \exp(-1.65 + 1.07 \times \ln(\mathrm{LSM}) \\ &+ 2.66 \times 10^{-8} \times \mathrm{CAP}^3 - 63.3 \times \mathrm{AST}^{-1})\}\end{aligned}$$

as described earlier (23). All biochemical parameters were evaluated in a centralized lab with NABH accreditation as per the regulatory requirement (Elbit Diagnostics, Bangalore). Safety was evaluated by laboratory hematological, urinary, and biochemical parameters and by monitoring any incidence of adverse events.

2.7 Statistical analysis

All the statistical analysis was carried out by an independent statistician who was blinded to the study. A descriptive univariate analysis of demographic and clinical variables was performed by STATA Software version 16.0. The data gathered was considered as either continuous or categorical variables. All the analysis was conducted on the study population who completed the study. For continuous variables, the normality of distribution was tested by a one-sample Shapiro–Wilk normality test. The quantitative variables were described either as mean and standard deviation or median and interquartile range based on the normality. The primary and secondary efficacy endpoints were compared between the groups by Mann–Whitney U tests. Repeated measures one-way ANOVA followed by Dunnett's *post-hoc* test were performed for comparing the parameters at every visit with baseline values, within the group to understand the trend in their change. The categorical variables were compared by Chi-square test and the frequency and percentage of the population were presented. The change in quantitative variables from baseline to end of the study was compared to differentiate the treatment effect between the treatment groups. The level of statistical significance is defined as $p < 0.05$.

3 Results

3.1 Demographic and baseline characteristics

A total of 72 patients were randomized to active and placebo groups at baseline. Nine patients were lost to follow-up, and 63 participants completed the study, 32 in active (14 male and 18 female) and 31 (22 male and 9 female) in the placebo group (Figure 3). The baseline characteristics of the two groups were comparable. All the participants were Asian and had no history of smoking, drug abuse, or alcoholism. The median ALT and AST values were 69.92 ± 1.1 and 61.9 ± 1.7 IU/L, respectively. As per ultrasound analysis, 38.1% ($n = 24$) patients had grade 1 fatty liver, and 61.9% ($N = 39$) had a grade 2 stage of fatty liver. The median LSM value was 11 (7.0–12) kPa, and 79.3% had a score higher than >7.0 kPa. The median CAp value was 266 (254–282) db/m, and 63.5% of patients showed over 34% fat deposition in the liver (Table 1). Five patients were given metformin at 500 mg/day (four in placebo and one in GCP) during the study. All the patients (both in placebo and GCP groups) enrolled in the study were asked to walk for 30 min, 5 days a week. The instruction for a healthy diet included avoiding fried and processed food and consuming home-cooked food. The patients were not treated with any pharmacological drug for NAFLD during the study period.

3.2 Primary endpoints

3.2.1 Change in liver transaminases

The primary efficacy endpoints were the changes in Fibroscan score for liver stiffness and fat accumulation and the change in serum levels of liver transaminases from baseline (day 0) to the end of the study (day 90). The mean reduction in AST in the GCP-supplemented patients from baseline to the end of the study was 9.53 IU/L (15.1%) which was significant ($p < 0.001$) compared to placebo (3.16 IU/L) (Table 2). The mean AST reduced from 60.74 ± 14.7 to 57.58 ± 19.2 in placebo and from 63.09 ± 16.9 to 53.56 ± 17.3, $p < 0.001$ in patients supplemented with GCP for 90 days. Similarly mean ALT values reduced from 69.16 ± 14.9 to 61.73 ± 22.8 in placebo and 70.69 ± 14.3 to 57.22 ± 18.9 in GCP, a mean reduction of 13.5 IU/L (19.1%) in 90 days, significantly ($p < 0.001$) better than placebo (7.4 IU/L, 10.6%) (Table 2).

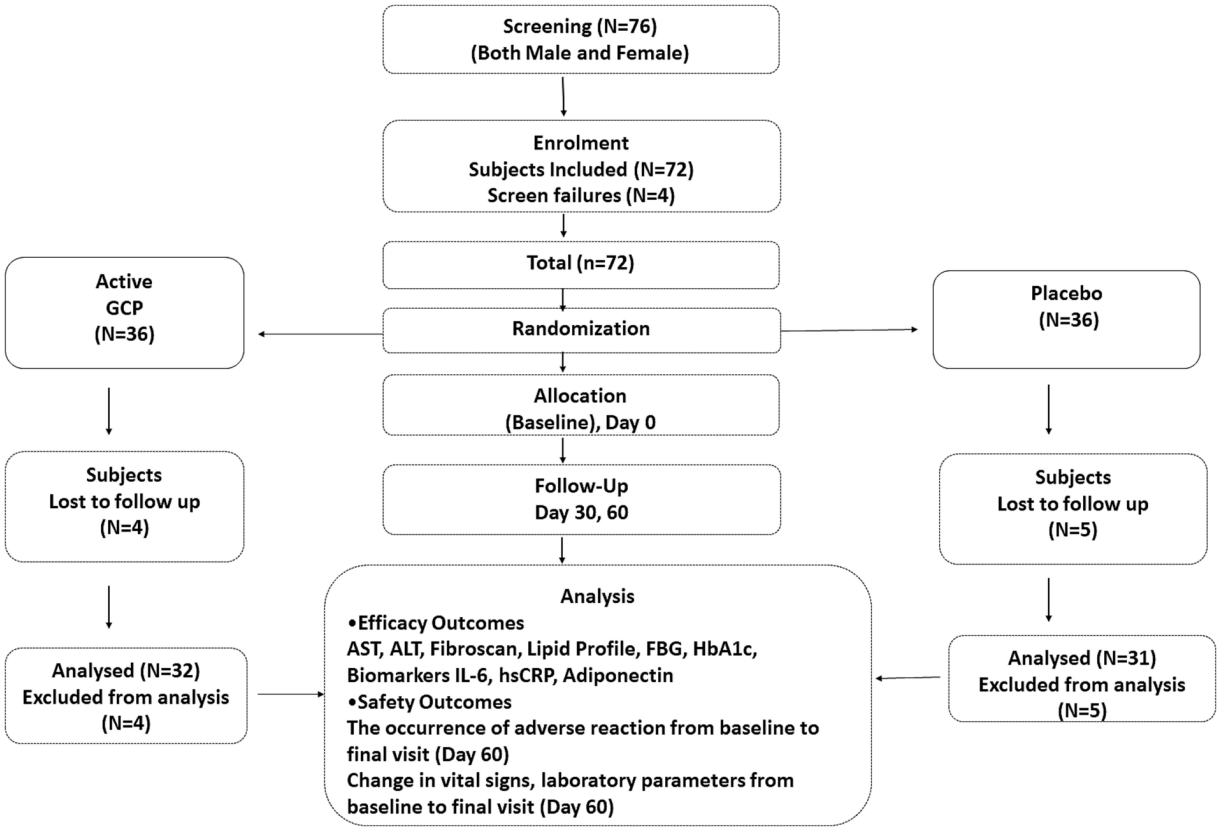

FIGURE 3
Consort diagram. Consort flow diagram showing the details of enrollment and disposition of participants.

TABLE 1 Demographic and baseline characteristics.

Characteristics	PLACEBO (*N* = 31)	GCP (*N* = 32)	Overall (*N* = 63)	*P*-value
Age (years)	48.74 ± 9.1	47.88 ± 8.5	48.30 ± 8.7	0.61
Gender				
Male	22 (70.9%)	14 (43.8%)	36 (57.1%)	0.04
Female	9 (29.03%)	18 (56.3%)	27 (42.9%)	
Height (cm)	167.29 ± 9.5	163.32 ± 9.9	165.27 ± 9.9	0.12
Weight (kg)	83.72 ± 9.6	79.03 ± 9.2	81.34 ± 9.6	0.052
BMI (kg/m^2)	29.75 ± 2.4	29.79 ± 2.5	29.77 ± 2.4	0.93
WC (cm)	98.40 ± 11.0	97.04 ± 9.5	97.71 ± 10.2	0.50
FBS	111.08 ± 25.1	109.48 ± 15.1	110.28 ± 20.8	0.82
Liver Enzymes (IU/L)				
AST	60.74 ± 14.7	63.09 ± 16.9	61.9 ± 1.7	0.19
ALT	69.16 ± 14.9	70.69 ± 14.3	69.92 ± 1.1	0.27
USG analysis				
Fatty liver grade I	10 (32.3%)	14 (43.8%)	24 (38.1%)	0.43
Fatty liver grade II	21 (67.7%)	18 (56.3%)	39 (61.9%)	
Fibroscan analysis				
CAP (db/m)	267.0 (255, 282)	264.0 (253.3, 286)	266 (254–282)	0.75
LSM (kPa)	11.10 (7.4, 12.0)	11.60 (7.5–12.5)	11 (7.0–12.0)	0.32

The values represent mean ± standard deviation for parametric and median and interquartile values for nonparametric data, except for gender and USG analysis where the number of participants and percentage are given. Continuous variables were compared by the Mann Whitney U test and categorical variables by the Chi-Squared test. WC, waist circumference; Hb1AC, Glycated hemoglobin; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; USG, Ultrasonography. *$p < 0.05$ between active and placebo.

TABLE 2 The change in liver transaminases, liver fat and stiffness from baseline to day 90 as primary end points.

Parameter	Group	Day 0	Day 90	Mean change D90- D0	P1
AST (IU/L)	Placebo	60.74 ± 14.7	57.58 ± 19.2	−3.16 (−6.78, 0.47)	<0.001
	GCP	63.09 ± 16.9	53.56 ± 17.3*	−9.53 (−12.79, −6.27)	
ALT (IU/L)	Placebo	69.16 ± 14.9	61.73 ± 22.8*	−7.43 (−13.12, −1.75)	0.002
	GCP	70.69 ± 14.3	57.22 ± 18.9*	−13.47 (−18.43, −8.51)	
LSM (kPa)	Placebo	9.80 ± 3.00	9.86 ± 2.56*	0.064 (−0.89, 1.03)	0.001
	GCP	10.23 ± 3.38	9.60 ± 2.87**	−0.56 (−1.4, 0.26)	
CAP (db/m)	Placebo	270.5 ± 22.36	260.1 ± 30.75	−10.42 (−21.59,0.75)	0.03
	GCP	269.6 ± 22.17	257.2 ± 21.42*	−12.38 (−17.37, −7.38)	
FAST Score	Placebo	0.54 ± 0.12	0.49 ± 0.17	−0.04 (−0.088, 0.008)	0.001
	GCP	0.55 ± 0.17	0.46 ± 0.17**	−0.08 (−0.12, −0.04)	

The Mean ± Standard deviation values are represented in the table. The mean change and 95% confidence interval of the mean are given for the difference from day 0. Paired T test was used to compare the changes in the parameters from days 0 to 90 within placebo and GCP. Mann–Whitney U tests was used for the comparison of change in the values from days 0 to 90 between the groups. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CAP, Controlled attenuation parameter (fat attenuation by Fibroscan); LSM, Liver stiffness measurement by Fibroscan; FAST, Fibroscan AST score. *$P < 0.05$, **$P < 0.01$, from baseline to end of the study within the groups. P1: The significance of the change in the parameter between placebo and GCP, Normal values of AST – 8–48 IU/L and ALT- 7–55 IU/L.

3.2.2 Change in liver fat and fibrosis

The Fibroscan data showed a significant ($p < 0.001$) mean reduction in liver stiffness of 0.57 kPa [10.23 ± 3.38 to 9.66 ± 2.87] in GCP compared to an increase of 0.06 kPa [9.80 ± 3.00 to 9.86 ± 2.56] in placebo. The reduction in liver stiffness was 5.5% in GCP compared to placebo, which was statistically significant ($p = 0.001$). The CAP score decreased by 12.4 db/m in GCP [269.6 ± 22.17 to 257.2 ± 21.42] versus 10.4 db/m in placebo [270.5 ± 22.36 to 260.1 ± 30.75]. The reduction in fat attenuation was 4.6% in GCP compared to 3.8% in placebo ($p = 0.03$). We calculated the FAST score, which identifies patients at risk of progressive NASH (23, 24). The FAST score showed a significant reduction in the GCP group [0.55 ± 0.17 to 0.46 ± 0.17 P,0.001], while it was [0.54 ± 0.12 to 0.49 ± 0.17], accounting for a 16.3% reduction by GCP which was significantly better than 9.2% in placebo ($p = 0.001$) (Table 2).

3.2.3 The trend in the decrease of liver enzymes within the groups

The liver transaminases were monitored every month to understand their change within the group. The decrease in liver enzymes was observed at day 30 and continued up to day 90 in GCP. The placebo group also showed a reduction in ALT and AST, which could be because of exercise, although the effect was lower than that observed in GCP (Figure 4).

3.2.4 Gender and concomitant medicine

Gender-specific analysis retained the effect of GCP in males, while in females, only liver stiffness was significantly better than placebo, which could be due to the lower number of females in the placebo (Supplementary Tables S1A,B). Five participants were taking metformin to control diabetes (4 in placebo and 1 in GCP). Excluding these participants from the analysis did not change the outcome of the study (Supplementary Tables S2A,B).

3.3 Secondary endpoints

3.3.1 Lipid profile

Interestingly we observed a reduction in total cholesterol (13.8 vs. 1.96 mg/dL), triglycerides (23.75 vs. 8.59 mg/dL), LDL-C (6.5 vs. 3.6 mg/dL), and VLDL-C (5.1 vs. 1.71 mg/dL) with GCP supplementation in comparison to placebo (Table 3). Repeated measure ANOVA analysis showed a significant trend in total cholesterol, triglycerides, and VLDL-C decrease in GCP ($p < 0.01$), but the trend in LDL-C decrease was not significant (Table 3). HDL-C levels were not affected by GCP and placebo (Table 3).

3.3.2 Body weight and metabolic markers

The mean body weight (2.28 vs. 0.3 kg), BMI (1.18 vs. 0.01), and waist circumference (1.45 vs. 0.35 cm) decreased significantly in GCP-supplemented patients compared to placebo. Body weight decrease was observed in 90.6% of patients in GCP (29/32) compared to 29% (9/31) in placebo. Similarly, waist circumference and BMI also decreased in over 90% of patients in GCP compared to 30% in placebo. Body weight, abdominal circumference, and BMI reductions were significantly better in GCP compared to placebo. Total bilirubin decreased, and we observed a nonsignificant and clinically irrelevant increase in alkaline phosphatase levels in patients supplemented with GCP. Fasting blood sugar showed a decrease in both groups and was not significant in GCP compared to placebo, while HbA1c did not change in both groups (Table 4).

3.3.3 Inflammatory markers

The inflammatory cytokine Interleukin-6 and C reactive protein showed a significant decrease by 0.64 pg./mL and 3 mg/L in GCP, which was significant compared to placebo. Adiponectin, the hormone which prevents lipid storage and protects the liver from inflammation and fibrosis, increased from the baseline values by 1.45 µg/mL (13.5%) in GCP, while placebo showed no effect on adiponectin levels (Figure 5).

3.4 Safety parameters

Statistical analysis of safety was performed for all the patients who completed the study. Mild adverse events were reported by three participants from the placebo group. The events were resolved without any medication and were not related to the study medication (Table 5). No adverse event was reported in the GCP group. There were no significant differences between the treatment groups for the clinical laboratory examination results. All the laboratory parameters were

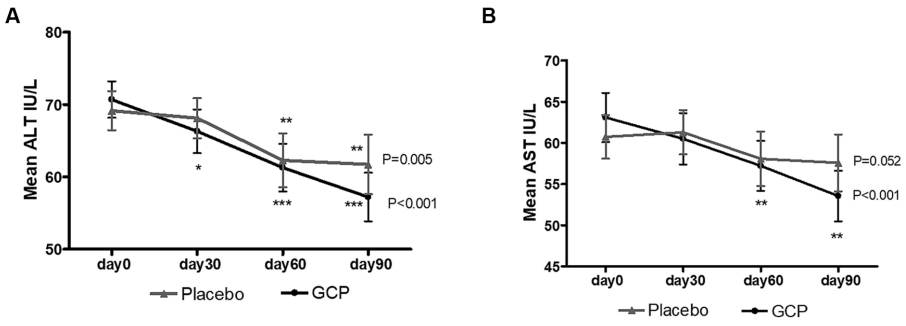

FIGURE 4
The trend in change in liver transaminases in **(A)** ALT and **(B)** AST GCP and Placebo during the study. The Mean ± Standard deviation values are represented for AST and ALT levels. Repeated measure ANOVA (*p*-value) and Dunnett's post-test (*), with day 0 values as control, were used to compare the changes in AST and ALT within placebo and GCP.

TABLE 3 Effect of GCP on lipid parameters.

Parameter	Group	Day 0	Day 30	Day 60	Day 90	Repeated measure ANOVA	*P*-value#
TC	Placebo	258.19 ± 37.45	248.77 ± 45.1	251.5 ± 42.45	256.23 ± 44.37	0.350	<0.001
	Difference from day 0		−9.42 (−21.88, 3.04)	−6.69 (−20.75, 7.36)	−1.96 (−17.46, 13.52)		
	GCP	261.21 ± 33.98	252.78 ± 37.2*	252.72 ± 31.46*	247.38 ± 27.32*	<0.001	
	Difference from day 0		−8.43 (−13.70, −3.16)	−8.49 (−14.18, −2.81)	−13.83 (−20.92, −6.75)		
TG	Placebo	192.68 ± 42.07	180 ± 45.52	187.57 ± 44.26	184.09 ± 56.20	0.597	<0.001
	Difference from day 0		−12.68 (−30.13, 4.77)	−5.11 (−19.7, 9.47)	−8.59 (−25.91, 8.73)		
	GCP	191.13 ± 40.72	182.34 ± 45.03	172.91 ± 49.19	167.38 ± 25.17	0.008	
	Difference from day 0		−8.79 (−21.65, 4.07)	−18.22 (−32.26,-4.20)	−23.75 (−40.16, −7.35)		
LDL	Placebo	142.26 ± 26.95	132.16 ± 24.36	133.21 ± 20.47	138.6 ± 27.33	0.099	0.001
	Difference from day 0		−10.10 (−20.05,- 0.13)	−9.05 (−20.57, 2.46)	−3.66 (−14.54, 7.22)		
	GCP	143.21 ± 19.97	137.94 ± 21.85	140.13 ± 19.13	136.66 ± 13.13	0.202	
	Difference from day 0		−5.27 (−10.94, 0.40)	−3.08 (−9.68, 3.51)	−6.55 (−13.38, 0.27)		
VLDL	Placebo	43.26 ± 8.85	40.16 ± 10.34	42.09 ± 9.47	41.55 ± 12.76	0.449	0.005
	Difference from day 0		−3.10 (−6.56, 0.37)	−1.17 (−4.10, 1.77)	−1.71 (−5.22, 1.82)		
	GCP	43.75 ± 8.6	41.91 ± 9.81*	39.59 ± 10.66*	38.69 ± 6.20*	0.003	
	Difference from day 0		−1.84 (−4.49, 0.80)	−4.16 (−6.96, −1.35)	−5.06 (−8.47,- 1.65)		
HDL	Placebo	39.29 ± 5.14	38.23 ± 3.87	36.79 ± 3.08	37.35 ± 3.66	0.008	0.050
	Difference from day 0		−1.06 (−2.12, −0.01)	−2.5 (−4.27, −0.72)	−1.94 (−3.43, −0.45)		
	GCP	40.22 ± 3.47	40.0 ± 3.09	39.47 ± 2.49	39.5 ± 2.23	0.215	
	Difference from day 0		−0.22 (−0.88, 0.75)	−0.75 (−1.82, 0.32)	−0.72 (−1.65, 0.21)		

The Mean ± Standard deviation values are represented for lipid levels. The mean change and 95% confidence interval of the mean are given for the difference from day 0. Repeated measure ANOVA and Dunnett's post-test (*), with day 0 values as control, were used to compare the changes in the parameters within placebo and GCP. Mann–Whitney U tests was used for the comparison of change in the values from day 0 to day 90 between the groups. TC, Total cholesterol; TG, triglycerides; LDL, Low-density lipoprotein cholesterol; HDL, High-density lipoprotein cholesterol. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ from baseline to end of the study within the group. #The significance of the change in the parameter between placebo and GCP at day 90. The normal ranges were TG < 150 mg dL^{-1}, TC < 200 mg dL^{-1}, LDL < 100 mg dL^{-1}, VLDL < 30 mg dL^{-1}, HDL 40–60 mg dL^{-1}.

within the specified normal range (Supplementary Tables S3–S5). These results suggest that Garcinol 20% (50 mg) with Curcuminoids 95% (250 mg) and piperine (5 mg) combination (GCP) is safe for use in NASH without liver cirrhosis patients.

4 Discussion

The present study reports the beneficial effects of a combination of garcinol and curcuminoids with piperine (GCP) supplementation in improving liver parameters, systemic inflammation, and dyslipidemia in patients with mild to moderate NASH. It is probably the first study to document the effect of the combination of garcinol and curcuminoids on liver fibrosis and steatosis assessed using transient elastography (FibroScan).

Currently, no specific pharmacological treatments for NAFLD and NASH are approved by the FDA, although numerous drugs are being evaluated (7). NAFLD is associated with several other comorbidities like diabetes, cardiovascular disease, kidney disease, and cancer and is considered the manifestation of metabolic syndrome

TABLE 4 Effect of GCP on Secondary efficacy outcomes.

Parameter	Group	Day 0	Day 90	Mean change	*P*-value[#]
Body Weight (kg)	Placebo	83.72 ± 9.61	83.42 ± 9.14	−0.3	<0.001
	GCP	79.03 ± 9.25	76.75 ± 8.92**	−2.28	
BMI (kg/m^2)	Placebo	29.75 ± 2.36	29.74 ± 2.15	0.01	<0.001
	GCP	29.79 ± 2.51	28.61 ± 1.67**	−1.18	
WC (cm)	Placebo	98.4 ± 11.00	98.05 ± 10.60	−0.35	< 0.001
	GCP	97.04 ± 9.46	95.59 ± 8.74**	−1.45	
Bilirubin (mg/dL)	Placebo	0.73 ± 0.26	0.78 ± 0.24	0.05	0.007
	GCP	0.82 ± 0.30	0.73 ± 0.21	−0.08	
ALP (IU/mL)	Placebo	78.29 ± 20.88	83.87 ± 19.52	5.58	0.88
	GCP	83.06 ± 20.17	93.36 ± 30.24	12.44	
FBS (mg/dL)	Placebo	111.09 ± 25.26	105.66 ± 18.29	−5.43	0.172
	GCP	109.51 ± 15.81	98.91 ± 13.21	−10.61	
HbA1c (%)	Placebo	6.42 ± 0.35	6.67 ± 0.14	0.25	0.18
	GCP	6.35 ± 0.49	6.34 ± 0.58	−0.01	

The Mean ± Standard deviation values are represented in the table. Paired *t*-test was used to compare the changes in the parameters from days 0 to 90 within placebo and GCP. Mann–Whitney U tests was used for the comparison of change in the values from days 0 to 90 between the groups. BMI, Body mass index; WC, Waist/Abdominal circumference; ALP, Alkaline phosphatase; FBS, Fasting blood sugar; HbA1c, glycosylated hemoglobin. *$P < 0.05$, **$P < 0.01$, ***P < 0.001 from baseline to end of the study within the group. [#]The significance of the change in the parameter between placebo and GCP at day 90. The normal ranges were ALP- 40 to 129 IU/L, Bilirubin- 0.1 to 1.2 mg dL^{-1} FBS < 100 mg/dL, and HbA1c < 5.7%.

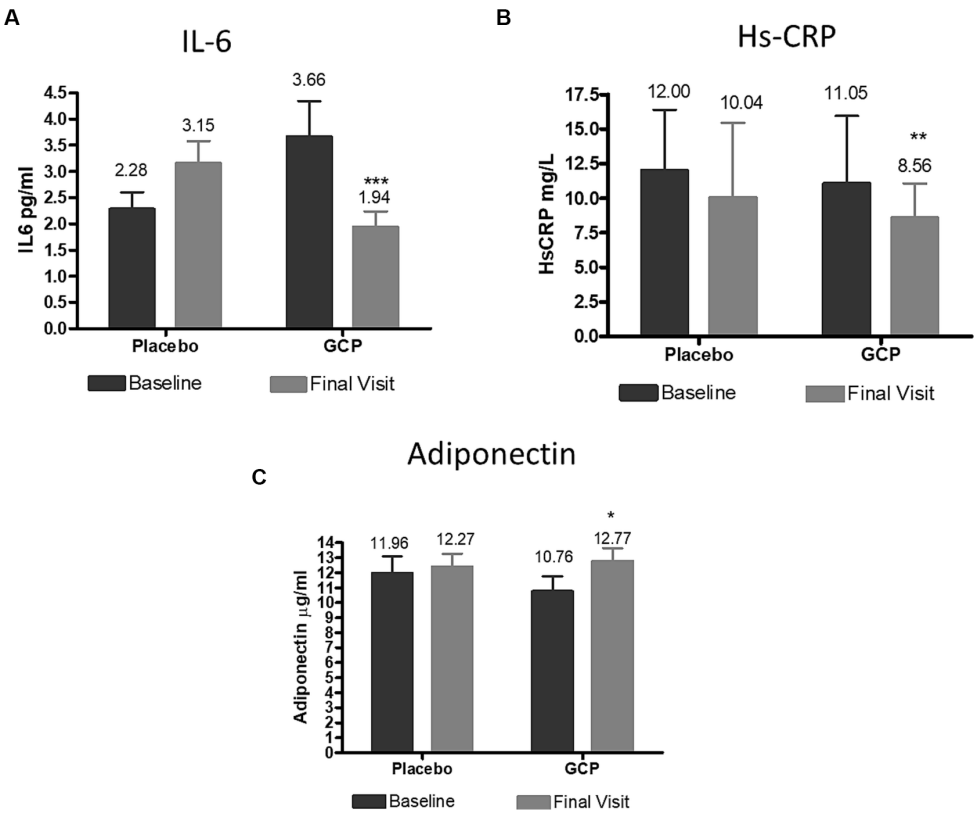

FIGURE 5
Effect of GCP on inflammatory markers **(A)** IL 6, **(B)** HsCRP and **(C)** Adiponectin. The Mean ± Standard deviation values are represented. The reduction IL6 and Hs-CRP were 0.80 and −1.72 ($p < 0.001$) and −1.8 and − 2.48 ($p = 0.02$) for placebo for GCP, respectively. The increase in adiponectin was 0.42 for placebo and 2.02 for GCP ($p < 0.001$) by Mann–Whitney test. IL6, Interleukin 6; HsCRP, High sensitivity C reactive protein. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ from baseline to end of the study within the group.

TABLE 5 List of adverse events and their resolution.

Participant no.	Gender- age	Group	Event term	Start date	End date	Outcome
4,013	M-59	Placebo	Diarrhea	12-Sep-21	14-Sep-21	Resolved
4,015	F-64	Placebo	Urinary tract infection	07-Oct-21	09-Oct-21	Resolved
4,016	M-47	Placebo	Lower abdominal pain	08-Oct-21	09-Oct-21	Resolved

Description of mild adverse events experienced by participants during the study. M, male; F, Female.

in the liver (25, 26). A few promising drugs in phase 3 evaluation are Obetocholic acid -a Farnesoid X or bile acid receptor agonist, Resmetirom, the thyroid hormone receptor beta agonist, Armachol, stearoyl coenzyme A desaturase inhibitor, and Semaglutide, glucagon-like peptide-1 agonist (27, 28). Lifestyle modification through a hypocaloric diet and exercise are the basis for the treatment of NAFLD in the absence of approved pharmacological agents.

Given the complex pathophysiology of NASH, a combination of different drugs with diverse mechanisms of action, therapeutic approaches targeting each stage of the disease may be required to manage the disease progression (29). Herbal and traditional medicine have garnered attention in recent years to effectively manage NAFLD (30). Plant polyphenols were found to have a protective effect in reducing inflammation, oxidative stress, and liver steatosis in animal models (31). In clinical trials, resveratrol supplementation was found to improve inflammatory profile in NAFLD patients, while hesperidin was reported to ameliorate steatosis, glycemia, and hepatic enzymes (32, 33). Flax seeds and Coenzyme Q10 supplementation were reported to reduce inflammation and improve liver function (34, 35). Curcumin is known to interact with multiple molecular targets and alter their gene expression and signaling pathways (36). In animals, curcumin supplementation was shown to reduce obesity and glucose intolerance, fibrosis, and intrahepatic accumulation of $CD4^+$ cells in association with NAFLD (37). Clinical trials with curcumin have shown a reduction in triglycerides, blood glucose, and liver enzymes in individuals with metabolic complications (38). Garcinol is an antiadipogenic agent which activates the AMPK pathway and reduces endoplasmic reticulum stress in adipose tissues (13, 14). Garcinol was also shown to modulate gut microbiota and control inflammation by increasing the intestinal commensal bacteria, *Akkermansia* (13).

We earlier reported that the combination of garcinol and curcuminoids reduced NAFLD activity score, fibrosis, and steatosis in a stelic animal model (STAM™) of NASH. The effect of the combination was significantly better than individual treatment with either curcuminoids or garcinol. Curcuminoids were found to reduce inflammation mediated by NF-κB and fibrosis, while garcinol was effective in modulating macrophage activity and reducing steatosis. The combination was effective in targeting both inflammation and steatosis (15). The current clinical study validates the preclinical data of GCP in a clinical setting.

All the study participants, irrespective of the study intervention were asked to walk for 30 min every day for at least 5 days in a week as a lifestyle modification and were not treated with any pharmacological drug. No specific diet was proposed as the participants were from different parts of the country with varied food habits. They were only instructed to avoid processed and fried food during the study. The moderate exercise performed by the participants could have had a positive effect on the liver health. Experimental studies have shown that moderate aerobic and anaerobic exercise for 20–60 min, 4–7 days a week for 6 months to improve liver histology ad reverse liver damage (39, 40). It is also possible that the observed results could be due to the combination of moderate exercise and GCP intervention. However, the participants consuming placebo along with moderate exercise, did not show a substantial change in the liver parameters and the difference between GCP and placebo for the outcome measures was significant.

The elevation of liver enzymes is a marker of hepatocyte injury observed in NAFLD patients. Recent studies suggest that a higher level of ALT may be a consequence and predictor for the development of NASH (41). Curcumin supplemented at a dose of 500 mg to 1,000 mg for 8–12 weeks was reported to reduce AST (−2.7 to −9.2 IU/L) and ALT (−2.9 to 2212-11.3 IU/L) in NAFLD patients in earlier studies (12, 42–44). We observed a reduction of 9.5 IU/L for AST and 13.5 IU/L for ALT with GCP, suggesting that the combination may be better than curcuminoids alone.

For NAFLD progressing to NASH, hepatic fibrosis is the standard endpoint. Although liver biopsy is the accepted diagnosis of fibrosis, the use of transient elastography (Fibroscan) to measure liver stiffness through the estimation of the velocity of propagation of a shear wave through liver tissue is emerging as a valid method for assessing fibrosis (45, 46). Our study demonstrated a reduction of 5.5% in LSM and 4.6% in CAP compared to an increase in LSM and a 3.8% decrease in CAP in placebo in 12 weeks, which could be considered moderate activity. However, all the patients (100%) consuming GCP showed a decrease in LSM, while it was observed in only 13% of patients in the placebo group (4/31). It is worthwhile to note that in an earlier clinical study with Saroglitazar, a dual peroxisome proliferator-activated receptor α/γ agonist approved for diabetic dyslipidemia, the reported reduction in LSM was 10.5% after 24 weeks, although the improvement in CAP was much superior (23.5%) (47). In a 10-year study to monitor fibrosis progression in NAFLD patients, an improvement was defined as a reduction in the LSM by more than 2 kPa in 10 years. ALT improvement by more than 30% and more than 1 unit change in BMI (kg/m^2) was found to contribute to LSM improvement (48).

Several non-invasive methods have been developed to diagnose NASH patients in recent years. The FAST score, which takes the LSM, CAP measurements by Fibroscan, and the AST values into consideration, provides an efficient non-invasive method to identify patients at risk of progressive NASH for clinical trials or treatments (23). The FAST score showed a significant decrease in the GCP group compared to the placebo, further reiterating that the herbal combination can potentially improve overall liver function. These results agree with the preclinical data in the mouse model, where we observed a reduction in fibrosis and steatosis by liver histopathology.

The prognosis of NASH depends on obesity, insulin resistance, and type 2 diabetes as risk factors, and addressing these conditions has been the focus of treatment. Weight loss by bariatric surgery has shown benefits in improving NAFLD (49). The American Association for the Study of Liver Diseases (AASLD) NAFLD practice guidelines also recommend a reduction in body weight by 3–5% to improve

hepatic steatosis, while (≥ 7%) reduction to improve fibrosis (50, 51). We observed a 3.8% reduction in weight, 1.8% in waist circumference, and 3.3% in BMI in the GCP group, which suggests an improvement in anthropometric parameters which could have a positive effect on NAFLD. Supplementation for a longer time might improve these parameters further. Cardiometabolic risks are higher in NASH patients, so control of dyslipidemia is another essential factor. Further, excess accumulation of fatty acids in the liver can cause lipotoxicity and hepatocyte injury (52). GCP was helpful in reducing TC (5.3%), TG (12.8%), LDL (4.6%), and VLDL (11.5%) levels while HDL was not affected. Although these are only minimal effects, cumulatively positive effects on multiple parameters would synergistically and effectively improve liver health.

Adequate control of diabetes is critical for NASH since hyperglycemia and insulin resistance contribute to hepatic steatosis. Although we observed some reduction in FBS, no effect was seen with HbA1c, suggesting a minimal effect of GCP supplementation in diabetic control. Several studies have shown a positive effect of curcumin on glycemic control in type 2 diabetic patients (53). Most of these studies used higher doses of curcumin for a longer duration compared to that used in GCP, which could explain the results observed in our study.

Prolonged lipotoxicity causes oxidative stress and ER stress culminating in the activation of toll-like receptors, inflammasomes, and transcription factors involved in the expression of inflammatory markers (51). Leptin and adiponectin secreted from the adipose tissue have a pro and anti-inflammatory effect on liver inflammation. Adiponectin reduces hepatic inflammation, and lipid accumulation and regulates oxidative stress and Kupffer cell polarization (54). A systematic review and meta-analysis of randomized clinical trials reported that curcumin could significantly improve adiponectin concentrations compared to placebo (55). Our results agree with this observation, although the curcumin was used at 1500 mg dose in RCTs compared to 250 mg in our study. An increase in adiponectin was also observed in our preclinical study with the same combination (15).

GCP supplement resulted in a significant reduction in inflammatory markers (IL6 and HsCRP) in the serum, suggesting an improvement in the inflammatory status in these patients. IL6 has been identified as one of the independent prognostic factors for liver steatosis in obese individuals (56). The cytokine has also been observed as a predictive biomarker for the development of NASH in NAFLD patients (57). Thus, a reduction in IL6 may be a significant observation in this study. In an earlier study, Saadati et al. observed a reduction in inflammatory markers in NAFLD patients supplemented with 1,500 mg curcumin for 12 weeks but the changes were not significantly different from placebo (58). Curcuminoids with piperine at 500 + 5 mg was reported to reduce the serum levels of inflammatory markers (TNF-α, MCP1) in another trial (11). Our results suggest that by combining curcumin with garcinol, the effective dose of curcumin could be reduced to get better benefit on inflammatory parameters.

Finally, we believe that the mechanism of action of GCP may be mediated through its action of oxidative stress, inflammation, and energy balance. Fatty liver is a consequence of increased free fatty acids imported into liver from adipocytes, diet and *de novo* hepatic lipogenesis. Earlier studies have shown that garcinol could reduce adipogenesis by reducing ER stress and activating AMPK, the cellular energy sensor. The progression to NASH is facilitated by an elevation of intracellular reactive oxygen species (ROS) that increase oxidative stress inflammation and cell death. Both Curcuminoids and Garcinol have antioxidant properties, with evidence of liver protection against damage induced by alcohol, drugs, and other agents (59–61). Curcuminoids are well studied for their anti-inflammatory activity and health benefits related to chronic inflammatory diseases. Thus, the beneficial effect observed in the present study could be due to the combined effect of reduction in oxidative stress, inflammation, and energy balance by GCP. Further, Garcinol has been observed to prevent gut dysbiosis which is one of the contributing factors for NAFLD (62). Since NAFLD progressing to NASH is a complex disease driven by multiple pathways, the combination of GCP may target some of these pathways at different levels resulting in a better outcome.

All the serum biochemical markers and vital signs were in the normal range throughout the study, and no serious adverse events were reported by any patients during the 90 days of supplementation, suggesting the safety of GCP formulation. The Curcumin C3 complex has been evaluated in more than 100 clinical studies and was found to be safe for consumption at a dose as high as 8 g per day. Garcinol at 40% was also proven to be safe in preclinical studies (63). The combination described in the present study contained much lower doses of curcumin c3 complex and Garcinol and hence could be regarded as a safe composition.

A few limitations, as well as strengths of our study, are worth mentioning. The study was conducted on mild to moderate NASH patients with mild diabetes or dyslipidemia in a relatively small population. We cannot generalize the results for NASH patients with other comorbidities. We did not conduct a liver biopsy to confirm our results which could be another limitation. However, we used validated non-invasive testing using FibroScan, which could be a study strength. The study was conducted in two sites in India, one in the south and the other in the eastern part of the country, which includes a diverse population and could be termed as a strength of the study. The gender distribution in the two groups was not equal, which is also a limitation and future studies need to be conducted in larger population in both male and females. The age distribution was comparable between the two groups and hence a specific age-related analysis was not carried out. The results observed were significant, but the percentage reductions were moderate, and the liver enzymes were not reduced to normal levels. The effects of dietary supplements could not be compared to pharmaceutical drugs, as many of them were conducted over a six-month duration compared to the three-month study detailed here. The study used food components as actives and was found to be safe as reported herein. The study had a gender difference in active and placebo, which could have affected the analysis and is a potential limitation of the study. In the future, we plan to conduct studies on a larger population of NASH patients of different age-groups, for a longer period of time. The development of NASH involves complex mechanisms and its resolution in terms of improvement in liver and serum biomarkers is also a slow process. A study duration of 6–12 months may help to understand the effect of GCP on liver function and other metabolic parameters. Further, GCP may also be combined with other known supplements, like Vitamin E, to optimize its effect in future clinical studies.

5 Conclusion

GCP as a dietary supplement with safe food ingredients was effective in improving liver enzymes, liver steatosis and fibrosis, inflammation, and lipid markers in NASH patients. Curcumin has been reported to have favorable effects on NAFLD, at high doses. By combining it with garcinol we observed positive effects at a much lower dose. Thus, GCP could be a

potential option as an adjunct supplement along with lifestyle management and other standard treatments for the management of NAFLD progressing to NASH. Future studies for a longer duration in comparison to standard treatment would benefit the clinical use of GCP as an adjunct supplement for the management of NASH.

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

All study documents were reviewed and the Study Protocol Number CPL/79/G&C_NASH/JUN/19 was approved by the Institutional Ethics committee of MS Ramaiah Medical College and Hospital, 24 December 2019, Dr. Satyprakash's Center for Digestive and liver diseases, 11 October 2019, College of Medicine and JNM Hospital, 22 June 2020 and Santosh Hospital, 21 January 2021. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MM: conceptualization and funding. KN: study design, review. MN: review. SP: clinical study coordination and data curation. CB and SS: principal investigator. LM: study design, data analysis, and writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MM, KN, LM, and SP were affiliated with Sami-Sabinsa Group Limited or Sabinsa Corporation. The authors declare that this study received funding from Sami-Sabinsa Group Limited. The funder had the following involvement in the study: These authors were involved in the conceptualisation, protocol development ,study design and coordination with principal investigators. The study was conducted by the principal investigators and the participants funders and the investigators were blinded to the study. MN has been on the advisory board/consultant for 89BIO, Altimmune, Gilead, cohBar, Cytodyn, Intercept, Pfizer, Novo Nordisk, Blade, EchoSens, Fractyl, Madrgial, NorthSea, Prespecturm, Terns, Sami-Sabinsa Group Limited, 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 is a shareholder or has stocks in Anaetos, Chrownwell, Ciema, Rivus Pharma and Viking. Curcumin C3 Complex®, Livinol®, and LivLonga® are registered US trademarks of Sabinsa Corporation.

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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1201186/full#supplementary-material

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New insights into metabolic dysfunction-associated steatotic liver disease and oxidative balance score

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Background: The relationship between oxidative stress and metabolic dysfunction-associated steatotic liver disease (MASLD) has not been studied, which remains inadequately recognized. This is a cross-sectional study in a US adult population to explore the relationship between MASLD and oxidative balance scores (OBS), which containing integrating dietary nutrition and lifestyle factors.

Methods: We analyzed data from National Health and Nutrition Examination Survey during 2017–2018. Multivariate logistic regression, restricted cubic spline curve (RCS) and subgroup analysis were used to investigate the association between OBS and MASLD. Cox regression analysis was utilized to assess the association between OBS and all-cause mortality among individuals.

Results: The multivariable-adjusted odds ratio (OR) and 95% confidence interval (CI) for the highest quartile of OBS (Q4) was 0.30 (0.12, 0.77) ($p = 0.012$) compared to the lowest quartile of OBS (Q1). The RCS regression and subgroup analysis indicated an inverted relationship between OBS and the development of MASLD. The OBS Q4 group (HR: 0.15, 95% CI: 0.03–0.87; $p = 0.035$) exhibited a lower risk of all-cause death than the Q1 group.

Conclusion: OBS is statistically significantly and negatively correlated with the risk of MASLD and all-cause mortality in US adults. More prospective investigations are required to substantiate our findings.

KEYWORDS

metabolic dysfunction-associated steatotic liver disease (MASLD), oxidative balance score (OBS), nutrition, NHANES, mortality

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinically common chronic liver disease that pathologically manifests as excessive lipid deposition in liver parenchymal cells (1). With changing lifestyles, NAFLD is becoming prevalent worldwide, and approximately 25% of adults are affected by this disease (2, 3). NAFLD can progress to the development of severe hepatic disease or even liver cell carcinoma, with a lack of effective treatment and a poor prognosis at late stages (4). NAFLD has an indisputable onset and is often asymptomatic in its early stages and is often not appreciated; therefore, early prevention, diagnosis and intervention of NAFLD are needed to slow its progression (5). Following the Delphi Consensus process, the term "metabolic dysfunction-associated steatohepatitis" (MASLD) became the successor to the term "NAFLD" (6) to enhance the precision of nomenclature.

MASLD is a highly heterogeneous disease in which multiple factors, such as genetics, environment, aging, and poor dietary practice, especially the over-intake of high-calorie diets abundant in saturated fatty acids or simple carbohydrates, may be involved in its pathophysiological mechanisms (7). Thus, changing dietary behavior and diet structure is a feasible strategy for the prevention and treatment of MASLD (8). It has been shown that changes in oxidative stress indicators occur in patients with NAFLD, including elevated oxidative stress marker 8-oxo-dG (9) and decreased coenzyme Q10 (10), suggesting that oxidative stress may exert an essential part in the pathophysiological process of MASLD. Therefore, antioxidants in food, including certain antioxidant vitamins and micronutrients, exhibit some antioxidant stress activity and may be effective in preventing and treating MASLD (11, 12). Although studies have shown a relationship between oxidative stress-related nutrients/foods and MASLD, it is possible that dietary interfactions may interfere with the effectiveness of integrated nutrients (13). Therefore, a new strategy based on dietary models related to oxidative and antioxidant modification is needed.

The oxidative balance score (OBS) evaluates an individual's level of oxidative stress by integrating dietary nutrition and lifestyle (14). The OBS was first proposed in 2002 in an epidemiologic study of a Belgian male smoking population, which used vitamin C and β-carotene as the antioxidant constituents and iron as the pro-oxidant constituent and showed that the lower the OBS score was, the higher the level of oxidative homeostasis in an individual, indicating that the lower the oxidative homeostasis score was, the higher the mortality rate in the population (15). Thereafter, numerous studies were conducted to investigate the relationship between OBS and a wide range of chronic disorders, such as type 2 diabetes mellitus (16), cardiovascular disease (17), and colorectal cancer (18). Recently, a study conducted in Iran showed that the greater the adherence to an OBS, the lower the odds of developing NAFLD (19).

Therefore, cross-sectional research based on the 2017–2018 National Health and Nutrition Examination Survey (NHANES) was carried out to evaluate the association between OBS and the degree of hepatosteatosis as measured by quantification of vibration-controlled transient elastography (VCTE) in US adults.

Materials and methods

Study design and data source

The current study is a retrospective, cross-sectional, population-based analysis of data extracted from the 2017–2018 US National Health and Nutrition Examination Survey (NHANES) database, which is intended to provide an assessment of the dietary state of health and nutrition using a sophisticated, multiphasic design to collect and analyze data that are representative of the nation as a whole. An extensive assessment process was required for participants, including a household interview and a mobile examination center (MEC) visit, which consisted of a medical physical examination, specialized measurements, and laboratory tests (20). In this study, liver ultrasound transient elastography (TE) examinations were first used, and participants supplied two 24-h periods of detailed dietary intake information, subsequently used to estimate energy, nutrient and other food component intake. Initial dietary memories were collected over the course of the NHANES visit, and the subsequent ones were gathered over the phone 3–10 days afterwards.

Study population

The present study was originally comprised of 5,856 participants aged ≥18 years. Of these, 4,745 underwent complete elastography. In addition, participants with the following conditions were excluded: (1) missing data (dietary intake information, exercise data, body mass index, waist circumference, cancer history, and education level);(2) MASLD with increased alcohol intake (MetALD), defined as a daily intake of 20–50 g of alcohol for females and 30–60 g daily for males; (3) alcoholic liver disease (ALD), defined as a daily intake of more than 50 g of alcohol for females and more than 60 g daily for males; (4) other underlying etiologies of hepatic disease. Finally, we recruited 2,854 participants in this study (Figure 1).

Study variables

Measurement of MASLD, MASH and liver fibrosis

The severity of liver steatosis was evaluated by the controlled attenuation parameter (CAP) derived from FibroScan®, which is available at the Mobile Examination Center (MEC) using vibration-controlled transient elastography (VCTE) technology (21). According to a previous study, the definition of SLD is a median CAP ≥263 dB/m (22). The SLD participants meet one of the 5 cardiometabolic risk factors as suggested on the recent Delphi consensus statement (23) and excluded MetALD, ALD, underlying etiologies of hepatic disease were defined as MASLD. The definition we used to classify metabolic dysfunction-associated steatohepatitis (MASH) is ALT >25 U/L or AST > 25 U/L on the basis of MASLD (24). Liver stiffness measurements (LSM) determined clinically significant and advanced fibrosis with cut-offs of 8.6 kPa and 13.1 kPa based on Youden's study, respectively (25).

Oxidative balance score

In the present study, we obtain the OBS for each individual participant. According to Azita's study (19), we selected 10 dietary components (selenium, fiber, β-carotene, folic acid, iron, vitamin D, vitamin C, vitamin E, saturated fatty acids, and polyunsaturated fatty acids) and lifestyle components (physical activity, smoking and obesity) for their association with oxidative stress (Table 1). Besides, the coffee/tea consumption and non-steroidal anti-inflammatory drug (NSAID) using participant as important modulator of oxidant balance (26, 27), we added them to the lifestyle components. The dietary components were categorized into quintiles. In the case of dietary antioxidants, the first through fifth quintiles were scored as 0–4. A reversed scoring method was used for dietary pro-oxidants. For NSAID using, we assigned 0: never, 4: regular user. For physical activity, we assigned 0: MET-minute/week≤400, 2: 400 < MET-minute/week ≤1000, and 4: MET-minute/week>1,000. For BMI and WC, we gave the following scores: 0 for BMI ≥ 30 kg/m^2 and male WC ≥ 102 cm/female WC ≥ 88 cm; 2 for BMI ≥ 30 kg/m^2 or male WC ≥ 102 cm/female WC ≥ 88 cm; and 4 for BMI < 30 kg/m^2 and male WC < 102/female WC < 88 cm. With regard to smoking, the scale was as follows: 0 for current smoker (smoked >100 cigarettes in a lifetime

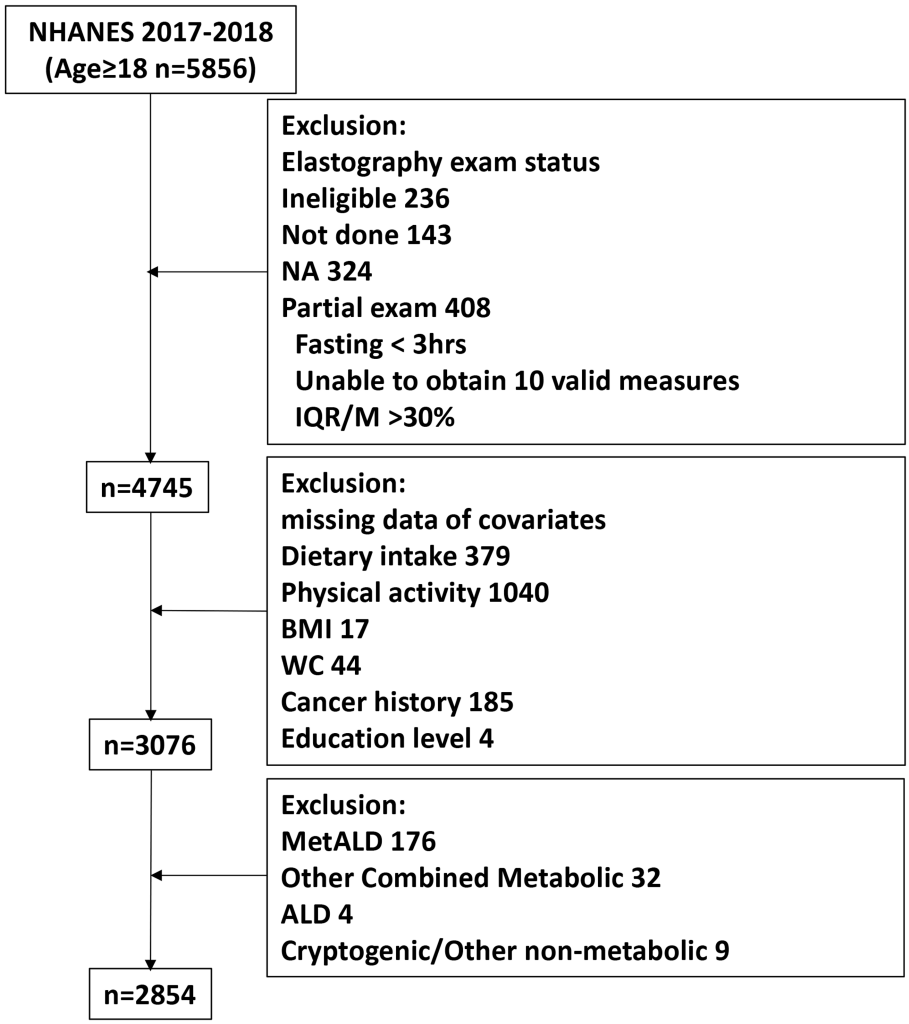

FIGURE 1
Flowchart of the study participants.

and smoked on several days or every day), 2 for ex-smoker (smoked >100 cigarettes in a lifetime and currently do not smoke), and 4 for never smoker (smoked <100 cigarettes in a lifetime). The scores were then added together to calculate the OBS for each subject.

Covariables

Drawing on existing publications and clinical considerations, a selection of covariates that may serve as potential confounders in the relationship between OBS and MASLD was made. Through standardized household interviews, we obtained demographic features, including age, gender, ethnicity, educational level, and financial condition. Educational level was classified into several categories: below high school, high school graduate/GED or equivalent, and college or above. Financial condition was categorized as low (PIR $\leq$ 1.0), medium (1 < PIR $\leq$ 4), and high (PIR > 4). To be evaluated for diabetes, subjects were required to fulfill one of the following criteria: (1) history of insulin use, (2) clinically previous diagnosis of diabetes, (3) taking glucose-lowering medication, and (4) laboratory data HbA1c $\geq$ 6.5% and fasting blood glucose $\geq$126 mg/dL (28). Inclusion criteria for hypertensive patients were as follows: (1) Subjects were required to answer at least one of the following questions by answering "(1) Be ever told on two or more occasions that they have hypertension or (2) Whether they have ever been told about their hypertension and taken prescribed medication" and obtaining at least one affirmative answer. (2) Subjects were identified as having hypertension by a clinical measurement of blood pressure with a mean of three consecutive systolic blood pressure measurements $\geq$140 mmHg or a mean of three consecutive diastolic blood pressure measurements $\geq$90 mmHg. Multiple imputation was performed by R for missing covariables measured at the baseline visit according to a previous study (29).

Statistical analysis

We used weighted samples, strata, and subgroups from the NHANES database and calculated weighted populations for this research using the SURVEY program in SAS. Continuous variables were presented in the form of weighted means and standard errors, with categorical variables presented in the form of unweighted

TABLE 1 Oxidative balance score assignment scheme.

OBS components	Property		Scoring assignment				
			0	1	2	3	4
Lifestyle components							
Physical activity (MET-minute/week)	Antioxidant		≤400		400–1,000		≥1,000
BMI and WC	Prooxidant	Male	BMI ≥ 30 + WC ≥ 102		BMI ≥ 30 or WC ≥ 102		BMI < 30 + WC < 102
		Female	BMI ≥ 30 + WC ≥ 88		BMI ≥ 30 or WC ≥ 88		BMI < 30 + WC < 88
Smoking	Prooxidant		current smoking		former smoking		never smoking
NSAID use	Antioxidant		Never				Regular
Caffeine (mg/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
Dietary components							
Dietary fiber (g/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
Carotene (RE/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
Total folate (mcg/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
Vitamin C (mg/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
Vitamin E (ATE) (mg/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
Selenium (mcg/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
Vitamin D (mcg/d)	Antioxidant		Quintile1	Quintile2	Quintile3	Quintile4	Quintile5
SFA (g/d)	Prooxidant		Quintile5	Quintile4	Quintile3	Quintile2	Quintile1
PUFA (g/d)	Prooxidant		Quintile5	Quintile4	Quintile3	Quintile2	Quintile1
Iron (mg/d)	Prooxidant		Quintile5	Quintile4	Quintile3	Quintile2	Quintile1

OBS, Oxidative Balance Score; MET, metabolic equivalent; BMI, body mass index; WC, waist circumference; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

numbers and weighted proportions. We performed logistic regression analysis using SURVEYLOGISTIC statements to examine the relationship between OBS and MASLD. We performed multivariate regression analyses to adjust for potential confounders identified in single-variable regressions, consisting of age in years, sex, race, education level, and comorbidities (history of diabetes, hypertension, and cancer). Statistical analyses of categorical variables were performed using weighted $\chi 2$ tests, and analyses of continuous variables were performed using weighted linear regression models to identify between-group differences. Stratified multivariate regression analysis was used for subgroup analysis. It is also recommended to use the RCS function when adjusting for continuous exposure to minimize residual confounding (30). In this study, a RCS model with four nodes was used to investigate the relationship between OBS and MASLD prior to and after adjustment for confounders. All analyses were performed using the R program and EmpowerStats. p values less than 0.05 were considered statistically significant.

Results

Participant characteristics

A total of 2,854 participants from NHANES (2017–2018) were included in this study, of whom 1,294 (45.34%) were diagnosed with MASLD based on a previous study. Among all participants with MASLD, the prevalence was higher in males (56.67%) than in females (43.33%) ($p = 0.019$). Patients who had MASLD were much older and were more likely to be non-Hispanic white, with no differences in educational attainment or income between these two groups (Table 2). Baseline comparison of characteristics indicated that patients with MASLD had a greater prevalence of diabetes mellitus (21.72% vs. 4.38%, $p < 0.001$), hypertension (62.12% vs. 37.03%, $p < 0.001$), and smoking (41.79% vs. 39.63%, $p = 0.009$). The MASLD patients also had higher BMIs and waist circumferences. Participants without MASLD had significantly greater OBS ($p < 0.001$) in comparison to participants suffering from MASLD. Comparing laboratory characteristics, we found that ALT, GGT, HSCRP, and cholesterol were significantly higher in the MASLD group, while serum albumin was significantly lower (Table 2). When we separate the MASLD into MASH and MASLD[1](MASLD without MASH)(27.44%) based on ALT and AST, the incidence of MASH was 17.90%. The OBS was decreased in MASH group (Supplementary Table S1). However, when compared participants with clinically significant fibrosis (12.75%) and advanced fibrosis (3.63%) to non-fibrosis, OBS showed no difference in the population ($p = 0.237$) (Supplementary Table S2).

Then, all 2,854 individuals were divided into four groups based on OBS quartiles: group 1 (OBS Q1, OBS: 14 to 25), group 2 (OBS Q2, OBS: 26 to 29), group 3 (OBS Q3, OBS: 30 to 33), and group 4 (OBS Q4, OBS: 34 to 47). Individuals in the 1st quartile (Q1) with lower OBS (as a reference group) were more likely to be younger, women, white, less educated, and with less income. Furthermore, participants in Q1 were more likely to smoke and be less physically active. As OBS quartiles progressively increase, BMI progressively decreases.

Compared with those in the lowest quartile of OBS, subjects in the highest quartile had lower levels of GGT, HSCRP and higher level of albumin (Table 3). Serum GGT (31) and albumin (32) are considered prooxidant and antioxidant indicators in previous studies, respectively. From OBS Q1 to Q4, GGT gradually decreased ($p = 0.019$) and albumin gradually increased ($p < 0.001$).

Relationship between OBS and MASLD/liver fibrosis

Multivariate logistic regression indicated that OBS was statistically correlated with MASLD (Table 4). In model 2, we found that each 1-unit increase in OBS resulted in a 7% reduction in the risk of MASLD (OR = 0.93, 95% confidence interval (CI) 0.90–0.95), suggesting that OBS was negatively associated with the risk of MASLD. In the crude model and model 1, the highest quartile was significantly associated with MASLD compared with the lowest quartile (p for trend<0.001, <0.001). In addition, adherence to the 4th quartile of OBS was also found to be correlated with MASLD after adjusting for confounders in model 2 (OR = 0.30, 95% CI 0.12–0.77; $p = 0.012$, p for trend = 0.001). However, OBS (continuous and categorical) was not associated with the risk of clinically significant fibrosis and advanced fibrosis in the all models (Supplementary Table S3).

Subgroup analysis

Group analyses were performed based on age, gender, race, education level, and history of diabetes, hypertension and cancer (Table 5). Regarding the race and education subgroup, statistical significance was found (interaction $p = 0.008$ and < 0.001). When stratified by race, among non-Hispanic whites, we found that OBS was associated with a lower prevalence of MASLD, whereas in other races, this trend was less pronounced than that in non-Hispanic whites. When considering education level, the negative association between OBS and MASLD was more pronounced among those with college or higher education. However, age stratification, gender subgroup, history of diabetes, HBP, and cancer had no significant effect on the results of the analysis (Table 5).

RCS analysis

We further assessed the relationship between OBS and MASLD with the use of RCS curves and multivariate logistic regression. We identified a nonlinear association between OBS and MASLD (nonlinear $p = 0.315$, 0.256, 0.077) in crude models, model 1 and model 2, respectively (Figure 2). We detected that the risk of MASLD gradually decreased as OBS increased.

Correlation between OBS and all-cause mortality

The relationship between OBS and all-cause mortality was further assessed by Cox proportional hazards models. We found

TABLE 2 Baseline characteristics of all participants by MASLD.

Weighted characteristics of the study population				
Characteristic	Total	MASLD		Value of p
		No	Yes	
N	2,854	1,560	1,294	
Weighted *N*	15,36,53,090	8,81,27,629	6,55,25,460	
Age (years)	46 ± 1	43 ± 1	50 ± 1	<0.001
Gender (%)				0.019
Male	51.61	47.84	56.67	
Female	48.39	52.16	43.33	
Race (%)				<0.001
Mexican American	9.15	6.58	12.59	
Other Hispanic	6.82	7.06	6.51	
Non-Hispanic White	62.49	63.88	60.61	
Non-Hispanic Black	10.62	12.24	8.44	
Others	10.92	10.24	11.84	
Education level (%)				0.165
<high school	9.11	8.31	10.18	
High school graduate/GED or equivalent	26.92	25.54	28.76	
College graduate or above	63.98	66.14	61.06	
Economic status (%)				0.292
Low (PIR ≤ 1)	12.01	12.43	11.45	
Middle (1 < PIR < 4)	48.31	46.33	51.00	
High (PIR ≥ 4)	39.68	41.24	37.55	
Diabetes (%)				<0.001
Yes	11.77	4.38	21.72	
No	88.23	95.62	78.28	
Hypertension (%)				<0.001
Yes	47.73	37.03	62.12	
No	52.27	62.97	37.88	
History of cancer (%)				0.026
Yes	8.99	7.61	10.85	
No	91.01	92.39	89.15	
Smoking status (%)				0.009
Current	16.94	18.57	14.74	
Former	23.62	21.06	27.05	
Never	59.44	60.37	58.21	
NSAID use				<0.001
Never	83.35	88.87	75.93	
Regular	16.65	11.13	24.07	
BMI (kg/m2)	29.1 ± 0.3	26.2 ± 0.3	33.0 ± 0.4	<0.001
Waist circumference (cm)	99.1 ± 0.7	91.2 ± 0.7	109.6 ± 0.8	<0.001

(Continued)

that continuous OBS resulted in an 6% reduction in the risk of all-cause mortality (OR = 0.94, 95% CI 0.86–1.03; $p = 0.204$). While OBS was assessed as a categorical variable, after adjusting for covariables, the OBS Q4 group (HR: 0.15, 95% CI: 0.02–0.87; $p = 0.035$) exhibited a lower risk of all-cause mortality than the OBS Q1 group in all participants (Table 6). When adjusting for liver fibrosis, the lower risk of all-cause mortality in OBS Q4 group still exist (Supplementary Table S4), which means mortality risk might be indirectly affected by OBS itself rather than the level of fibrosis.

TABLE 2 (Continued)

Weighted characteristics of the study population				
Characteristic	Total	MASLD		Value of *p*
		No	Yes	
Physical activity (Met.h/wk)	1,377 ± 54	1,423 ± 64	1,315 ± 59	0.091
OBS	30.10 ± 0.33	31.03 ± 0.44	28.85 ± 0.28	<0.001
OBS				<0.001
Q1	20.87	16.32	26.99	
Q2	26.54	24.89	28.77	
Q3	23.76	24.46	22.82	
Q4	28.83	34.33	21.43	
Laboratory features				
ALT (U/L)	23 ± 1	20 ± 1	27 ± 1	<0.001
AST (U/L)	22 ± 0	22 ± 1	23 ± 0	0.403
GGT(IU/L)	27 ± 1	23 ± 1	33 ± 1	<0.001
Total Bilirubin (mg/dL)	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.150
Albumin (g/dL)	4.14 ± 0.01	4.16 ± 0.02	4.10 ± 0.02	0.006
HSCRP (mg/L)	3.58 ± 0.23	2.82 ± 0.35	4.58 ± 0.18	<0.001
Cholesterol(mg/dL)	190 ± 2	187 ± 1	194 ± 3	0.025

All values represented are weighted means ± SE or weighted percentage. The value of *p* was calculated by a weighted linear regression model and weighted chi-square test, respectively. SE, standard error; PIR, poverty income ratio; BMI, body mass index; WC, waist circumference; MET, metabolic equivalent; OBS, oxidative balance score; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transferase; HSCRP, hypersensitive c-reactive protein.

Discussion

We assessed the association between OBS and MASLD in this large cross-sectional study on the basis of NHANES (ranging from 2017 to 2018). A 15-component-based OBS was used to extensively evaluate exposure through dietary and lifestyle-related exposure to antioxidant and pro-oxidant components, which allowed us to adequately evaluate the complexity of oxidative homeostasis in individuals. We demonstrated in this study that participants without MASLD had significantly higher OBS than those with MASLD. After adjusting for confounders, the effect of OBS on MASLD was shown to be significantly dependent on race and education level.

Extensive studies have suggested that impaired oxidative stress is associated with the occurrence and progression of MASLD/MASH through the overproduction of reactive oxygen species (ROS) to mediate lipid metabolism, insulin signaling, and innate immune signaling pathways (33–35). Either pro-oxidants or antioxidants may act antagonistically or synergistically, while antioxidants may be pro-oxidants at high doses (36). In addition, supplementation with a multitude of dietary antioxidants (vitamin E, vitamin C), as well as lifestyle changes (exercise) may improve clinical markers in part by reducing oxidative stress in NAFLD patients (37). Consequently, there is a need for a comprehensive assessment of index-OBS to evaluate oxidative balance in individuals and to investigate the impact of this balance on MASLD. Prior investigations have examined the potential benefit of this index on NAFLD through inflammation, oxidative stress, and glycolipid metabolism (38). By the way, OBS is not associated with progression of liver fibrosis. A randomized controlled clinical trial showed that vitamin E was associated with improvement in NAFLD but not liver fibrosis (39), while other studies have found that antioxidants are associated with improved fibrosis in NAFLD patients (40, 41). The reason for this consideration may be that OBS is a composite index that includes pro-oxidants and antioxidants, and therefore its combined effect cannot be explained by the alleviation of fibrosis by antioxidants alone.

The traditional OBS contains 20 items related to oxidative stress (16), but a recent study on OBS in NAFLD contained only 13 items (19). In our study, we calculated OBS contained 13 items and added caffeine intake and NSAID using to get a new OBS. In addition, there is evidence suggesting that micronutrients such as vitamins, selenium, carotenoids, and magnesium, which have antioxidant, immunomodulatory, and lipoprotective properties, may play protective roles in NAFLD (42). Polyunsaturated fatty acids (PUFA) are susceptible to lipid peroxidation implicated in NAFLD, while vitamin E has been shown to protect against non-enzymatic lipid peroxidation (43). The accumulation of excess saturated fatty acids (SFA) induces cellular lipotoxic damage and increases the risk of MASLD (44). In our study, the lower intake of antioxidant properties (carotene, vitamin C and vitamin E) and higher intake of prooxidant properties (SFA, PUFA) could be associated with the onset of MASLD. It is important that n-3 and n-6 PUFA have a differing role in oxidative stress. The n-3 PUFA have been shown to be antioxidative. However, higher intakes of n-6 PUFAS have been associated with an increased oxidative stress (45). It's a limitation that we did not categorize PUFAs into n-3 PUFAs and n-6 PUFAs to calculate OBS.

According to our study, lifestyle may have a significant impact on the development of MASLD. In addition to dietary nutrients, there is an important role of a healthy lifestyle in avoiding MASLD. Physical activity, body mass index control and smoking cessation might be effective, which is consistent with previous studies showing the impact of obesity, sedentary lifestyle, smoking and other individual variables and situational factors on NAFLD (46). Studies showed that caffeine could improved fatty liver in ApoE KO mice with NAFLD *in vivo* (47) and NSAID-activated gene-1 (NAG-1), or growth differentiation factor-15 (GDF15), is associated with NAFLD (48). This is the first study to identify OBS included caffeine intake and NSAID as a MASLD protective factor in US adults and find a significant negative association between OBS and MASLD.

The study has both strengths and weaknesses. First, this is the first study evaluating the relationship between OBS and MASLD in US

TABLE 3 Baseline characteristics of all participants by the OBS quartile.

Characteristic	Total	Q1	Q2	Q3	Q4	Value of *p*
		14–25	26–29	30–33	34–47	
N	Total	678	687	674	815	
Age (years)	46 ± 1	43 ± 1	45 ± 1	48 ± 1	48 ± 1	0.001
Gender (%)						0.003
Male	51.61	41.73	51.31	51.63	59.01	
Female	48.39	58.27	48.69	48.37	40.99	
Race (%)						<0.001
Mexican American	9.15	10.20	8.21	10.31	8.29	
Other Hispanic	6.82	4.73	6.19	6.25	9.40	
Non-Hispanic White	62.49	58.20	65.30	62.38	63.08	
Non-Hispanic Black	10.62	16.81	10.84	10.32	6.19	
Others	10.92	10.06	9.46	10.74	13.04	
Education level (%)						<0.001
<high school	9.11	13.58	10.23	7.33	6.30	
High school graduate/GED or equivalent	26.92	38.92	26.41	26.75	18.82	
College graduate or above	63.98	47.49	63.36	65.91	74.88	
Economic status (%)						<0.001
Low(PIR ≤ 1)	12.01	16.45	14.39	11.62	7.08	
Middle(1 < PIR < 4)	48.31	53.95	50.89	44.95	44.71	
High(PIR ≥ 4)	39.68	29.60	34.73	43.43	48.22	
Diabetes (%)						0.254
Yes	11.77	14.73	10.36	9.53	12.79	
No	88.23	85.27	89.64	90.47	87.21	
Hypertension (%)						0.245
Yes	47.73	48.69	50.84	49.01	43.12	
No	52.27	51.31	49.16	50.99	56.88	
History of cancer (%)						0.279
Yes	8.99	8.35	7.19	9.34	10.83	
No	91.01	91.65	92.81	90.66	89.17	
Smoking status (%)						<0.001
Current	16.94	32.93	21.26	11.79	5.63	
Former	23.62	27.86	22.32	24.45	21.05	
Never	59.44	39.22	56.42	63.76	73.32	
NSAID use						<0.001
Never	83.35	94.81	85.44	80.98	75.08	
Regular	16.65	5.19	14.56	19.02	24.92	
BMI(kg/m2)	29.1 ± 0.3	32.2 ± 0.6	30.0 ± 0.5	28.2 ± 0.4	26.8 ± 0.3	<0.001
Waist circumference (cm)	99.1 ± 0.7	106.0 ± 1.1	100.9 ± 1.2	97.4 ± 1.2	93.7 ± 0.8	<0.001
Physical activity (Met.h/wk)	1,377 ± 54	1,228 ± 63	1,325 ± 75	1,294 ± 83	1,601 ± 91	0.009
Laboratory features						
ALT (U/L)	23 ± 1	23 ± 1	26 ± 2	21 ± 1	23 ± 1	0.086
AST (U/L)	22 ± 0	21 ± 1	24 ± 1	21 ± 0	23 ± 1	0.112
GGT(IU/L)	27 ± 1	31 ± 2	29 ± 2	27 ± 2	24 ± 1	0.019
Total Bilirubin (mg/dL)	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.002
Albumin (g/dL)	4.14 ± 0.01	4.05 ± 0.03	4.14 ± 0.02	4.15 ± 0.01	4.18 ± 0.02	<0.001
HSCRP (mg/L)	3.58 ± 0.23	4.95 ± 0.34	3.98 ± 0.29	3.72 ± 0.71	2.11 ± 0.13	<0.001
Cholesterol(mg/dL)	190 ± 2	190 ± 6	190 ± 2	194 ± 2	187 ± 3	0.341

All values represented are weighted means ± SE or weighted percentage. The value of *p* was calculated by a weighted linear regression model and weighted chi-square test, respectively.
SE, standard error; PIR, poverty income ratio; BMI, body mass index; WC, waist circumference; MET, metabolic equivalent; OBS, oxidative balance score; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transferase; HSCRP, hypersensitive c-reactive protein.

TABLE 4 Association of the OBS with MASLD.

OBS	OR (95% CI); value of *p*					
	Crude model		Model 1		Model 2	
Continuous	0.94(0.92,0.96)	<0.001	0.92(0.89,0.94)	<0.001	0.93(0.90,0.95)	0.003
Q1	1.00 (ref)		1.00 (ref)		1.00 (ref)	
Q2	0.70(0.51,0.95)	0.043	0.63(0.47,0.85)	0.014	0.68(0.36,1.26)	0.073
Q3	0.56(0.38,0.83)	0.014	0.45(0.30,0.67)	0.004	0.53(0.22,1.27)	0.052
Q4	0.38(0.26,0.55)	<0.001	0.27(0.18,0.41)	<0.001	0.30(0.12,0.77)	0.012
p for trend		<0.001		<0.001		0.001

Model 1: adjusted for age, gender, and race.
Model 2: Model 1+ education level, diabetes, hypertension, cancer history and serum albumin.
The OBS was converted from a continuous variable to a categorical variable (quartiles). Weighted logistic regression was used to obtain OR, 95% CI and *p* for trend.
Crude model was adjusted with no covariates.

TABLE 5 Subgroup analyses of the association between OBS and MASLD.

Subgroup analyses of the association between OBS and NAFLD, NHANES 2017–2018						
Variables	Q1	Q2	Q3	Q4	*p* for trend	*p* for interaction
Age group						0.178
<60	Ref	0.80 (0.60, 1.07)	0.61 (0.40, 0.93)	0.37 (0.24, 0.56)	0.011	
≥60	Ref	0.39 (0.19, 0.78)	0.43 (0.17, 1.11)	0.24 (0.14, 0.42)	0.017	
Gender						0.316
Male	Ref	0.81 (0.54, 1.23)	0.82 (0.52, 1.31)	0.42 (0.26, 0.69)	0.034	
Female	Ref	0.64 (0.45, 0.92)	0.42 (0.23, 0.76)	0.29 (0.16, 0.52)	0.014	
Race						0.008
Non-Hispanic White	Ref	0.63 (0.39, 1.00)	0.59 (0.34, 1.01)	0.22 (0.14, 0.37)	0.007	
Others	Ref	0.81 (0.55, 1.20)	0.55 (0.39, 0.77)	0.63 (0.38, 1.04)	0.096	
Education level						<0.001
<high school	Ref	0.66 (0.26, 1.63)	0.66 (0.31, 1.40)	0.49 (0.19, 1.25)	0.259	
High school graduate/GED or equivalent	Ref	0.42 (0.23, 0.75)	0.89 (0.49, 1.63)	0.43 (0.20, 0.92)	0.215	
College graduate or above	Ref	0.85 (0.54, 1.33)	0.46 (0.28, 0.77)	0.30 (0.19, 0.48)	0.012	
Diabetes						0.944
Yes	Ref	0.82 (0.32, 2.06)	0.67 (0.24, 1.86)	0.42 (0.19, 0.92)	0.079	
No	Ref	0.69 (0.53, 0.91)	0.57 (0.37, 0.90)	0.34 (0.22, 0.50)	0.008	
Hypertension						0.879
Yes	Ref	0.78 (0.56, 1.09)	0.61 (0.40, 0.93)	0.35 (0.23, 0.53)	0.013	
No	Ref	0.63 (0.41, 0.98)	0.56 (0.28, 1.12)	0.34 (0.21, 0.56)	0.019	
History of cancer						0.141
Yes	Ref	0.69 (0.10, 4.68)	0.19 (0.04, 0.85)	0.16 (0.04, 0.67)	0.034	
No	Ref	0.70 (0.56, 0.89)	0.64 (0.45, 0.93)	0.37 (0.24, 0.57)	0.015	

Data are presented as OR (95% CI). Adjusted for age, gender, race, education level, diabetes, hypertension, cancer history, and albumin. In different stratified policies, stratified variables were not adjusted themselves.

adults. Second, based on the stratified and multistage nature of the NHANES data, our findings have a high generalization among the mobile community. Third, we endeavored to account for various confounders through the use of a questionnaire and massive adjustment for variables. However, there are some limitations to this study. The present study could not effectively assess causality in the nature of the cross-sections. Although we controlled for potential confounders, we still cannot rule out the effect of unknown indexes. Another weakness of this study may be that no examination of alcohol intake was performed. However, since heavy drinkers were excluded, examination of this component was unlikely to influence the outcomes. Further validation of the predictive value of OBS for MASLD through prospective studies is needed to improve the utility of our findings.

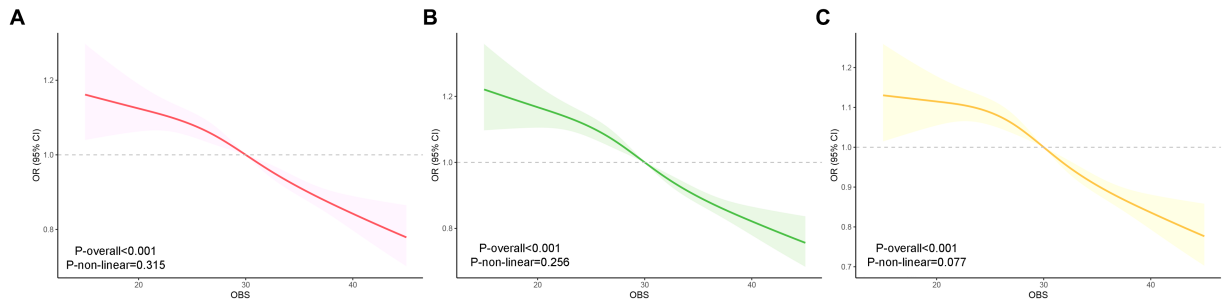

FIGURE 2
RCS analysis of the association between OBS and MASLD. Crude model was adjusted with no covariates. Model 1: adjusted for age, gender and race. Model 2: in addition to the variables in Model 1, education level, diabetes, hypertension, cancer history, and albumin were added. RCS curve of the association between OBS and MASLD of crude model **(A)**, Model 1 **(B)** and Model 2 **(C)**. RCS, restricted cubic spline.

TABLE 6 Associations of OBS with all-cause mortality.

OBS	HR (95% CI); value of *p*					
	Crude model		Model 1		Model 2	
Continuous	0.96(0.88,1.04)	0.337	0.93(0.84,1.01)	0.096	0.94(0.86,1.03)	0.204
Q1	1.00 (ref)		1.00 (ref)		1.00 (ref)	
Q2	0.99(0.42,2.35)	0.987	0.68(0.30,1.58)	0.371	0.88(0.33,2.30)	0.789
Q3	1.82(0.50,6.67)	0.364	1.16(0.33,4.03)	0.820	1.63(0.47,5.65)	0.442
Q4	0.30(0.07,1.22)	0.092	0.17(0.05,0.63)	0.008	0.15(0.03,0.87)	0.035

Model 1: adjusted for age, gender, and race.
Model 2: Model 1+ education level, diabetes, hypertension, cancer history and serum albumin.
The OBS was converted from a continuous variable to a categorical variable (quartiles). Weighted cox regression analysis was used to obtain HR, 95% CI.
Crude model was adjusted with no covariates.

Conclusion

To summarize, it is suggested in this cross-sectional study that OBS is negatively associated with the incidence of MASLD and all-cause mortality in US adults. An negative association was observed between OBS and the prevalence of MASLD in a nationally representative sample of adults in the United States. Our findings confirm the role of healthy food and lifestyle choices in the prevention of MASLD. In addition, they can be used as a strategy to arrest the course of MASLD.

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 studies involving humans were approved by National Health and Nutrition Examination Survey. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LP: Conceptualization, Formal analysis, Funding acquisition, Validation, Visualization, Writing – original draft, Writing – review & editing. LL: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. JL: Investigation, Software, Validation, Writing – original draft. YL: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

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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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1320238/full#supplementary-material

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Hepatoprotective effects of peach gum polysaccharides against alcoholic liver injury: moderation of oxidative stress and promotion of lipid metabolism

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Natural polysaccharides extracted from plants have received increasing attention due to their rich bioactivity. In our study, peach gum polysaccharides (PGPs) were extracted by water extraction-alcohol precipitation method. PGPs are typical pyranose polysaccharides with a mean molecular weight of 3.68×10^6 g/mol. The antioxidant activity and hepatoprotective capacity of PGPs were studied. *In vitro*, assays showed that PGPs scavenged DPPH, OH, and O_2^- in a dose-dependent manner. PGPs exhibited antioxidative properties against alcohol-induced HL7702 cells, as evidenced by the normalization of MDA, SOD, ROS, and GSH levels. To further elucidate the hepatoprotective mechanism of PGPs, we carried out *in vivo* experiments in male mice. PGPs exerted hepatoprotective effects in alcohol liver disease (ALD) mice by exerting antioxidant effects, decreasing the inflammatory response and modulating lipid metabolism. In addition, metabolomic analysis indicated that PGPs mainly regulate D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, and arginine biosynthesis to promote hepatic metabolism and maintain body functions. Overall, this study revealed that the hepatoprotective mechanism of PGPs against ALD might be associated with the regulation of oxidative stress and lipid metabolism.

KEYWORDS

peach gum polysaccharides, structural analysis, antioxidant activity, alcoholic liver damage, metabolomic

1 Introduction

Peach gum (PG), a gelatinous substance secreted by the bark of the peach tree, is recorded in the *Compendium of Materia Medica* as alleviating pain (1). Generally, the key component of PG is a macromolecule polysaccharide (2). PGPs consist of (1→3) and (1→6) linked Galp unit backbones and highly branched macromolecular structures (3). PGPs with highly branched macromolecular structures show favorable bioactivity, emulsifying performance, and antioxidant and antibacterial activity (1). Modern scientific research has found that PGPs exert hypoglycemic and hypolipidemic properties, as well as improve intestinal flora and enhance human immunity (4).

Alcoholic liver disease (ALD) is a clinical form of liver injury due to excessive alcohol intake. The latest epidemiological survey found that ALD is still one of the most common diseases that seriously endangers public health worldwide, showing an increasing yearly trend (5). Alcohol intake increases the amount of nicotinamide adenine dinucleotide/NAD+ produced by the oxidation of ethanol to acetaldehyde in hepatocytes, thereby disrupting fatty acid oxidation. The natural course of alcoholic liver disease follows the pattern of steatosis - alcoholic hepatitis - fibrosis - cirrhosis - hepatocellular carcinoma. Hepatic steatosis, defined histologically as intracellular fat deposition, is an early reversible stage of ALD due to ethanol exposure (6–8). The liver, the main site of ethanol metabolism, contains high levels of alcohol dehydrogenase (ADH), which catalyzes the oxidation of ethanol to the toxic metabolite (9). Oxidative metabolites produced by alcohol metabolism, including acetaldehyde and free radicals, dominate the pathological progression of the alcoholic liver (10). The large morbidity and mortality rates associated with ALD are primarily prevented through political measures to reduce the availability of alcohol (7). However, a limited number of drugs with low adverse effects are available to mitigate alcoholic liver injury (5). Therefore, current research is focused on finding and utilizing natural active ingredients with low toxicity and high efficiency.

Polysaccharides derived from some organisms exhibit excellent anti-ALD activity (5, 11). The hepatic metabolic burden can be attenuated due to the antioxidative and hypolipidemic properties of polysaccharides. Furthermore, the anti-ALD characteristics of polysaccharides were attributed to hepatoprotective effects in regulating fatty acid metabolism and reducing reactive oxygen species production (12). Nepali et al. reported that polysaccharides inhibited the mRNA expression of CYP2E1 and NADPH oxidase in ethanol-fed mice, which was considered to moderate oxidative stress and lipid accumulation induced by alcohol intake (13). Unlike drugs, natural PGPs can be developed as functional foods for daily intake to provide preventive control of ALD. Additionally, the biological activity and high yield of PGPs that have been characterized make them broad prospects for development. However, the alleviation of ALD by PGPs has never been reported. The great exploitation potential of PGPs provides more profitable possibilities for farmers. Therefore, we focused on the protective mechanism of ALD to investigate the bioactivity of PGPs.

In our study, PGPs were extracted with hot water, and their monosaccharide composition and molecular weight distribution were characterized. The hepatoprotective activity of PGPs on ALD models was evaluated *in vitro* and *in vivo*. Metabolomics techniques were used to explore potential mechanisms of action. Our results suggest that PGPs exert protective effects against ALD in terms of decreasing oxidative stress and regulating lipid metabolism. We preliminarily explored the protective mechanism of PGPs against ALD, aiming to provide a theoretical basis for the development of related functional products as in **Figure 1**.

2 Materials and methods

2.1 Materials and chemicals

Raw peach gum powder was provided by Suizhou Peach Gum Industry, Hubei, China. Methanol, acetonitrile, and formic acid were chromatographically pure from Merck Chemical Co. (Darmstadt, Germany). Trichloromethane, anhydrous ethanol, sulfuric acid and standard monosaccharides were purchased from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China). The chemicals and reagents used in our experiment were analytically pure.

Malonaldehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), γ-gamma glutamyl transpeptidase (γ-GGT), and triglyceride (TG) assay kits were purchased from Nanjing Jiancheng Biological Engineering Institute. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alcohol dehydrogenase (ADH), and acetaldehyde dehydrogenase (ALDH) assay kits were purchased from Solarbio Science and Technology Co. Reactive oxygen species (ROS) assay kits and ELISA kits were purchased from Beyotime Biotechnology Institute.

2.2 Preparation of PGPs

After passing through a100 mesh sieve, the raw peach gum powder was added to Milli-Q water at 1:100 (g/mL), stirred at 90 °C for 2 h, extracted twice, and centrifuged at 4500 rpm for 15 min. The supernatant was concentrated to 1/5 using a rotary evaporator, and four times the volume of anhydrous ethanol was added. The peach gum-ethanol solution was placed at 4 °C overnight to

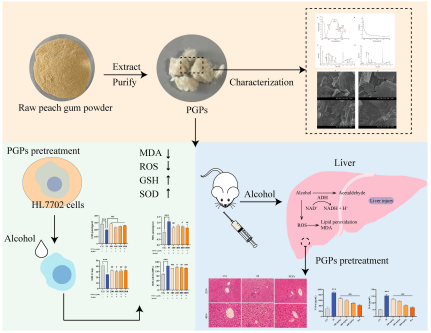

FIGURE 1
Preliminarily explored the protective mechanism of PGPs against ALD.

precipitate the crude polysaccharides. The crude polysaccharide was redissolved in Milli-Q water at 1:25 (g/mL) and deproteinized by Sevage reagent (chloroform: n-butyl alcohol = 4:1, V/V). Dialysis was performed using a dialysis bag with a molecular weight cutoff of 14000 Da for 48 h. Vacuum freeze-drying was used to obtain PGPs.

2.3 Characterization of PGPs

Carbohydrates in PGPs were determined by the phenol-sulfate method using glucose as a standard. The structure of PGPs was observed by scanning electron microscope (SEM). A total of 1 μg/mL PGPs-water solution was dropped on a clean and flat mica substrate, naturally air-dried and observed using an atomic force microscope equipped with a silicon SPM probe (AFM; Nanosurf, CH) (14).

The molecular weight (M_w) of PGPs was estimated by high-performance size-exclusion chromatography (HPSEC) (11). High-performance anion-exchange chromatography (HPAEC) equipped with a pulsed amperometric detector (PAD; Dionex ICS 5000 + system, USA) was used to determine the monosaccharide composition according to the method of Wang et al. (15).

The PGPs (2 mg) were fully ground with 200 mg of KBr under infrared light irradiation drying conditions and scanned in the wavelength range of 400–4000 cm^{-1} using a Fourier transform infrared spectrometer (FTIR; PerkinElmer, USA). An ultraviolet-visible spectrophotometer (UV-Vis; Thermo, USA) was used to scan at 190–400 nm.

2.4 *In vitro* free radical scavenging assay

2.4.1 2-Diphenyl-picryl hydrazine free radical (DPPH) scavenging ability

The 2-diphenyl-picryl hydrazine free radical (DPPH) scavenging ability was determined according to the method of Yao et al. (16) with slight modifications. PGPs solutions ranging from 1 to 10 mg/mL were prepared. Then, 0.2 mL DPPH radical solution (0.4 mol/L in anhydrous ethanol) and 2 mL deionized water were successively added to 1.0 mL PGPs solution. The solution was mixed and left in the dark for 30 min at room temperature. The control (deionized water instead of PGPs solutions) was used as a calibrator, and the absorbance was measured at 517 nm wavelength. Vitamin C was used as a positive control.

$$\text{DPPH scavenging ability} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100\% \quad (1)$$

where A_0 represents the blank absorbance (water instead of PGPs solution), A_1 represents the absorbance of the complete reflection system, and A_2 represents the absorbance of the reaction system without DPPH solution (using water instead).

2.4.2 Hydroxyl radical (OH) scavenging ability

Hydroxyl radical (OH) scavenging ability was determined according to the method of Long et al. (17) with slight modifications. Two milliliters of $FeSO_4$ solution (6 mmol/L), 2 mL of H_2O_2 solution (6 mmol/L) and 2 mL of salicylic acid solution (6 mmol/L) were successively added to 2 mL of PGPs solution (1–10 mg/mL). After standing for 30 min, the absorbance was measured at a wavelength of 510 nm. Vitamin C was used as a positive control.

$$\text{OH scavenging ability} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100\% \quad (2)$$

where A_0 represents the blank absorbance (water instead of PGPs solution), A_1 represents the absorbance of the complete reflection system, and A_2 represents the absorbance of the reaction system without H_2O_2 solution (using water instead).

2.4.3 Superoxide anion free radical (O_2^-) scavenging ability

Referring to the method of Zhang et al. (18), the O_2^- scavenging ability was determined. Then, 4.5 mL of Tris-HCl buffer solution (50 mmol/L, pH 8.2) and 0.4 mL of pyrogallol solution (25 mmol/L) were successively added to 2 mL of PGPs solution (0.1–1.0 mg/mL). After a 25-min water bath at room temperature, 1 mL of HCl (8 mmol/L) solution was added, and the absorbance was quickly measured at 325 nm. Vitamin C was used as a positive control.

$$O_2^- \text{ scavenging ability} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100\% \quad (3)$$

where A_0 represents the blank absorbance (water instead of PGPs solution), A_1 represents the absorbance of the complete reflection system, and A_2 represents the absorbance of the reaction system without pyrogallol solution (using water instead).

2.5 *In vitro* cell experiments

2.5.1 Cell culture and treatment

HL7702 human hepatocytes were obtained from the China Center for Type Culture Collection. HL7702 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution in sterile culture flasks. Cells were maintained at 37 °C with 5% CO_2 in a CO_2 incubator (Memmert, Germany).

2.5.2 Cell viability assay

The methyl thiazolyl tetrazolium (MTT) method was used to individually determine cell viability after PGPs and alcohol intervention. Then, 0 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, 800 μg/mL, and 1000 μg/mL PGP solutions and 0, 0.5, 1, 2, 3, and 4% alcohol were added to RPMI 1640 medium, respectively. Cells were seeded into 96-well plates and cultured for 24 h to confirm cell apposition. The old medium was discarded and the cells were washed with PBS before the addition of configured PGPs solution or alcohol. After incubation in PGPs solution for 24 h or in ethanol for 12 h, 10 μL/well of MTT solution was added to the plate for 4 h of incubation. Absorbance values were measured at 570 nm after full dissolution with 100 μL/well of methyl saliva.

2.5.3 Detection of intracellular MDA, GSH, SOD, and ROS

According to the MTT experiment results, 200 μg/mL, 400 μg/mL, 800 μg/mL, and 1000 μg/mL were selected as the PGPs solution concentrations for the experimental group, and 3% alcohol was used for modeling. Cells were seeded into 6-well plate, treated with PGPs solution for 24 h, removed and washed with PBS, followed by replacement with 3% alcohol for 12 h. Cells were collected. The intracellular MDA, GSH, SOD, and ROS contents were determined according to the manufacturer's protocol. The control, model, and PGPs pretreatment groups were named CG, M, 200, 400, 800, and 1000, respectively.

2.6 Hepatoprotective effects of PGPs *in vivo*

2.6.1 Animal treatment and modeling

Acute alcohol exposure after polysaccharide intervention is a widely used model of ALD (19–21). Therefore, we selected this type of acute ALD model for experiments to better simulate the body's resistance to ALD after daily intake of PGPs. Forty-eight SPF KM male mice (No. 42000600048880) were obtained from Hubei Laboratory Animal Research Center. Mice were maintained in a specific pathogen-free environment with room temperature at 25 ± 4°C, 60 ~ 70% relative humidity, and 12 h light alternated. After 1 week of adaptive feeding, the mice were randomly divided into 6 groups, including the control group (CG), model group (M), 150 mg/kg bifendate positive control group (Pos), 200 mg/kg PGPs group (200), 400 mg/kg PGPs group (400), and 800 mg/kg PGPs group (800), with 8 mice in each group. Gavage was administered daily at 10 am for 14 consecutive days at the above dose, and mice in the CG and M groups were treated with 0.9% saline instead of PGPs or bifendate. Two hours after the last gavage, mice in the model and PGP groups were administered 50% ethanol (12 mL/kg body weight), and 6 h later, 50% ethanol was gavaged again at a dose of 10 mL/kg (22). Equal amounts of 0.9% normal saline were given to the control group. Experimental mice were sacrificed after overnight fasting, and blood and liver were collected. The experimental design and operating procedures were approved by the Animal Welfare Ethics Committee of Wuhan Polytechnic University (WPU202209001). The experimental plan was shown in **Figure 2**.

2.6.2 Biochemical assays

Precooled saline was added to the liver tissue at a ratio of 9:1, and the homogenate was prepared using a high-throughput tissue grinder. Blood was centrifuged at 3000 rpm for 10 min at 4 °C and the supernatant serum was collected.

The contents of ALT, AST, and γ-GGT were measured in the serum. The oxidative stress indicators GSH, MDA, CAT, and SOD and the alcohol-metabolizing enzyme (ADH, ALDH) and TG were measured in liver homogenates using analytical kits. The levels of lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) in liver homogenate were measured by ELISA.

2.6.3 Histopathological observation

Liver tissue of 5 mm × 5 mm × 3 mm size was harvested from the middle-left lobe of the mouse liver and fixed in tissue fixative solution (4% paraformaldehyde) for hematoxylin-eosin (H&E) staining and oil red staining.

2.6.4 Untargeted metabolomics analysis

A mixture of organic solvent (acetonitrile: methanol: water = 2:2:1, v/v/v) was added to 50 mg of liver tissue in a 2 mL EP tube and fully ground for 5 min using a high-throughput tissue grinder. Tissue homogenates were vortexed for 10 min followed by 30 min of sonication in an ice water bath. After centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatant was aspirated, and nitrogen was blown to dryness using an automatic nitrogen blower at room temperature. The dried sample was redissolved in 200 μL of acetonitrile-water (1:1, v/v), centrifuged at 15,000 rpm for 10 min at 4 °C to obtain the supernatant, and transferred to an injection bottle equipped with a lined tube for measurement. QC samples, consisting of all liver samples mixed in equal volumes, were used to test the stability of the analytical method.

Untargeted metabolomics analysis was performed on an Orbitrap Exploris 120 mass spectrometer coupled with a Vanquish UHPLC system (Thermo Fisher Scientific). Metabolites were separated using a Waters ACQUITY HSS T3 column at a flow rate of 0.4 mL/min. The column temperature was 45 °C, and the injection volume was 8 μL. Mobile phase A was 0.1% formic acid water, and mobile phase B was acetonitrile. The gradient elution program was as follows: 5% B for 0~1 min, 5–30% B for 1~3 min, 30–80% B for 3~5 min, 80–100% B for 5~13 min, 100–5% B for 13~13.1 min, and 5% B for 15 min. Mass spectra were obtained in full scan-ddms2 mode with an H-ESI ion source. Mass spectrometry conditions were as follows: spray voltage, 4000 V in the positive ion mode and 3500 V in the negative ion mode; sheath gas: 30 Arb; ion transfer tube temp: 320 °C; vaporizer temp: 350 °C; orbitrap resolution: 12000. For sequence acquisition, a QC sample was interspersed every six needles.

2.7 Statistical analysis

Data analysis was conducted by using Microsoft Office Excel 2016 (Microsoft., USA) and SPSS Statistics 27 (Microsoft., USA). MS-DIAL 4.7 was used to perform peak alignment, retention time correction, and peak area extraction, parameters were shown in **Supplementary Table 1**. SIMCA-P 14.1 and R 4.2.3 software were used for multivariate statistical analysis. Metabolic pathway analysis was performed in Metaboanalyst 5.0.

3 Results

3.1 Molecular weight and monosaccharide composition of PGPs

In our study, PGPs contained 92.12 ± 0.85% total carbohydrate. HPSEC analysis showed that PGPs contained three fractions, with molecular weights estimated to be 5.010 × 10^6 g/mol (68.5%),

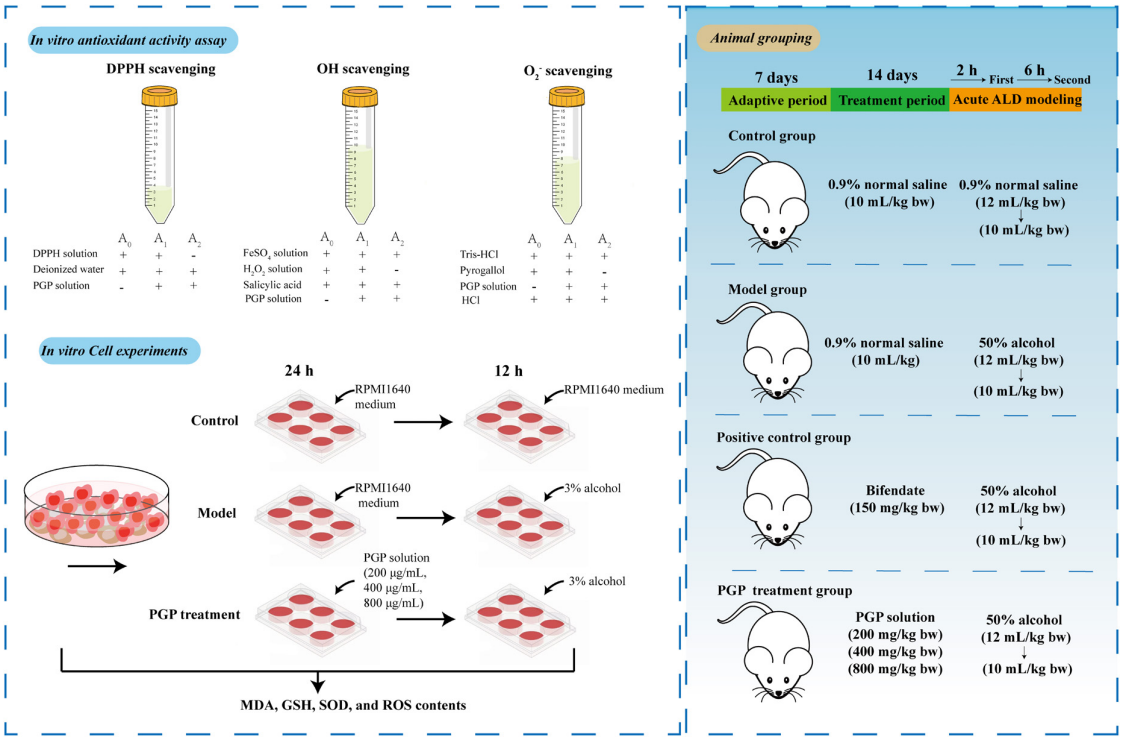

FIGURE 2
Scheme for the study of the protective mechanism of PGPs against alcoholic liver injury.

7.888 × 10^5 g/mol (28.3%), and 7.098 × 10^5 g/mol (3.2%) (Supplementary Figure 2). The mean molecular weight of PGPs was 3.68 × 10^6. The monosaccharide composition of the PGPs is shown in Table 1, consisting mainly of Ara, Gal, Xyl, Glc-UA, and Man (Figures 3A,B).

3.2 Spectral analysis and morphology of PGPs

The FT-IR spectrum of PGPs is shown in Figure 3C. The characteristic peak at 3400.6 cm^{-1} was assigned to the O-H stretching vibration of saccharide. The weak absorption peaks at 2924.0 cm^{-1} attributed to the tensile vibration of C-H (CH, CH2, CH3) are typical of polysaccharides. In addition, the weak vibrations at 1611.6 cm^{-1} and 1422.3 cm^{-1} were attributed to C = O(-COOH) symmetric stretching vibrations. The characteristic peak at 1039.9 cm^{-1} indicated the presence of pyranoside in PGPs (23). The characteristic absorption peak at approximately 895 cm^{-1} indicated that the glycosyl residues of PGPs are mainly β-type glycosidic bonds (24). In conclusion, PGPs are typical pyranose polysaccharides linked by a β-glycosidic bond. Figure 3D shows the UV-vis spectrum of PGPs at 190–400 nm, without obvious absorption peaks appearing at 200–280 nm, indicating the absence of nucleic acids and proteins in PGPs (4).

Peach gum polysaccharides are lamellar structures with a relatively smooth surface containing pore-like structures resulting from water volatilization (Supplementary Figure 1). AFM images showed that the chain morphology of PGPs was irregular spherical conformations with the height about 1.0 nm (Figure 3E). This result is consistent with previously study that reported on the structure of PGPs (25). Due to van der Waals forces between polysaccharide chains or hydrogen bond interactions between molecules, polysaccharide chains are wound to form a spherical structure (14).

TABLE 1 Monosaccharide contents of PGPs.

Monosaccharide composition	Content (μ g/mg)	Percentage (%)
Arabinose (Ara)	208.93	43.90
Galactose (Gal)	167.60	35.12
Xylose (Xyl)	53.98	11.47
Glucuronic acid (Glc-UA)	17.52	3.72
Mannose (Man)	16.41	3.49
Glucose (Glu)	2.29	0.49
Galacturonic acid (Gal-UA)	1.94	0.41
Ribose (Rib)	1.81	0.38
Fucose (Fuc)	0.21	0.04

3.3 Scavenging ability for DPPH, OH, and O_2^-

The DPPH, OH and O_2^- scavenging abilities of PGPs are shown in Figure 4, and excellent antioxidant ability was observed. The ability of PGPs to scavenge free radicals was dose-dependently

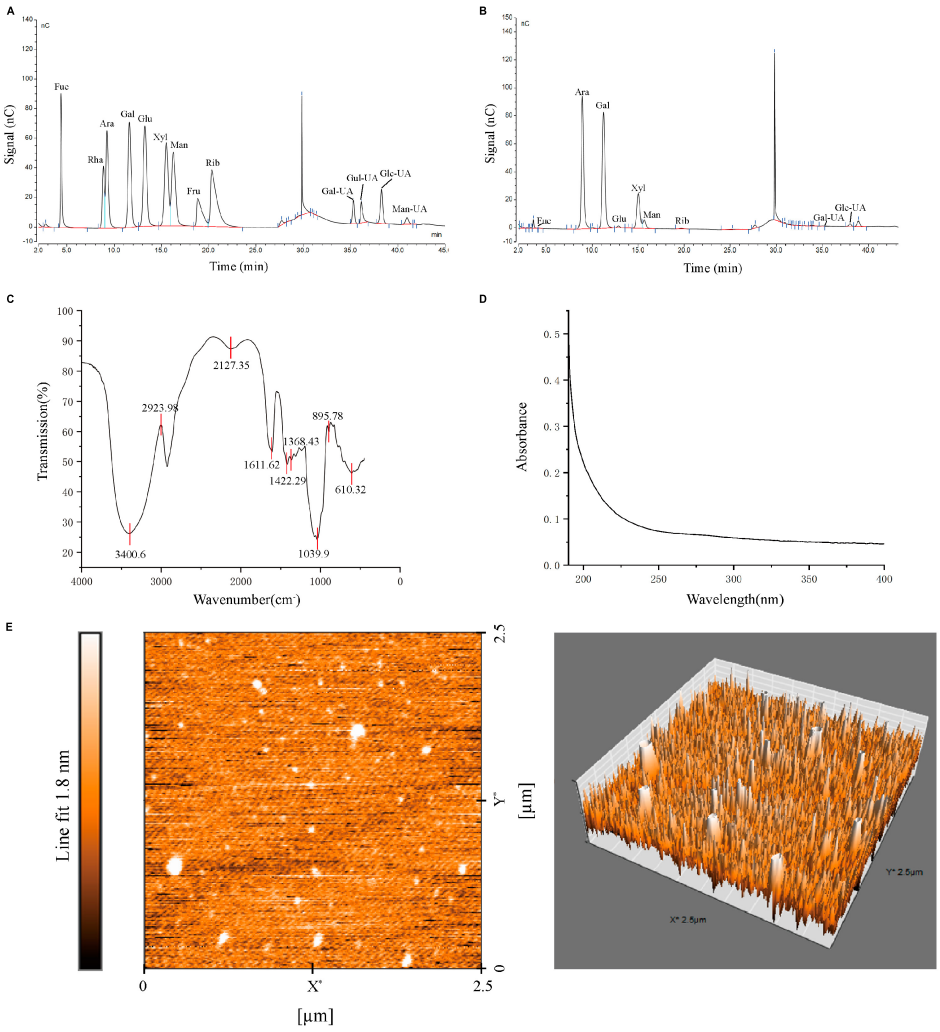

FIGURE 3
Characterization of PGPs. **(A)** Monosaccharide composition of standard. **(B)** Monosaccharide composition of PGPs. **(C)** FT-IR spectra. **(D)** UV-Vis spectra. **(E)** AFM images of PGPs.

enhanced. As the concentration of PGPs gradually increased from 1 mg/mL to 10 mg/mL, the DPPH scavenging rate increased from 24.59 ± 2.17% to 64.48 ± 2.06%, and the OH scavenging rate increased from 21.44 ± 2.52% to 61.11 ± 3.39%. For O_2^-, the free radical scavenging ability of PGPs increased from 16.77 ± 4.39% to 52.56 ± 6.73% at concentrations ranging from 0.1 mg/mL to 1.0 mg/mL. The IC_{50} values of scavenging ability for DPPH, OH, and O_2^- were 6.93 mg/mL, 6.45 mg/mL, and 0.99 mg/mL, respectively. This result confirms the antioxidant ability of PGPs *in vitro*.

3.4 Effects of PGPs and alcohol on the viability of HL7702 cells

As shown in Figure 5A, there was no significant difference in the viability of HL7702 cells exposed to different concentrations of PGPs (12.5, 25, 50, 100, 200, 400, 800, and 1000 μg/mL), indicating that the interference of PGPs on cell activity was limited. The cytotoxic effects induced by alcohol on cell viability were evaluated (Figure 5B) to select the intervening concentration of alcohol in the cell assay. The percentage of cell vitality decreased from 92.33% to 71.88% when the alcohol content was steadily increased from 0.5% to 4%, and a significant ($P < 0.01$) dose-dependent decrease was observed. The cell viability was 81.25% at 3% alcohol concentration, which could effectively establish an acute ALD cell model without causing a large number of cell death. Therefore, a 3% alcohol concentration and incubation for 12 h was selected as the *in vitro* alcohol damage to HL7702 cells to guarantee the quantity of cells for further tests.

3.5 Effect of PGPs on oxidative stress in HL7702 cells

HL7702 cells were cultured with 3% alcohol medium for 12 h. Compared with the CG group, a significant decrease ($P < 0.001$) of 29.09% and 56.57% in the intracellular GSH and SOD contents was observed in the M group (Figures 5C, E). The MDA and ROS

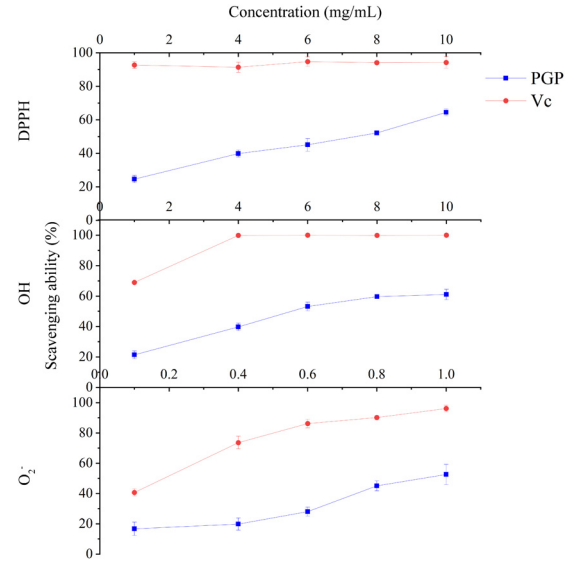

FIGURE 4
In vitro antioxidant capacity of PGPs.

contents in M group were significantly increased ($P < 0.001$) by 84.41% and 51.06%, respectively (Figures 5D, F). Cells showed significant oxidative damage in the presence of 3% alcohol. Under the protective influence of PGPs, the contents of GSH ($P < 0.001$) and SOD ($P < 0.05$) increased significantly, and the contents of MDA ($P < 0.05$) and ROS ($P < 0.05$) decreased significantly, demonstrating a considerable antioxidative stress effect.

3.6 Hepatoprotective effect of PGPs in mice

Serum levels of ALT, AST, and γ-GGT were used to assess liver function (Figures 6A–C). The contents of ALT, AST, and γ-GGT in M group were significantly increased under alcohol intervention ($P < 0.001$), indicating liver injury in the mice. Although the effects of PGPs on ALT and γ-GGT levels were not significant, PGPs exhibited a dose-dependent reduction in alcohol-induced dysregulation of ALT, AST, and γ-GGT levels. The AST levels in the serum of alcohol-induced mice were significantly reduced under the effect of 400 and 800 mg/kg PGPs ($P < 0.05$).

The HE-stained sections of liver tissue from each group are shown in Figure 6D. In the CG group, the hepatocytes had normal structure and regular morphology, and were neatly arranged around the central vein, with regular round nuclei and visible cell gaps. In the M group, the hepatocytes were disordered, swollen or even disintegrated, the boundary was fused, and the hepatocyte blisters degenerated into honeycomb-like structures. Hepatocyte damage was significantly ameliorated in the PGPs and Pos groups, and the degree of hepatocyte swelling, disintegration, and vacuolar degeneration was reduced. The PGPs-800 group showed less damage than the low-dose group, and the hepatocytes were relatively neatly arranged with clear cell gaps.

The oil red-stained sections of liver tissue from each group are shown in Figure 6E. No obvious red lipid droplets were observed in the CG group, while dense and extensive red lipid droplets were observed in the livers of M group, indicating that lipid metabolism disorder occurred in the ALD mice. In contrast, the accumulation of lipid droplets in the livers of PGPs-pretreated mice was mitigated and the degree of denseness was also reduced. Liver TG levels were examined to further confirm the protective effect of PGPs against alcohol-induced disorders of hepatic lipid metabolism (Figure 7A). The TG contents were significantly decreased ($P < 0.05$) in the PGPs and Pos groups, with reductions of 35.53% and 48.03% in the PGPs-800 and Pos groups, respectively. PGPs decreased TG contents in a dose-dependent manner, and TG content in PGPs-800 group was 104.30% of that in CG group. TG content of Pos group decreased to 84.13% of CG group. From histopathologic observations, the number of red lipid droplets in the PGPs-800 group approached that of the CG group, while the Pos group contained fewer lipid droplets than the CG group. The drug bifendate (Pos), which is directed at reducing aminotransferases, increases hepatocyte activity and accelerates lipid droplet metabolism.

3.7 Alcohol metabolizing enzymes, oxidative stress markers and inflammatory factors in mouse livers

Under alcohol intervention, significant differences ($P < 0.001$) in alcohol metabolizing enzymes (ADH, ALDH), oxidative stress indices (MDA, GSH, SOD, CAT), and inflammatory factors (TNF-α, IL-6, LPS) were observed in M group compared with CG group (Figures 7, 8). Pretreatment with PGPs and bifendate significantly attenuated ($P < 0.01$) alcohol-induced abnormal increases in MDA, TNF-α, IL-6, and LPS and abnormal decreases in GSH, SOD, and CAT. A dose-dependent trend was observed in the changes in TNF-α, IL-6, LPS, GSH, SOD, and CAT contents. Compared with M group, the contents of MDA, TNF-α, IL-6 and LPS in PGPs-800 group were decreased by 44.67%, 43.64%, 26.16% and 36.70%, respectively. The contents of GSH, SOD, and CAT in the PGPs-800 group were increased by 71.73%, 33.21%, and 34.49%, respectively. The ADH and ALDH contents were significantly decreased by 56.22% and 68.3% in M group compared to CG group ($P < 0.001$). In contrast, the intervention of 200 mg/kg and 400 mg/kg PGPs in improving ethanol metabolism's enzymatic activity were insignificant ($P > 0.05$). ALDH content was significantly ($P < 0.01$) increased by the intervention of 800 mg/kg of PGPs.

3.8 Metabolomics was used to verify the hepatoprotective effect of PGPs

Metabolites with coefficient of variation (CV) values greater than 30% in QC samples were eliminated from statistical analysis for data quality control. The results of the multivariate statistical analysis are shown in Table 2. The principal component analysis (PCA) model was established to preliminarily understand the overall difference in metabolites and reflect the variation within each group. R^2X was 0.70 and Q^2 was 0.41 in the positive ion mode, and R^2X was 0.79 and Q^2 was 0.50 in the negative ion mode. Both

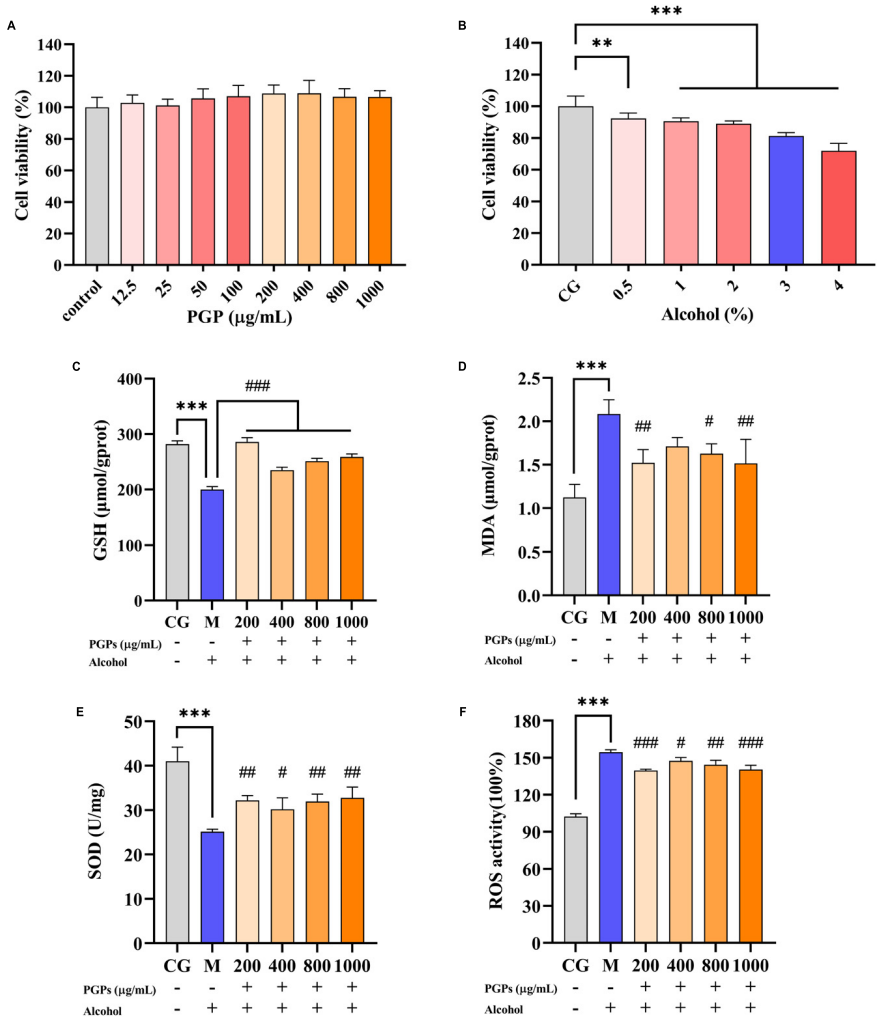

FIGURE 5
Protective effect of PGPs on alcohol-induced HL7702 cells. **(A)** Viability of HL7702 cells cultured with PGPs for 24 h. **(B)** Viability of HL7702 cells cultured with different concentrations of alcohol for 12 h. **(C)** GSH contents. **(D)** MDA contents. **(E)** SOD contents. **(F)** ROS contents. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, for Group M compared with group CG. #, ##, and ### indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, for group PGPs compared with group M.

R^2X and Q^2 were greater than 0.4 in the positive and negative ion modes, indicating the reliability of the statistical model. Orthogonal partial least squares discriminant analysis (OPLS-DA) was used for pairwise comparison to observe the separation between groups and aggregation within groups (Figures 9A, B). R^2X was 0.55 to 0.85, R^2Y was 0.94 to 1, Q^2 was 0.62 to 0.96, and all parameters were greater than 0.5, indicating satisfactory stability and prediction ability. The intercepts of Q^2 on the Y-axis after 200 permutation tests ranged from −0.8 to −0.28, which were less than 0, indicating that OPLS-DA was not overfitting (Figures 9C, D). The variable importance in projection (VIP) values in the OPLS-DA test of the control group (CG), model group (M), and high-dose PGPs group (800) were used to further screen differential metabolites.

The amplitude of changes in metabolites is shown as volcano plots (Figures 9E, F). According to the criteria of VIP > 1, P value < 0.05 of Student's t test, and fold change (FC) ≥ 1.5 or FC ≤ 0.5, twenty differential metabolites (8 up and 12 down) were screened between CG and M groups. Eighteen differential metabolites (16 up and 2 down) were screened between M and 800 groups. All these differential metabolites were identified by matching with the PubChem (PubChem (nih.gov)), KEGG (KEGG: Kyoto Encyclopedia of Genes and Genomes), and HMDB (Human Metabolome Database (hmdb.ca)) databases. The 10 differential metabolites common to CG vs. M and M vs. 800 are summarized in Table 3. The levels of these common differential metabolites were significantly downregulated in M group compared with CG group, while an upregulation trend was observed after pretreatment with a high dose (800 mg/kg) of PGPs (Figure 9G).

The pathway analysis is shown in Figure 9H and Supplementary Table 2. Pathway impact represents the ratio of the number of differential metabolites in a pathway to the total number of metabolites annotated to this pathway; a higher value indicates a greater degree of enrichment. According to the principle of pathway impact > 0.1 and $P < 0.05$, a total of three metabolic pathways were screened, including D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, and arginine biosynthesis.

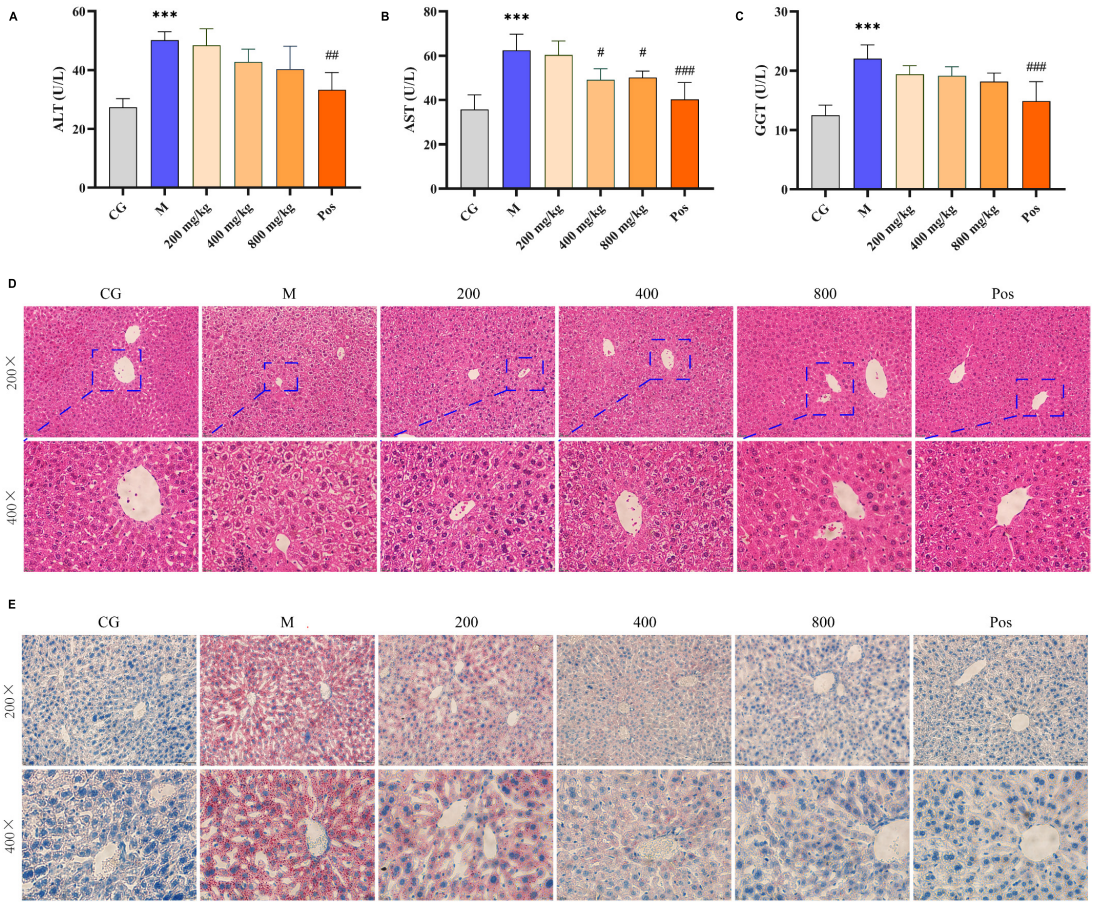

FIGURE 6
Serum levels of AST **(A)**, ALT **(B)**, and γ-GGT **(C)**. Morphological observation of the liver in alcohol-induced mice. **(D)** H&E staining. **(E)** Oil red staining. *** indicate $P < 0.001$, respectively, for Group M compared with group CG. #, ##, and ### indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, for group PGPs compared with group M.

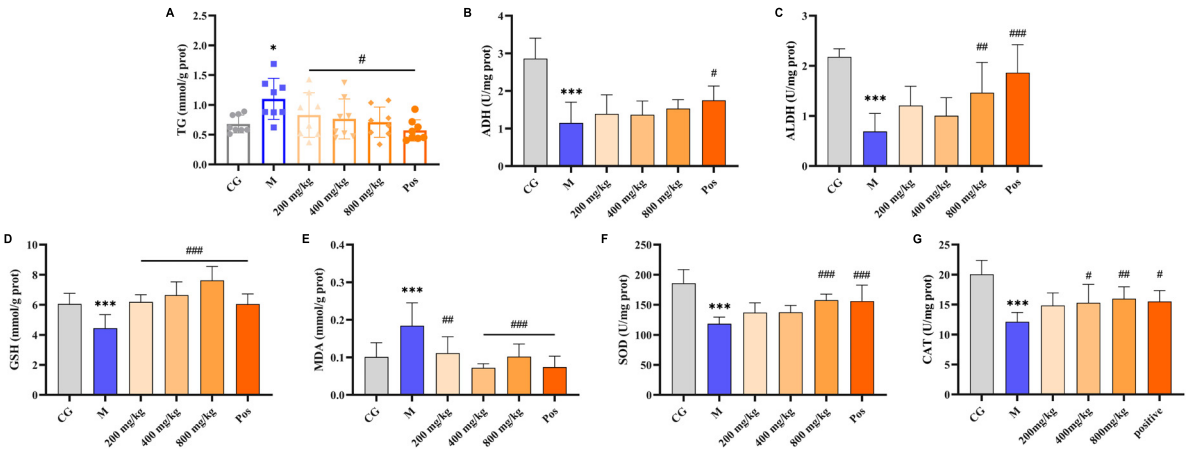

FIGURE 7
Effects of PGPs on biochemical constituents in mouse livers. **(A)** TG contents. **(B)** ADH contents. **(C)** ALDH contents. **(D)** GSH contents. **(E)** MDA contents. **(F)** SOD contents. **(G)** CAT contents. * and *** indicate $P < 0.05$ and $P < 0.001$, respectively, for Group M compared with group CG. #, ##, and ### indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, for group PGPs compared with group M.

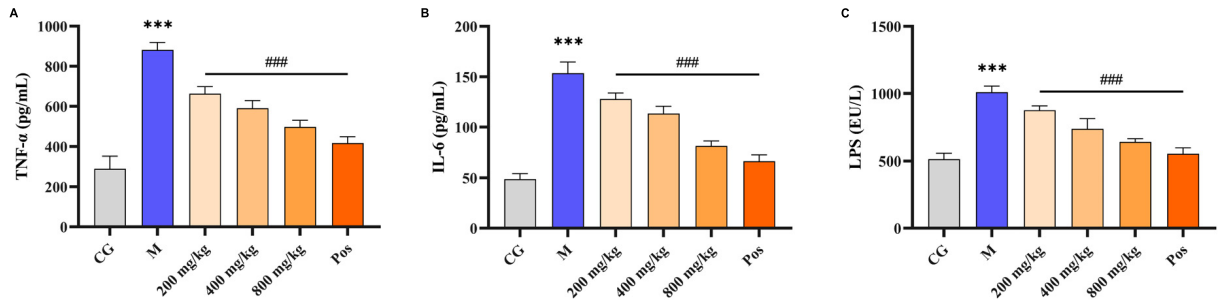

FIGURE 8
Effects of PGPs on biochemical constituents in mouse livers. **(A)** TNF-α contents. **(B)** IL-6 contents. **(C)** LPS contents. *** indicate $P < 0.001$, for Group M compared with group CG. ### indicate $P < 0.001$, for group PGPs compared with group M.

TABLE 2 **The results of multivariate statistical analysis.**

Ion mode	Statistical model type	Group	R^2X	R^2Y	Q^2	Int
Positive	PCA		0.70		0.41	
	OPLS-DA	CG vs. M	0.62	0.99	0.82	−0.55
		M vs. 200	0.61	0.98	0.62	−0.47
		M vs. 400	0.65	0.99	0.96	−0.43
		M vs. 800	0.55	0.98	0.71	−0.70
		M vs. Pos	0.61	0.99	0.93	−0.55
Negative	PCA		0.79		0.50	
	OPLS-DA	CG vs. M	0.85	0.94	0.83	−0.80
		M vs. 200	0.80	0.99	0.91	−0.72
		M vs. 400	0.82	1	0.85	−0.31
		M vs. 800	0.76	0.97	0.76	−0.45
		M vs. Pos	0.66	0.99	0.80	−0.28

Int is the intercept of Q^2 on the Y-axis after 200 permutation tests.

4 Discussion

4.1 PGPs exhibit favorable antioxidant ability

Evaluating the antioxidant ability *in vitro* is an effective method to investigate the hepatoprotective activity of PGPs on ALD in the early stage. In our study, the antioxidant ability of PGPs was preliminarily evaluated by measuring the DPPH, OH and O_2^- scavenging rates. Although the effect of PGPs on free radical scavenging was relatively mild compared to the positive control, the dose-dependent free radical scavenging exhibited by PGPs remained excellent. In addition, *Sepia esculenta* ink polysaccharides (SEPs) have been reported to have a favorable antioxidant ability, with IC_{50} values of 20.09 mg/mL for DPPH and 0.33 mg/mL for OH (26). Chestnut polysaccharides (CPs) scavenged free radicals in a dose-dependent manner, and the scavenging rates of DPPH and OH were 88.9% and 41.4% at 10 mg/mL (27). The OH scavenging ability of SEPs and the DPPH scavenging ability of CPs were superior to that of PGPs. PGPs demonstrated a more stabilizing antioxidant ability in DPPH and OH scavenging, with similar IC_{50} values of 6.93 mg/mL and 6.45 mg/mL, respectively. The free radical scavenging ability of bitter gourd polysaccharides have been reported, and the results showed that the highest scavenging rate of DPPH, OH and O_2^- was 39.7%, 36.7%, and 55.0%, respectively, in the concentration range of 0.1 to 3.2 mg/mL (28). Su et al. (29) evaluated the scavenging rate of DPPH, OH and O_2^- by polysaccharides extracted from *Auriculariales* at 0 to 1.2 mg/mL, and the highest scavenging rate of DPPH, OH and O_2^- was 37.8%, 29.7%, 42.4%. In comparison with other polysaccharides, we found that PGPs had superior O_2^- scavenging ability (IC_{50}: 0.99 mg/mL), while PGPs had no significant advantage for DPPH and OH scavenging. In summary, antioxidant is an important mechanism for polysaccharide to exert biological activity, so it is necessary to establish ALD cell model to further verify the antioxidant ability of PGPs.

Superoxide dismutase is a major enzymatic antioxidant defense system responsible for scavenging free radicals (30). GSH is an important cellular antioxidant in the body due to its ability to scavenge ROS or as an important cofactor for glutathione S-transferases and peroxidases (31). ROS have the potential to oxidize macromolecules and cause damage to cell membranes,

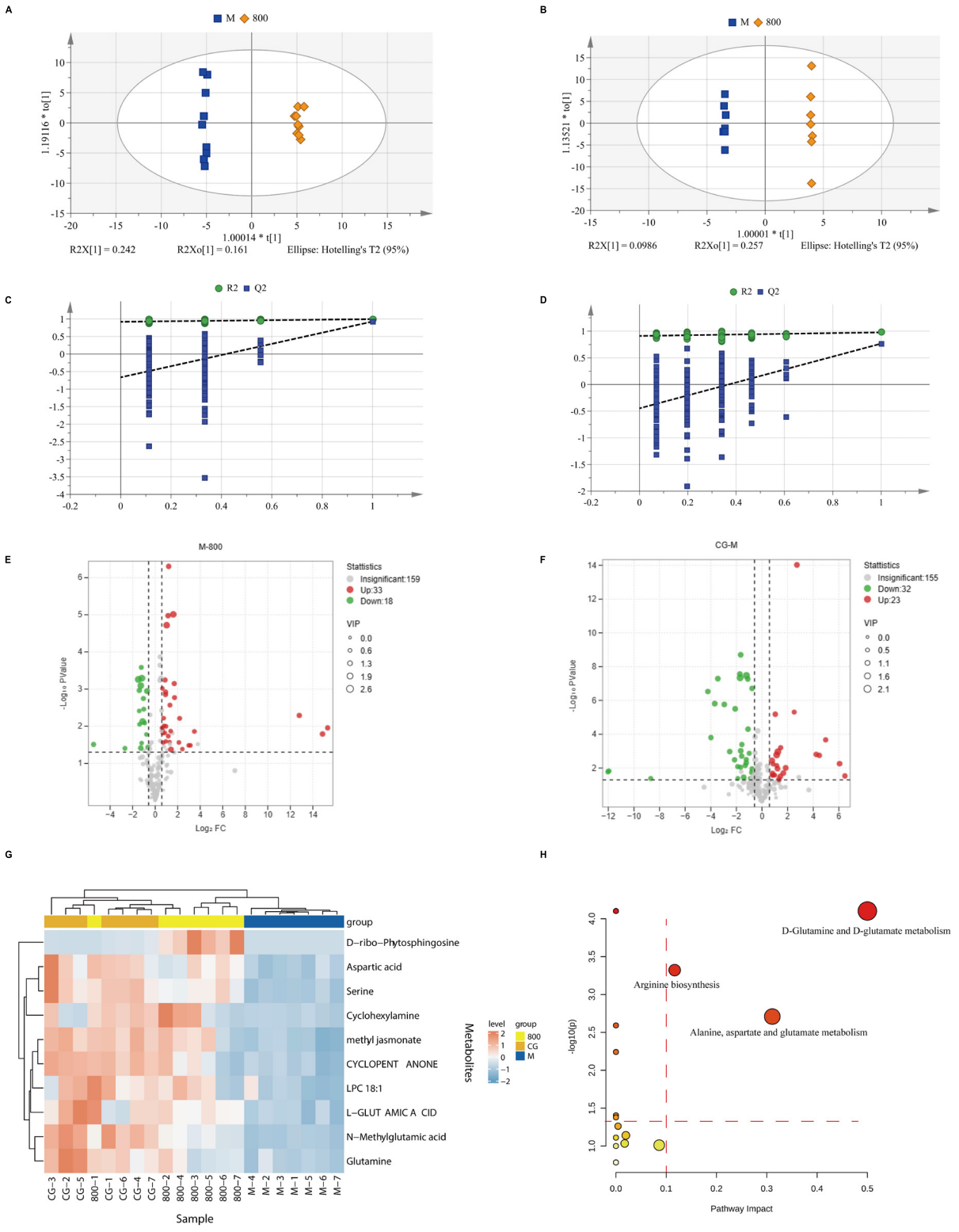

FIGURE 9
Multivariate statistical analysis and pathway analysis of metabolomics. OPLS-DA scores plot of M vs. 800 in positive **(A)** and negative **(B)**. Permutation tests of M vs. 800 in positive **(C)** and negative **(D)**. Volcano plot of M vs. 800 **(E)** and CG vs. M **(F)**. **(G)** Heatmap of differential metabolites. **(H)** Bubble plots for pathway analysis.

leading to the production of highly reactive peroxides, such as MDA. MDA can cause a cascade of oxidative reactions that inactivate cellular proteins, impair membrane fluidity and elasticity, and ultimately lead to cell death (32). Large amounts of ROS and MDA are released under alcohol intervention, while GSH and SOD react with the free radicals generated by

TABLE 3 Differential metabolites.

Metabolite name	RT (min)	Adduct type	Mz	Formula	ID	CG vs. M			M vs. 800		
						VIP	P-value	FC	VIP	P-value	FC
D-ribo-Phytosphingosine	1.144	[M+Na]+	340.2817	C18H39NO3	HMDB0004610	1.204951	0.041784	0.002424↓	1.609718	0.005109	7086.62↑
LPC 18:1	14.19	[M+H]+	522.3555	C26H52NO7P	HMDB0002815	1.282675	0.000406	0.335708↓	1.078373	0.026597	2.435479↑
L-Glutamic acid	0.708	[M+H]+	148.0603	C5H9NO4	HMDB0000148	1.241711	0.008268	0.268119↓	1.513421	0.000708	3.227889↑
Cyclopentanone	14.335	[M+NH4]+	102.0913	C5H8O	HMDB0031407	1.49943	2.00E-09	0.315009↓	1.05168	0.02543	1.929284↑
Cyclohexylamine	0.584	[M+H]+	100.1121	C6H13N	HMDB0031404	1.380665	0.003229	0.226286↓	1.212821	0.02728	4.303002↑
Methyl jasmonate	14.306	[M+H]+	225.1487	C13H20O3	HMDB0036583	1.504154	2.72E-08	0.301038↓	1.403339	0.002697	2.47412↑
Serine	2.176	[M+H]+	106.0499	C3H7NO3	HMDB0062263	1.522073	0.000963	0.315705↓	1.723755	1.06E-05	2.204454↑
N-Methylglutamic acid	14.106	[M+H]+	162.0761	C6H11NO4	HMDB0062660	1.559933	3.14E-06	0.234048↓	1.75374	4.96E-07	2.288777↑
Glutamine	0.724	[M+H]+	147.0764	C5H10N2O3	HMDB0000641	1.386633	0.001057	0.174219↓	1.445632	0.001691	3.284965↑
Aspartic acid	2.077	[M+H]+	134.0447	C4H7NO4	HMDB0062186	1.404119	0.004921	0.425436↓	1.137284	0.009719	1.721903↑

oxidation, resulting in a reduction in their content (Figure 5). Pretreatment with PGPs reduced ROS and MDA production and increased SOD and GSH levels, indicating that the oxidative stress caused by alcohol metabolism was modified. The excellent antioxidant ability conferred the effective preventive potential of PGPs against ALD. It is worth noting that the restorative effect of PGPs on GSH, MDA, SOD, and ROS levels was only dose-dependent at 400–1000 μg/mL. PGPs at 200 μg/mL showed better antioxidant capacity against oxidative stress in the HL7702 cells. Interestingly, Jin et al. reported that *schisandra* polysaccharide increased glucose consumption of buffalo rat liver (BRL) cells in a parabolic fashion at concentrations ranging from 12.5 to 400 μg/mL (33). Maximal glucose consumption was obtained at 100 μg/mL, followed by 200 μg/mL. Glucose consumption is the basis of energy metabolism (34). Natural polysaccharides have also been reported to exert bioactive effects by regulating energy metabolism (35). We hypothesized that with the intervention of 200 μg/mL polysaccharide, cells obtained higher energy metabolism level and thus increased antioxidant ability. However, we have not been able to find more studies to confirm this hypothesis. A deeper research idea should be considered in the future, that is, to explore the mechanism of polysaccharide in alleviating ALD from the perspective of energy metabolism.

4.2 Hepatoprotective effects of PGPs in ALD mice are associated with a reduction in inflammatory factors, mitigation of oxidative stress, and regulation of lipid metabolism

ALT, AST, and γ-GGT are metabolic enzymes that function primarily in the cytoplasm of hepatocytes. When the hepatocyte membrane is damaged, ALT, AST and γ-GGT are released into the circulation; therefore, elevated levels in the serum can be used as a marker of liver damage (36). These metabolic enzymes were observed to decrease in the intervention group, indicating that PGPs have the potential to maintain the membrane integrity of hepatocytes damaged by alcohol metabolism. This statement can be confirmed by histopathological observation that the structure of hepatocytes was improved by the intervention of PGPs (Figure 6D).

Liver lipid homeostasis is usually strictly controlled by the metabolic system, and excessive alcohol consumption can easily disturb lipid metabolism and cause rapid reactions such as steatosis (37). Under the interference of alcohol, there was a serious accumulation of lipid droplets in the liver of mice, and TG content increased significantly, $P < 0.05$ (Figures 6E, 7A). PGPs maintained the homeostasis of lipid metabolism in a dose-dependent manner, and the TG content was maintained at a normal level after 800 mg/kg PGPs pretreatment. Polysaccharides can promote fatty acid oxidation to regulate liver lipid metabolism and maintain hepatic stability. In a previous study, Bian et al. (38) used lipidomics to find that *Mori Fructus* polysaccharide had a significant effect on the synthesis and degradation of fatty acids and the metabolism of glycerophospholipids in ALD mice. As one of the phospholipids, glycerophospholipids are the main component of membrane (39). In terms of lipid metabolism, our metabolomic results enriched sphingolipid metabolic pathway and glycerophospholipid metabolic pathway, although $P > 0.05$ (Supplementary Table 2). Our results coincide with the study of *Mori Fructus* polysaccharide, indicating that natural polysaccharide alleviates ALD by regulating lipid metabolism. Furthermore, consistent with *in vitro* experiments, PGPs improved the antioxidant ability of ALD mice, as evidenced by the increase in GSH, SOD, and CAT content and the decrease in MDA content.

In addition, our study showed that the presence of ALD was accompanied by the production of inflammatory factors (Figure 8). Excessive alcohol consumption leads to increased levels of endotoxins, which may be due to altered intestinal flora disrupting the intestinal barrier. Increased intestinal permeability caused by chronic alcohol intake further induces cascading biological effects, including alcoholic liver disease (40). The levels

of inflammatory factors (TNF-α, IL-6, LPS) in ALD mice were significantly ($P < 0.001$) decreased by pretreatment with PGPs. Polysaccharides enter the organism and are used as a carbon source by microbial fermentation to maintain homeostasis of the intestinal environment. Wei et al. found that PGPs can be used by intestinal microbiota and can significantly increase the level of short-chain fatty acids during fermentation. The relative abundances of *Bacteroidetes* in PGP groups were increased (2). Astragalus polysaccharides have also been reported to increase the relative abundance of *Bacteroidetes* in ALD mice (41). In addition to *Bacteroidetes*, polysaccharides increased the relative abundance of *Lactobacillus* and *Firmicutes* to alleviate alcohol-induced intestinal barrier damage (42, 43). The improvement of gut microbiota suggests that PGPs can protect ALD mice by reducing the release of endotoxins through maintaining intestinal homeostasis.

Analyzing the alcohol-metabolizing enzymes ADH and ALDH content, we found that there was an increase in the content of ADH and ALDH with the intervention of PGPs, however, it was not significant. We speculated that PGPs alleviated liver damage and thus enhanced the activities of alcohol-metabolizing enzymes, but the protective effect of PGPs on ALD mice was not achieved by increasing the activity of alcohol metabolizing enzymes. In summary, PGPs should be exploited as an excellent anti-ALD bioactive substance, and their hepatoprotective mechanism may be the mitigation of oxidative stress, regulation of lipid metabolism, and reduction of inflammatory factors.

4.3 PGPs improve small molecule metabolism in ALD mice

Metabolomics was used to characterize the improvement in the metabolic profile in ALD mice by PGPs pretreatment. In our study, we found that the hepatoprotective effect of PGPs in ALD mice was generally dose-dependent and therefore mainly screened for differential metabolites in the M vs. CG and PGPs-800 vs. M. Pathway analysis was conducted on the ten screened metabolites (**Table 3**), and three metabolic pathways were screened with a pathway impact > 0.1, as follows: D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, and arginine biosynthesis.

Two metabolites, L-glutamate and L-glutamine, were captured in the D-glutamine and D-glutamate metabolism pathways. L-Glutamine is essential as a metabolic precursor to satisfy the requirements of cell proliferation for peptides and proteins, amino sugars, nucleic acids and nucleotides (44). L-Glutamate is the immediate product of L-glutamine metabolism, which is involved in hepatocyte gluconeogenesis (45). Glutamine and glutamate are involved in a number of biochemical reactions that contribute to tissue repair. In our study, glutamine and glutamate levels were significantly increased ($P < 0.01$) in the PGPs-800 group compared with the M group. We speculate that PGPs regulate glutamine and glutamate metabolism, enhance the functional stability of hepatocytes, and maintain cell membrane integrity, thereby attenuating liver injury. Jiang et al. (43) found that *Echinacea purpurea* polysaccharide could reduce oxidative stress and maintain intestinal permeability in mice with ALD through the D-glutamine and D-glutamate metabolism pathway. In addition, Wang et al. (46) reported that regulation of D-glutamine and D-glutamate metabolism and alanine, aspartate and glutamate metabolism pathways in fatty liver mice was involved in reducing oxidative stress and inflammation, thereby alleviating liver damage. Glutamine exerts potential functions against the harmful effects of oxidative stress (47). D-Glutamine and D-glutamate metabolism pathway is an important metabolic pathway for hepatoprotection. This result verified that the regulation of oxidative stress was a critical mechanism of PGPs against ALD from the metabolic level. Notably, the alanine, aspartate and glutamate metabolism pathways were also enriched in our study. Dai et al. (48) found that alanine, aspartate and glutamate metabolism is an important pathway in the metabolic regulation of *Hedyotis diffusa* in mice with liver injury. Changes in liver metabolism, especially alanine, aspartate and glutamate, will inevitably affect the growth and development of the body (43). In addition, arginine is a multifunctional amino acid, and reduced arginine levels are considered a specific biomarker following liver injury (49). Significant expression of the arginine synthesis pathway indicated that PGPs attenuated liver inflammation in ALD mice. Pretreatment with PGPs alleviates alcohol-induced liver injury by regulating D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, and the arginine biosynthesis pathway to improve liver metabolism.

5 Conclusion

Pretreatment with PGPs showed a protective effect against alcohol-induced liver injury. Characterization analysis revealed that PGPs are pyranose polysaccharides mainly composed of arabinose, galactose, xylose, glucuronic acid, mannose, etc. *In vitro* analysis demonstrated that PGPs reduce alcohol-induced oxidative stress in HL7702 cells. In addition, *in vivo* experiments further showed that the hepatoprotective effects of PGPs against alcoholic liver injury were achieved by exerting antioxidant effects, reducing the inflammatory response and regulating lipid metabolism. This study points out that PGPs have a favorable protective effect against ALD, and more in-depth studies such as targeting metabolomics, gut microbiota analysis and transcriptomics deserve to be employed in future studies of PGPs. Overall, PGPs have broad development prospects as highly bioactive and low-cost substance.

Data availability statement

The original contributions presented in this study are included in this article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements

because only commercially available established cell lines were used. The animal study was approved by Animal Welfare Ethics Committee of Wuhan Polytechnic University (WPU202209001). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BZ: Formal analysis, Investigation, Writing –original draft. PL: Conceptualization, Investigation, Methodology, Writing –review and editing. XY: Writing –review and editing. HC: Writing –review and editing. HZ: Writing –review and editing. QW: Writing –review and editing. YL: Writing –review and editing. MF: Writing –review and editing. YW: Writing –review and editing. ZG: Writing –review and editing.

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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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Supplementary material

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The association between diverse serum folate with MAFLD and liver fibrosis based on NHANES 2017–2020

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Background: Metabolically Associated Fatty Liver Disease (MAFLD) marks a progression from the previous paradigm of Non-Alcoholic Fatty Liver Disease (NAFLD), presenting a redefined diagnostic framework that accentuates metabolic factors while recognizing non-alcoholic contributors. In our investigation, our principal aim was to scrutinize the conceivable correlation between diverse serum folate levels and the prevalence of MAFLD and liver fibrosis.

Methods: In our investigation, we conducted an extensive analysis utilizing data derived from the National Health and Nutrition Examination Survey (NHANES) across the years 2017–2020. We aimed to investigate the association between different serum folate concentrations and the prevalence of MAFLD and liver fibrosis by comprehensive multivariate analysis. This analytical approach considered various variables, encompassing sociodemographic characteristics, lifestyle factors, hypertension, and diabetes. By including these potential confounders in our analysis, we aimed to ensure the stability of the findings regarding the association between different serum folate concentrations and the development of MAFLD and liver fibrosis.

Results: In our investigation, we utilized multiple linear regression models to thoroughly analyze the data, revealing noteworthy insights. Evidently, elevated levels of both total folate and 5-MTHF exhibited a distinct negative correlation with CAP, while 5-MTHF demonstrated a notable negative correlation with LSM. Furthermore, multiple logistic regression models were employed for an in-depth examination of the data. As the concentrations of total folate and 5-MTHF in the serum increased, a substantial decrease in the likelihood of MAFLD and liver fibrosis occurrence was observed.

Conclusion: The findings of this investigation robustly suggest the prevalence of MAFLD and liver fibrosis decreased significantly with the increase of serum concentrations of total folate and 5-MTHF.

KEYWORDS

NHANES, MAFLD, liver fibrosis, total folate, 5-MTHF

1 Introduction

In 2020, a noteworthy paradigmatic transition transpired in the categorization of hepatic maladies with introduction of Metabolically Associated Fatty Liver Disease (MAFLD) (1, 2). This revolutionary framework augments the delineation of hepatic disorders by assimilating indicators of metabolic aberrations, encompassing insulin resistance, heightened susceptibility to C-reactive protein (Hs-CRP), and diverse other concomitant metabolic predisposing factors (1, 2). Deviating from Non-Alcoholic Fatty Liver Disease (NAFLD), which explicitly omits conditions such as viral hepatitis, alcoholic liver disease, and other hepatic disorders, the diagnostic criteria for MAFLD embrace a notably more pragmatic standpoint (3). Through the incorporation of metabolic markers, MAFLD endeavors to discern individuals exhibiting fatty liver conditions. These individuals not only meet the conventional criteria for NAFLD but also demonstrate elevated risks of disease progression (4). This conceptual progression underscores a nuanced comprehension of the intricate interplay between metabolic factors and hepatic well-being, ushering in a more comprehensive and clinically pertinent approach to the diagnosis and treatment of fatty liver disorders within the scientific milieu (4).

Folate, a water-soluble B9 vitamin crucial for one-carbon metabolism and methylation reactions exists in three main forms: 5-Methyltetrahydrofolate (5-MTHF), folic acid, and tetrahydrofolate (THF), each serving distinct functions and roles (5, 6). 5-MTHF, an active form used directly by the body, plays a crucial role in cellular processes like gene regulation, neurotransmitter synthesis, and detoxification (7). Folic acid, a synthetic form of folate found in supplements and fortified foods, needs to be converted into active forms like 5-MTHF in the body but is essential in prenatal supplements to prevent neural tube defects during pregnancy (8). THF is a middleman in the folate pathway, transporting one-carbon units crucial for creating nucleotides and amino acids. The body converts THF into different forms, including 5-MTHF, through enzymatic processes (9). Experimental studies have implicated folate in processes such as oxidative stress, hepatic lipid metabolism, and chronic inflammation in the liver, all recognized as risk factors for NAFLD (10). In addition, certain animal investigations have indicated that supplementing with folic acid may mitigate hepatic steatosis (11, 12). Furthermore, human studies have demonstrated that serum folate could potentially exert a significant role in averting or retarding the progression of Non-Alcoholic Steatohepatitis (NASH), along with the potential to reverse liver inflammation and fibrosis (13). Prior epidemiological analyses have also indicated an inverse association between higher concentrations of serum total folate, 5-MTHF and the prevalence of NAFLD, liver fibrosis (14).

To the best of our knowledge, prior investigations examining the association between various serum folate levels and NAFLD and liver fibrosis have utilized diverse analytical approaches. These analyses primarily relied on blood biomarker indicators, such as the United States Fatty Liver Index (USFLI), Fatty Liver Index (FLI), Fibrosis-4 score (FIB-4), and NAFLD Fibrosis Score (NFS) (14–16). Earlier studies have also explored the impact of serum total folate on MAFLD using Vibration-Controlled Transient Elastography (VCTE). However, these studies encompassed only a single cycle from 2017 to 2018, resulting in a relatively limited number of population samples (17). Our primary objective was to augment the sample size by incorporating participants who underwent VCTE from the 2017–2020 cycles of the National Health and Nutrition Examination Survey (NHANES). Subsequently, we delved into the exploration of cross-sectional associations between various serum folate levels and both MAFLD as well as liver fibrosis. The hypothesis underlying this investigation is that there may be a significant negative association between various serum folate levels and MAFLD, as well as liver fibrosis.

2 Materials and methods

2.1 Study population

Conceived under the auspices of the National Center for Health Statistics (NCHS) and endorsed by the Centers for Disease Control and Prevention (CDC), NHANES stands as an intricately executed nationwide survey. Providing detailed insights into population characteristics, socioeconomic status, personal behaviors, individual health conditions, and pertinent health indicators derived from clinical laboratory assessments, NHANES serves as an invaluable instrument for acquiring nuanced insights into the multifaceted landscape of public health. The acquisition and dissemination of data within the NHANES database adhered to the Helsinki principles, with the requisite approval from the Ethics Committee to guarantee the ethical integrity of the data employed in this investigation. The dataset used in this investigation originates from the NHANES cycle spanning the years 2017–2020. From the initial cohort of 15,560 participants, several exclusions were implemented, outlined as follows: (1) individuals lacking comprehensive liver elastography measurements ($n = 6,513$), (2) those for whom the confirmation of MAFLD presence was inconclusive ($n = 37$), and (3) participants with incomplete data regarding serum folate ($n = 3,595$). Consequently, the definitive sample size was refined to 5,415 participants. For a more detailed representation, we refer the reader to Figure 1.

2.2 Measurement of various serum folate

For the analytical procedures, blood specimens were procured during the examination, subsequently frozen, and preserved at −30°C before being dispatched to CDC in Atlanta, Georgia. The quantification of five distinct folate forms (5-MTHF, folic acid, THF, 5-formyl-tetrahydrofolate, and 5,10-methenyl-tetrahydrofolate) was conducted using isotope-dilution high-performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS; detailed procedures can be found at https://wwwn.cdc.gov/Nchs/Nhanes/2017-2018/P_FOLFMS.htm). As expounded on the NHANES website, in instances where the measured value falls below the lower limit of detection divided by the square root of 2 lower limits of detection (LLOD), a standard protocol was applied to impute the value by dividing the LLOD by the square root of 2. Due to a substantial number of measurements for 5-formyl-tetrahydrofolate and 5,10-methenyl-tetrahydrofolate falling below LLOD, these values were excluded from further analysis. Comprehensive information regarding the LLOD for various serum folate levels and the proportions above LLOD can be found in Supplementary Table 1. To facilitate the analysis, participants were stratified into three cohorts based on tertile concentrations of diverse serum folate levels.

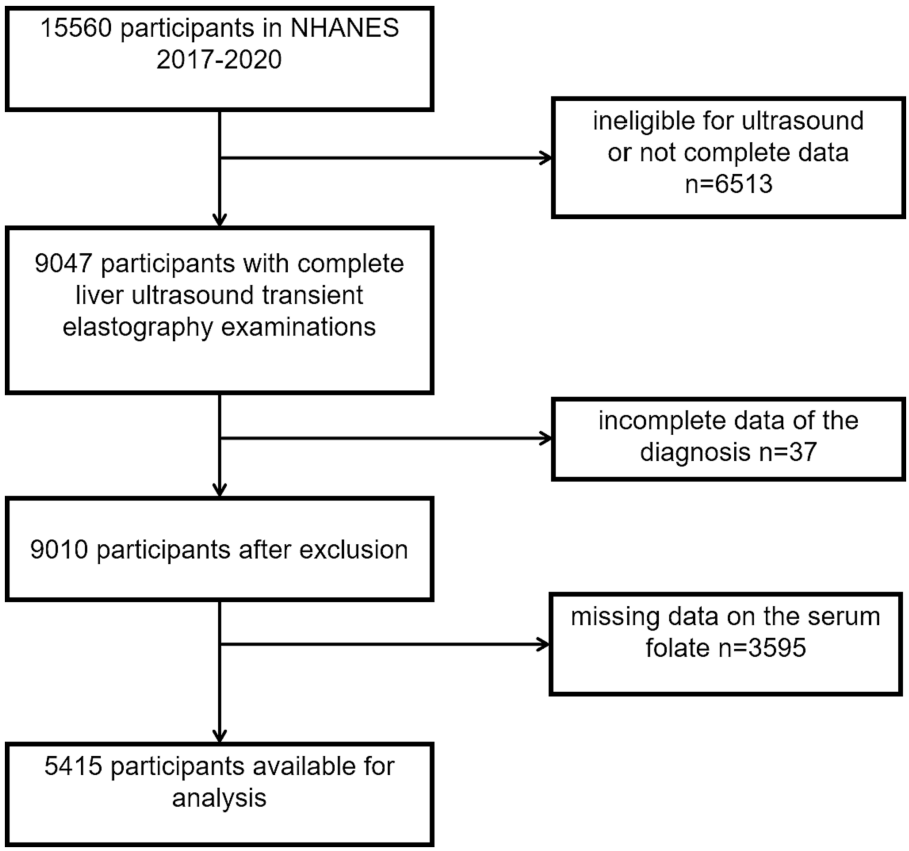

FIGURE 1
Flow chart of participants screening.

2.3 Definition of MAFLD

The diagnosis of MAFLD was established when hepatic steatosis coexisted with any of the following criteria: being categorized as overweight or obese, indicated by a body mass index (BMI) ≥ 25, having diabetes mellitus, or manifesting signs of metabolic irregularities. These metabolic irregularities were specifically recognized when a patient exhibited at least two of the following metabolic risk abnormalities: (1) systolic/diastolic blood pressure surpassing 130/85 mmHg or the use of specific antihypertensive medications; (2) waist circumference (WC) ≥102 cm for males and ≥ 88 cm for females; (3) plasma high-density lipoprotein cholesterol (HDL) levels below 40 mg/dL for men and below 50 mg/dL for women, or the use of specific medications; (4) plasma triglyceride (TG) levels exceeding 150 mg/dL or the use of specific medications; (5) homeostasis model assessment of insulin resistance (HOMA-IR) score surpassing 2.5; (6) fasting plasma glucose (FPG) within the range of 5.6–6.9 mmol/L or Glycohemoglobin A1c (HbA1c) ranging from 5.7 to 6.4%; and (7) plasma Hs-CRP levels ≥2 mg/L (2).

2.4 Non-invasive assessment of hepatic steatosis and liver fibrosis

The assessment of hepatic steatosis at the NHANES Mobile Examination Center (MEC) involved employing elastography through VCTE. To maintain assessment accuracy, participants were obligated to observe a fasting period of no less than 3 h preceding the examination. Moreover, they were mandated to acquire more than 10 liver measurement values, ensuring an interquartile range/median ratio below 30% to bolster the reliability of the evaluation. Consistent with prior research endeavors, we defined liver steatosis as Controlled Attenuation Parameter (CAP) ≥ 238 dB/m (18, 19), while Liver Stiffness Measurement (LSM) ≥ 7 kPa indicated the presence of liver fibrosis (18).

2.5 Covariate assessment

The subsequent laboratory parameters encompassed WC, TG, total cholesterol (TC), low-density lipoprotein cholesterol (LDL), HDL, FPG, HbA1c, HOMA-IR, and Hs-CRP. Demographic data, including age, gender, race, BMI, educational attainment, marital status, poverty income ratio (PIR), alcohol consumption, smoking habits, and medication usage, were collected through standardized self-reported questionnaires. Educational achievements were categorized into three groups: "less than high school," "high school or equivalent," and "higher than high school." Marital status was classified as "never married," "married/cohabiting," or "separated/divorced/widowed." PIR was divided into three cohorts: <1.30, 1.30–3.50, and > 3.50 (20). Alcohol consumption was delineated into three groups: individuals who never consumed alcohol, moderate drinkers

(1–2 drinks per day for males, 1 drink per day for females), and heavy drinkers (≥3 drinks per day for males, ≥2 drinks per day for females) (21). Smoking habits were categorized into three tiers: low (serum cotinine <0.015 ng/mL), moderate (0.015–3 ng/mL), and high level (serum cotinine >3 ng/mL) (22). According to the American Diabetes Association criteria, diabetes is characterized by the presence of any of the following criteria: FPG ≥ 126 mg/dL, HbA1c ≥ 6.5%, self-reported clinician-diagnosed diabetes, or initiation of drug treatment (23). Hypertension, as per the 2017 guidelines by the American Heart Association, is defined as a blood pressure reading surpassing 130/80 mm Hg or the initiation of specific drug treatment (24).

2.6 Statistical analysis

Continuous variables were expressed as mean values along with standard deviations (mean ± SD), while categorical variables were presented as percentages. The comparison of continuous variables utilized a weighted t-test, and for categorical variables, a chi-squared test was applied, with outcomes reported as counts (n) and percentages (%). Utilizing a linear regression model, we investigated the relationship between diverse serum folate levels and CAP as well as LSM. To assess the correlation between covariates and the analytical outcomes, three distinct models were formulated. Each model in the analysis progressively incorporated additional adjustments for covariates. The initial model remained unadjusted, while the second model included partial adjustments for age, race, gender, PIR, marital status, and educational level. Model 3 represents the fully adjusted model, encompassing additional variables such as BMI, smoking habits, alcohol consumption, diabetes, and hypertension. To assess the relationship between various serum folate levels and MAFLD as well as liver fibrosis, a multivariable logistic regression model was applied, utilizing the previously detailed three-level model. Following this, subgroup analyses were executed to explore potential modifications in effect measures, including gender, age, BMI, hypertension, and diabetes as potential influential factors. Moreover, a restricted cubic spline (RCS) analysis was performed to examine potential nonlinear associations between diverse serum folate levels and both MAFLD and liver fibrosis. All statistical analyses adhered to a significance level of less than 0.05 for a two-tailed *p* value. The software tools employed included STATA v16.0 (StataCorp LLC, College Station, TX, United States) and R (version 4.1.0, Vienna, Austria).

3 Results

3.1 Baseline characteristic

In our primary analysis, a total of 5,415 participants were included and Supplementary Table 2 furnishes further insights into the baseline attributes of these individuals. Comparative analysis between the non-MAFLD population and MAFLD patients revealed significant differences in gender, race, drinking habits, diabetes, hypertension, and BMI. Moreover, MAFLD patients exhibited higher levels of age, WC, TG, TC, LDL, FPG, Hb1Ac, HOMA-IR, and Hs-CRP, along with lower levels of HDL and 5-MTHF. Similar patterns were observed in patients with liver fibrosis compared to those without liver fibrosis, with notable distinctions in PIR, smoking habits, total folate, and THF. However, levels of TC and LDL did not show significant variations in the liver fibrosis group.

3.2 Association between various serum folate levels and CAP

The outcomes presented in Table 1 emanate from a series of multiple linear regression models aimed at exploring the potential association between concentrations of various serum folate levels and CAP. In Model 1, a noteworthy significant negative correlation was evident between serum total folate and 5-MTHF with CAP ($p = 0.047$, 0.015; respectively). Notably, Model 2 sustained a significant negative relationship between serum total folate and 5-MTHF with CAP ($p = 0.002$, <0.001; respectively). Lastly, in Model 3, the analysis consistently indicated a significant negative association between serum total folate and 5-MTHF with CAP ($p = 0.016$, 0.009; respectively). The outcomes of the linear regression analysis exploring the relationship between folic acid and THF with CAP are presented in Supplementary Table 3.

Additionally, we conducted an analysis utilizing serum total folate and 5-MTHF concentrations as categorical variables. In Model 1, in comparison to the reference group (T1), elevated levels of serum total folate (T2 and T3 groups) and 5-MTHF (T2 and T3 groups) demonstrated a significant negative association with CAP (p all <0.05). Transitioning to Model 2, the highest serum total folate and 5-MTHF level exhibited a significant negative association with CAP ($p = 0.004$, 0.002; respectively). Analogously, in Model 3, the highest serum total folate and 5-MTHF concentrations continued to exhibit a significant negative association with CAP ($p = 0.029$, 0.023; respectively).

3.3 Association between various serum folate levels and LSM

The outcomes presented in Table 2 elucidate findings from a series of multiple linear regression models devised to explore the potential association between concentrations of various serum folate levels and LSM. In Model 1, a noteworthy significant negative correlation was observed between serum 5-MTHF and LSM ($p = 0.031$). Notably, Model 2 sustained a significant negative relationship between 5-MTHF and LSM ($p = 0.011$). Finally, in Model 3, the analysis consistently indicated a significant negative association between serum 5-MTHF and LSM ($p = 0.019$). However, in all three models, we did not find a significant correlation between serum total folate and LSM. The results of the linear regression analysis exploring the relationship between folic acid and THF with LSM are presented in Supplementary Table 4. Additionally, we conducted an analysis using serum total folate and 5-MTHF concentrations as categorical variables. Nonetheless, in this analysis, we did not identify a significant correlation between serum total folate, 5-MTHF concentrations, and LSM.

3.4 Association between various serum folate levels and MAFLD

Table 3 encapsulates the outcomes derived from multiple logistic regression models scrutinizing the potential independent

TABLE 1 Linear regression model between serum total folate, 5-MTHF, and CAP.

		Model 1		Model 2		Model 3	
		β (95% CI)	*p* trend	β (95% CI)	*p* trend	β (95% CI)	*p* trend
Total folate	Continuous	−0.099 (−0.197, −0.001)	0.047	−0.155 (−0.253, −0.057)	0.002	−0.097 (−0.175, −0.018)	0.016
	T1	ref	ref	ref	ref	ref	ref
	T2	−7.215 (−13.630, −0.800)	0.028	−4.734 (−10.919, 1.451)	0.134	−2.130 (−6.799, 2.539)	0.371
	T3	−8.901 (−15.124, −2.679)	0.005	−9.096 (−15.250, −2.943)	0.004	−5.393 (−10.233, −0.552)	0.029
5-MTHF	Continuous	−0.136 (−0.246, −0.026)	0.015	−0.203 (−0.312, −0.093)	<0.001	−0.117 (−0.205, −0.029)	0.009
	T1	ref	ref	ref	ref	ref	ref
	T2	−7.661(−14.092,-1.230)	0.020	−5.273(−11.468,0.922)	0.095	−2.469(−7.130,2.191)	0.299
	T3	−9.624(−15.839,-3.408)	0.002	−9.998(−16.172,-3.825)	0.002	−5.612(−10.447,-0.777)	0.023

TABLE 2 Linear regression model between serum total folate, 5-MTHF, and LSM.

		Model 1		Model 2		Model 3	
		β (95% CI)	*p* trend	β (95% CI)	*p* trend	β (95% CI)	*p* trend
Total folate	Continuous	−0.002 (−0.009, 0.005)	0.589	−0.004 (−0.012, 0.004)	0.351	−0.003 (−0.010, 0.004)	0.406
	T1	ref	ref	ref	ref	ref	ref
	T2	0.072 (−0.389, 0.533)	0.761	0.117 (−0.352, 0.586)	0.625	0.051 (−0.420, 0.523)	0.831
	T3	−0.296 (−0.672, 0.080)	0.123	−0.307 (−0.711, 0.096)	0.135	−0.307 (−0.700, 0.086)	0.126
5-MTHF	Continuous	−0.006 (−0.011, −0.001)	0.031	−0.008 (−0.014, −0.002)	0.011	−0.007 (−0.013, −0.001)	0.019
	T1	ref	ref	ref	ref	ref	ref
	T2	−0.034 (−0.496, 0.429)	0.887	0.015 (−0.459, 0.489)	0.951	0.063 (−0.411, 0.537)	0.796
	T3	−0.363 (−0.749, 0.023)	0.065	−0.375 (−0.795, 0.046)	0.081	−0.303 (−0.697, 0.091)	0.132

associations between serum total folate and 5-MTHF concentrations and MAFLD. A significant negative association between the highest level of serum total folate and 5-MTHF and MAFLD was observed in Model 1 ($p = 0.008$, 0.003; respectively). Model 2 revealed a significant negative association between the highest level of serum total folate and 5-MTHF ($p = 0.025$, 0.012; respectively) with MAFLD. According to Model 3, the likelihood of MAFLD decreased significantly as total folate and 5-MTHF ($p = 0.042$, 0.024; respectively) concentrations in the serumincreased. The outcomes of the logistic regression analysis examining the association of folic acid and THF with MAFLD are presented in Supplementary Table 5. We found a significant positive correlation between THF and MAFLD in Model 1 and Model 2 (p <0.001,= 0.006; respectively).

Following comprehensive multivariable adjustments in Model 3, no evident nonlinear relationship between serum total folate, 5-MTHF, and MAFLD was observed in RCS analysis (p overall = 0.0245, 0.0466; p nonlinear = 0.1779, 0.3208) (refer to Figures 2A,B).

3.5 Association between various serum folate levels and liver fibrosis

Table 4 presents the outcomes derived from multiple logistic regression models scrutinizing the potential independent associations between serum total folate and 5-MTHF concentrations and liver fibrosis. A significant negative association between blood levels of total folate (T2 and T3 group) and 5-MTHF (T2 and T3 group) and liver fibrosis was observed in Model 1 (p all<0.05). In Model 2, serum total folate levels (T3 group, $p = 0.013$) and 5-MTHF levels (T3 group, $p = 0.006$) were significantly negatively associated with liver fibrosis. Furthermore, in the final model, Model 3, the analysis demonstrated that as the concentration of serum total folate and 5-MTHF increased, the likelihood of liver fibrosis also decreased significantly (T3 group, $p = 0.010$, 0.005; respectively). The outcomes of the logistic regression analysis assessing the association of folic acid and THF with liver fibrosis are presented in Supplementary Table 6. A significant negative correlation between folic acid and liver fibrosis was identified solely in Model 3 ($p = 0.014$).

Additionally, we conducted a RCS analysis based on Model 3 to explore potential nonlinear relationships between serum total folate, 5-MTHF, and the occurrence of liver fibrosis. We found a significant nonlinear relationship between serum total folate and liver fibrosis(p overall = 0.0029; p nonlinear = 0.0115); meanwhile, no evident nonlinear relationship between serum 5-MTHF, and liver fibrosis was observed in the RCS analysis (p overall = 0.0017; p nonlinear = 0.0565) (refer to Figures 2C,D). A discernable reduction in the risk of developing liver fibrosis was noted with an elevation in total folate levels surpassing a critical inflection point, estimated

TABLE 3 Logistic regression model between serum total folate, 5-MTHF level, and MAFLD.

		MAFLD					
		Model 1		Model 2		Model 3	
		OR (95%CI)	*p* trend	OR (95%CI)	*p* trend	OR (95%CI)	*p* trend
Total Folate	T1	ref	ref	ref	ref	ref	ref
	T2	0.819 (0.651, 1.030)	0.088	0.929 (0.738, 1.170)	0.532	0.934 (0.723, 1.206)	0.600
	T3	0.736 (0.587, 0.923)	0.008	0.768 (0.609, 0.968)	0.025	0.758 (0.581, 0.990)	0.042
5-MTHF	T1	ref	ref	ref	ref	ref	ref
	T2	0.818 (0.651, 1.028)	0.085	0.870 (0.683, 1.108)	0.467	0.932 (0.722, 1.204)	0.590
	T3	0.706 (0.563, 0.886)	0.003	0.689 (0.536, 0.886)	0.012	0.736 (0.564, 0.961)	0.024

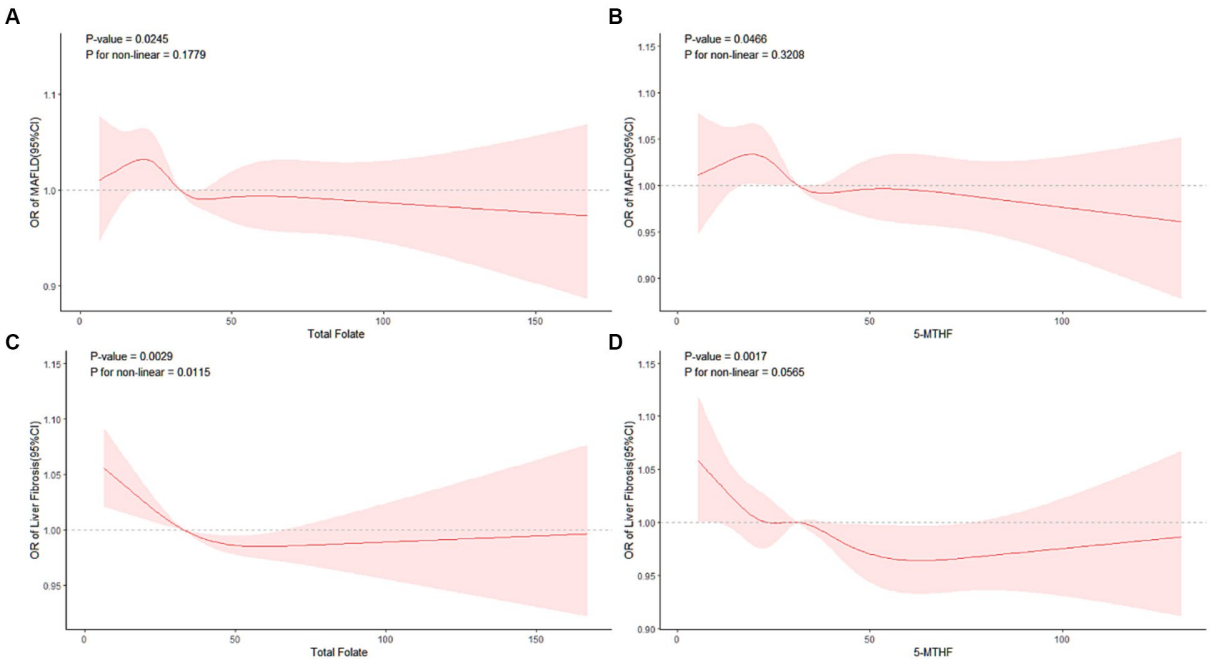

FIGURE 2
RCS analysis of the association of MAFLD with **(A)** total folate, **(B)** 5-MTHF, liver fibrosis with **(C)** total folate, and **(D)** 5-MTHF.

at approximately 31.19 nmol/L. Beyond this threshold, total folate demonstrated a protective role against the progression of liver fibrosis, underscoring its potential importance in mitigating this hepatic pathology.

3.6 Subgroup analysis

We performed stratified multivariate regression analysis to investigate the association between serum total folate, 5-MTHF, MAFLD, and liver fibrosis within distinct population subgroups categorized by gender (male/female), age (<60, ≥60), BMI (<28, ≥28), hypertension, and diabetes. As depicted in Figure 3, no significant significance was observed in all subgroup analyses of serum total folate and 5-MTHF with MAFLD and liver fibrosis (p all >0.05).

4 Discussion

Previous investigations have primarily focused on exploring the associations between serum total folate levels and the probability of NAFLD and liver fibrosis. However, to the best of our knowledge, there is a conspicuous dearth of epidemiological evidence pertaining to the potential correlations between various serum folate levels and MAFLD (15–17). Furthermore, prior investigations employing CAP and LSM to assess the impact of distinct serum folate types on hepatic steatosis and fibrosis were limited to a single survey cycle, resulting in a smaller study population (25). To address these knowledge gaps, we conducted an expansive study involving participants in the United States, leveraging data obtained from NHANES cycles spanning from 2017 to 2020 to encompass a more extensive cohort. This investigation exhibits distinctiveness through methodological advancements. In contrast to antecedent studies, which predominantly

TABLE 4 Logistic regression model between serum total folate, 5-MTHF level, and liver fibrosis.

		Liver fibrosis					
		Model 1		Model 2		Model 3	
		OR (95%CI)	*p* trend	OR (95%CI)	*p* trend	OR (95%CI)	*p* trend
Total folate	T1	ref	ref	ref	ref	ref	ref
	T2	0.756 (0.587, 0.973)	0.030	0.868 (0.666, 1.133)	0.298	0.847 (0.641, 1.117)	0.239
	T3	0.722 (0.560, 0.932)	0.012	0.695 (0.522, 0.925)	0.013	0.677 (0.503, 0.910)	0.010
5-MTHF	T1	ref	ref	ref	ref	ref	ref
	T2	0.859 (0.659, 1.121)	0.263	0.848 (0.642, 1.118)	0.242	0.859 (0.659, 1.121)	0.263
	T3	0.666 (0.499, 0.888)	0.006	0.651 (0.483, 0.878)	0.005	0.666 (0.499, 0.888)	0.006

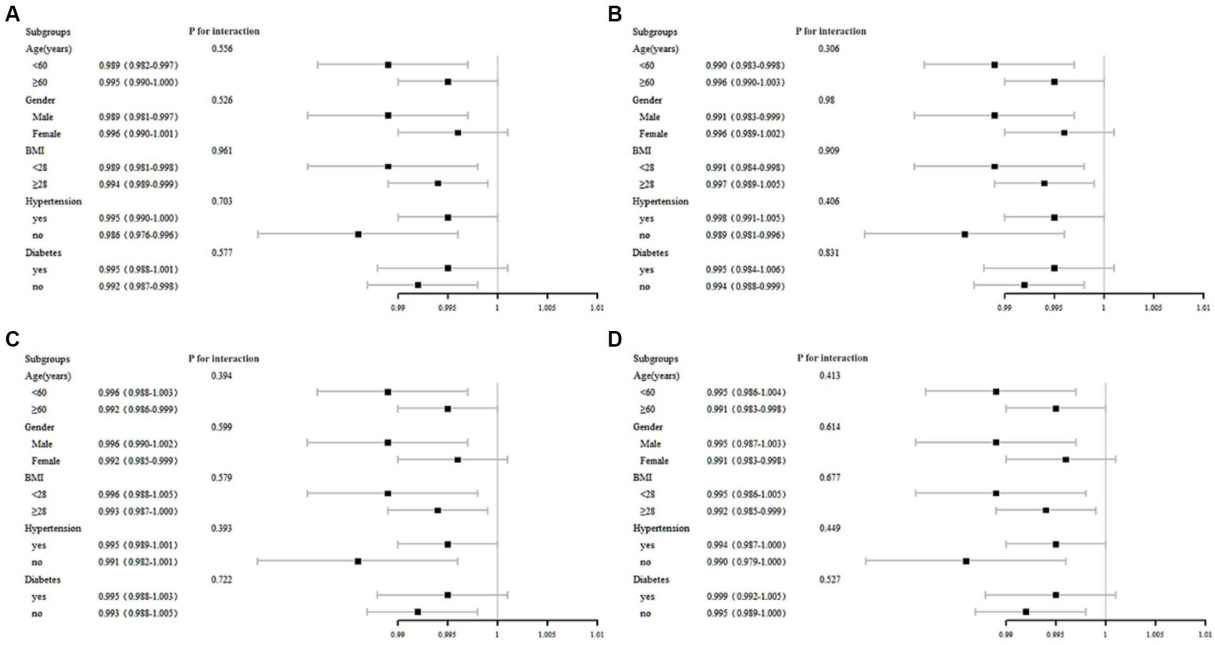

FIGURE 3
Subgroup analysis of the association between MAFLD with **(A)** total folate, **(B)** 5-MTHF, liver fibrosis with **(C)** total folate, and **(D)** 5-MTHF.

scrutinized predictors without employing VCTE, our approach integrates the latest VCTE data, thereby elevating the precision of our analysis (14–16). Furthermore, diverging from earlier inquiries focused on MAFLD that solely considered serum total folate levels from a single cycle (17), our study spans the period from 2017 to 2020. This allows us to not only examine the correlation between diverse serum folate levels and MAFLD but also results in a substantial augmentation of the sample size. This enlargement contributes to a more robust and generalizable investigation, facilitating a more accurate comprehension of the relationship between various serum folate levels and the occurrence of MAFLD, along with its implications for liver fibrosis. Throughout our inquiry, we discerned a noteworthy and persistent inverse correlation between serum total folate, 5-MTHF, and MAFLD, liver fibrosis. These observations align with antecedent investigations in the domain and retain their robustness even following adjustments for diverse potential confounding factors, encompassing sociodemographic characteristics, lifestyle elements, hypertension, and diabetes (14, 25). Nevertheless, our subgroup analyses revealed no significant distinctions in variables, a finding that aligns with previous studies (14, 16).

Research has substantiated the potential of folate to ameliorate MAFLD through diverse pathways. Primarily, folate assumes a crucial role in purine and thymidylate synthesis as well as DNA methylation. Folate deficiency, in turn, is implicated in the heightened expression of genes associated with hepatic fat synthesis, consequently leading to the development of hepatic steatosis (26). Furthermore, inadequate folate levels may disrupt the fibroblast growth factor (FGF) pathways. FGF concentrations have been correlated with insulin resistance, and the regulation of visceral adiposity, by FGF stimulates adipokines and inflammatory factors, thereby fostering the progression of lipotoxic liver disease (27, 28). Thirdly, folate exerts regulatory control over the transcription of NADPH oxidase, effectively mitigating the oxidative stress induced by a high-fat diet (29). This action results in increased adenosine monophosphate (AMP) levels and the activation of liver

kinase B1 (LKB1), subsequently restoring adenosine monophosphate-activated protein kinase (AMPK) activation in the liver. Consequently, this regulatory mechanism contributes to the enhancement of cholesterol and glucose metabolism (30). Fourthly, folate, through the modulation of hepatic microRNA expression, has the potential to reduce blood glucose and lipid concentrations, enhance insulin sensitivity, and ameliorate liver function (31). These findings align with our study, indicating a correlation between elevated serum concentrations of both total folate, 5-MTHF and a decreased prevalence of MAFLD.

Our investigation revealed no substantial correlation between the presence of folic acid, THF and the prevalence of MAFLD. Similarly, another study focusing on young adults did not identify a significant association between folic acid and MAFLD (25). In contrast, Yang et al. (14) reported a significant positive correlation between serum folic acid concentration and the prevalence of NAFLD in their study. Elevated serum folic acid levels were linked to an increase in pro-inflammatory cytokines (IL-8 and TNF-α) and a decrease in natural killer cell cytotoxicity, potentially contributing to the onset of NAFLD (32, 33). Notably, the definition of NAFLD varied between studies, with Yang et al. relying on the USFLI or the FLI, while our study and Wen et al. defined NAFLD/MAFLD using CAP obtained through VCTE. This discrepancy in defining NAFLD/MAFLD may account for the observed inconsistencies in results. Specifically, our study identified a significant inverse association between THF and MAFLD only in Model 1 and 2. In preceding murine investigations, enhancements in intestinal microbiota have demonstrated a concurrent mitigation of steatosis and elevation in hepatic THF content (34). Nonetheless, a paucity of precise experimental or epidemiological inquiries has been undertaken to elucidate the association between THF and hepatic steatosis. Our present study did not reveal a noteworthy correlation between folic acid, THF, and MAFLD. Our hypothesis posits that the underlying cause may be attributed to the comparatively limited proportion of folic acid and THF, constituting a minor fraction of total folate, typically accounting for less than 5% on average. Further studies are warranted to validate our findings, and additional investigations into potential underlying mechanisms are essential.

Beyond its demonstrated efficacy in mitigating liver steatosis, investigations have substantiated the potential of folate to ameliorate liver fibrosis through diverse pathways. A pivotal mediator in hepatocellular injury is hepatic oxidative stress. Folate, with its antioxidant attributes, has been proposed to play a crucial role by directly scavenging Reactive Oxygen Species (ROS), enhancing hepatic lipid peroxidation, and impeding the activities of various antioxidant enzymes (10). Moreover, an investigation has revealed that folate supplementation induces an elevation in the hepatic expression of the fusion protein Syntaxin 17, a crucial factor in counteracting the advancement of liver inflammation and fibrosis (13). The one-carbon units linked to folates serve dual roles—they can undergo oxidation, contribute to the remethylation of homocysteine to generate methionine, or act as substrates for *de novo* purine and thymidine synthesis. Consequently, folate deficiency may disrupt *de novo* phosphatidylcholine synthesis and perturb purine signaling, hastening the progression of liver fibrosis (5). The liberation of inflammatory cytokines orchestrated by macrophages is implicated in the progression of vascular disease. Folate has been demonstrated to mitigate homocysteine-induced pro-inflammatory cytokine expression and impede the recruitment and activation of Kupffer cells, consequently diminishing the likelihood of liver fibrosis (10). In our investigation, a significant negative association between serum total folate and 5-MTHF concentration and the occurrence of liver fibrosis was observed, except THF. While our study revealed a substantial negative correlation between folic acid and liver fibrosis in Model 3, this association was not evident in Models 1 and 2, or in the linear regression analyses. Therefore, we cautiously conclude that the current evidence for this relationship is not highly feasible. Further research examining the impact of various serum folate forms on liver fibrosis is warranted for a more comprehensive understanding of these relationships.

This study marks the inaugural investigation into the correlation between diverse serum folate levels and MAFLD. Notably, it encompasses the largest NHANES population with CAP and LSM data within the 2017–2020 cycle. These findings underscore the importance of elevating public awareness regarding the influence of heightened serum folate concentrations on MAFLD and liver fibrosis. Public health initiatives can amplify awareness by emphasizing the impact of serum folate on metabolic health, targeting the general population. These campaigns should underscore the necessity of maintaining optimal folate levels. In clinical practice, healthcare providers should personalize treatments for individuals with metabolic disorders, recommending folate-rich foods or medications to mitigate associated risks. Moreover, this study prompts consideration for future research directions on serum folate in MAFLD and liver fibrosis. Potential investigations include exploring the dose–response correlation between folate intake and MAFLD as well as liver fibrosis, discerning the impact of diverse folate levels on outcomes. Additionally, evaluating the feasibility and efficacy of incorporating routine folate into clinical practice. This may enhance the precision of dietary or supplemental recommendations, contributing to more personalized and effective strategies for managing metabolic health.

Employing a comprehensive approach that integrates multifactor regression analysis and subgroup analysis, we assessed the influence of various serum folate levels on MAFLD and liver fibrosis. Despite these strengths, certain limitations merit consideration. The cross-sectional design of the NHANES dataset inherently precludes the establishment of causal relationships. Despite meticulous covariate control, the potential influence of unaccounted variables on the association between diverse serum folate levels and MAFLD, liver fibrosis remains a consideration. Genetic factors, specific dietary patterns, or unaccounted lifestyle habits not addressed in the study may serve as confounding variables. For example, variations in participants' exercise habits related to liver conditions could introduce biases, thereby complicating the accurate attribution of observed effects solely to serum folate levels. Additionally, the absence of standardized diagnostic thresholds for liver steatosis and fibrosis through VCTE poses a significant challenge. Researchers often employ diverse criteria, rendering cross-study comparisons challenging. Consequently, the precision of estimates may be compromised. Moreover, the study recognizes the impact of variability in diagnostic criteria for comorbid conditions, such as diabetes, alcohol consumption, and cotinine exposure. The adoption of diverse criteria across studies results in heterogeneous participant categorization, creating challenges in drawing uniform conclusions. For example, our

study uses cotinine levels, while other employs self-reported smoking numbers. These methodological differences may introduce discrepancies that could affect the accuracy of assessing the association between serum folate levels and liver health. Finally, concerns surrounding MAFLD have emerged due to its heterogeneous etiologies, and the term "fatty" has faced criticism for its potential stigmatizing effects (35). In 2023, an international panel of experts recommended transitioning from MAFLD to metabolic dysfunction-associated steatotic liver disease (MASLD). The updated diagnostic criteria for MASLD require the fulfillment of at least one of the five cardiometabolic risk factors, contingent upon the presence of liver steatosis (36). MAFLD's limitations stem from evolving diagnostic criteria, possibly leading to heterogeneity in study populations.

5 Conclusion

In this expansive cross-sectional investigation encompassing a substantial population, we have established a significant negative correlation between heightened serum concentrations of total folate, 5-MTHF, and CAP. Simultaneously, elevated serum 5-MTHF levels are significantly associated with lower LSM. Furthermore, we have discerned a significant relationship between increased serum concentrations of both total folate and 5-MTHF and a decreased prevalence of MAFLD and liver fibrosis. Our findings have undergone meticulous adjustments for various potential confounders, reaffirming the robustness of these associations. These results underscore the significance of enhancing public awareness regarding the impact of elevated serum folate concentrations on metabolic health.

Data availability statement

The data utilized in this investigation were sourced from NHANES, and detailed information can be accessed at: https://wwwn.cdc.gov/nchs/nhanes/Default.aspx.

Ethics statement

The studies involving humans were approved by Ethical approval for the study was granted by the Research Ethics Review Board of the National Center for Health Statistics. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

JC: Conceptualization, Data curation, Formal Analysis, Methodology, Software, Writing – original draft, Writing – review & editing. DC: Software, Supervision, Writing – review & editing. WL: Writing – review & editing. FX: Writing – review & editing. XF: Writing – review & editing. LZ: Writing – review & editing. HL: Writing – review & editing. JS: Funding acquisition, Project administration, Supervision, Writing – review & editing. HY: Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

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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

Supplementary material

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Hypercaloric low-carbohydrate high-fat diet protects against the development of nonalcoholic fatty liver disease in obese mice in contrast to isocaloric Western diet

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Objective: Obesity and metabolic complications, such as type 2 diabetes and nonalcoholic fatty liver disease (NAFLD), are one of the greatest public health challenges of the 21st century. The major role of high sugar and carbohydrate consumption rather than caloric intake in obesity and NAFLD pathophysiology remains a subject of debate. A low-carbohydrate but high-fat diet (LCHFD) has shown promising results in obesity management, but its effects in preventing NAFLD need to be detailed. This study aims to compare the effects of a LCHFD with a high-fat high-sugar obesogenic Western diet (WD) on the progression of obesity, type 2 diabetes, and nonalcoholic fatty liver disease.

Methods: Male C57BL/6J mice were initially fed a WD for 10 weeks. Subsequently, they were either switched to a LCHFD or maintained on the WD for an additional 6 weeks. Hepatic effects of the diet were explored by histological staining and RT-qPCR.

Results: After the initial 10 weeks WD feeding, LCHF diet demonstrated effectiveness in halting weight gain, maintaining a normal glucose tolerance and insulin levels, in comparison to the WD-fed mice, which developed obesity, glucose intolerance, increased insulin levels and induced NAFLD. In the liver, LCHFD mitigated the accumulation of hepatic triglycerides and the increase in Fasn relative gene expression compared to the WD mice. Beneficial effects of the LCHFD occurred despite a similar calorie intake compared to the WD mice.

Conclusion: Our results emphasize the negative impact of a high sugar/carbohydrate and lipid association for obesity progression and NAFLD development. LCHFD has shown beneficial effects for NAFLD management, notably improving weight management, and maintaining a normal glucose tolerance and liver health.

KEYWORDS

obesity, non-alcoholic fatty liver disease, low-carbohydrate diet, Western diet, insulin

1 Introduction

Over the past decades, the prevalence of obesity has substantially increased, reaching 17% of the adult population in France and 42.4% in the United States (1, 2). If several factors have been attributed to the increased obesity rate, the major contributor is diet composition and particularly the Western diet (WD) (3). The WD is characterized by being hypercaloric, rich in saturated fats, refined carbohydrates, and added sugars and salt (4). The consumption of a WD increases the risk of obesity and metabolic comorbidities, such as type 2 diabetes and nonalcoholic fatty liver disease (NAFLD) (5, 6). Currently, type 2 diabetes affects approximately 463 million adults, while the prevalence of NAFLD is estimated to be between 25 and 30% in the world population (7, 8). The increase of NAFLD prevalence is a major challenge of the 21st century, as NAFLD is the contributor to liver mortality and morbidity which has the fastest growth (9).

Health care guidelines promote lifestyle changes for weight loss and comorbidity reduction (10, 11). Therefore, low-calorie diets have become increasingly popular and widespread, even if their long-term weight loss effectiveness are limited due to a low adherence rate (12, 13). The carbohydrate-insulin model of obesity has been proposed to explain the limited effect of caloric restriction on weight loss; it suggests that the adiposity increase is not due to caloric excess but rather to hyperinsulinemia induced by the consumption of high amounts of sugar and refined carbohydrates (i.e., the WD) (14). Thus, the adverse consequences of elevated sugar and carbohydrate consumption within the WD continue to be a subject of debate and are still not fully understood. In this context, studying a low carbohydrate-high-fat diet (LCHFD) represents a good strategy to better understand whether carbohydrates, not an excess of calories, are indeed the trigger for obesity development. Indeed, the LCHFD is a diet in which carbohydrate intake is largely reduced (10% of daily consumption) while overall caloric intake is unchanged and lipid intake is consequently largely increased (by 70 to 80%) (15, 16). In human, the LCHFD diet, popularly known as the ketogenic diet, has been demonstrated to be safe and effective for obesity management and its related complications, by promoting weight loss and improving glucose metabolism despite maintaining a high caloric amount (17–21).

However, the effects of a LCHFD diet in preventing NAFLD remain poorly understood. Pre-clinical studies have demonstrated that the transition from a WD to a hypercaloric LCHFD diet resulted in a modification of the hepatic lipid metabolism genes expression in favor of an increase in those involved in the fatty acid oxidation and a decrease in those involved in fatty acid storage (22–24). Nevertheless, none of these studies directly focused on the LCHFD diet effects on NAFLD markers, including histological explorations.

The objective of this study was to compare the effects of a LCHFD and WD on the progression of obesity, glucose intolerance, and NAFLD development in diet-induced obese mice. We hypothesize that the LCHFD has the potential to impede the progression from obesity to the development of NAFLD suggesting that it is the association of a high sugar and a high calorie amount consumption, and not simply an excess of calories, that is responsible for the worsening of the metabolic complications. We report here that after WD feeding, the LCHFD mice stopped weight gain and prevented glucose intolerance and NAFLD development. These effects seemed to involve a decrease insulin production in comparison to the Western Diet, preventing the liver from *de novo* lipogenesis activation.

2 Materials and methods

2.1 Animals and study approval

All experiments were approved by our local ethics committee (CREMEAS, agreement numbers: 2018042013495170) and performed following the Guide for the Care and Use of Laboratory Animal Experiments. Thirty-five 8 weeks-old C57BL/6J male mice (ENVIGO, Gannat, France) were maintained at 22 ± 2°C on a 12 h day/night cycle and housed twice in conventional open-top cages enriched with cotton sticks, shredded paper, and wooden chew sticks. Water and food were provided *ad libitum*.

2.2 Experimental design and mouse studies

The experimental design is detailed in Figure 1A. Animals were divided into 3 groups: (1) the control group, fed a standard control chow diet (SD) for 16 weeks (SD; 20.5% fat, 15.5% protein and 64% carbohydrates, 3.82 kcal/g, Safe® Diets, $n = 8$); (2) mice fed a high-fat high-sugar WD for 16 weeks (WD 16w; 58.6% fat, 14.4% protein and 27% carbohydrate, 5.52 kcal/g, Safe® Diet, $n = 9$); and (3) LCHFD group, fed a WD for 10 weeks and then fed a LCHFD for 6 weeks (LCHFD; 77% fat, 18.9% protein and 4.2% carbohydrates, 5.55 kcal/g, Safe® Diets, $n = 10$). Body weight and food intake were measured weekly.

Moreover, 9 mice were also fed a WD for 10 weeks and then sacrificed by cervical dislocation, only to establish a baseline for comparison prior to the dietary change (WD 10w). Adipose tissue and liver were harvested and weighted, and the data are available in the Supplementary Figure S1.

Intraperitoneal glucose tolerance tests (IPGTTs) were performed at the 10th week for the WD 10w group and at the 16th week for the SD, WD 16w and LCHFD groups. After 4 h of fasting, glucose (1.5 g/kg iv) was administered and blood glucose concentrations were measured at $t = 0$,15, 30, 45, 60, and 120 min from the tail vein using a glucose meter (AccuChek Performa, Roche, Basel, Switzerland). The area under the curve (AUC) was calculated from the values of IPGTT.

At the end of the 16th week and after 4 h of fasting, the SD, WD and LCHFD mice were placed in a hermetic cage and anesthetized through inhalation of 4% isoflurane (Aerrane, CSP, Cournon, France). Then, mice were euthanized by cervical dislocation and exsanguinated. Blood was collected into a heparinized tube, and plasma was separated

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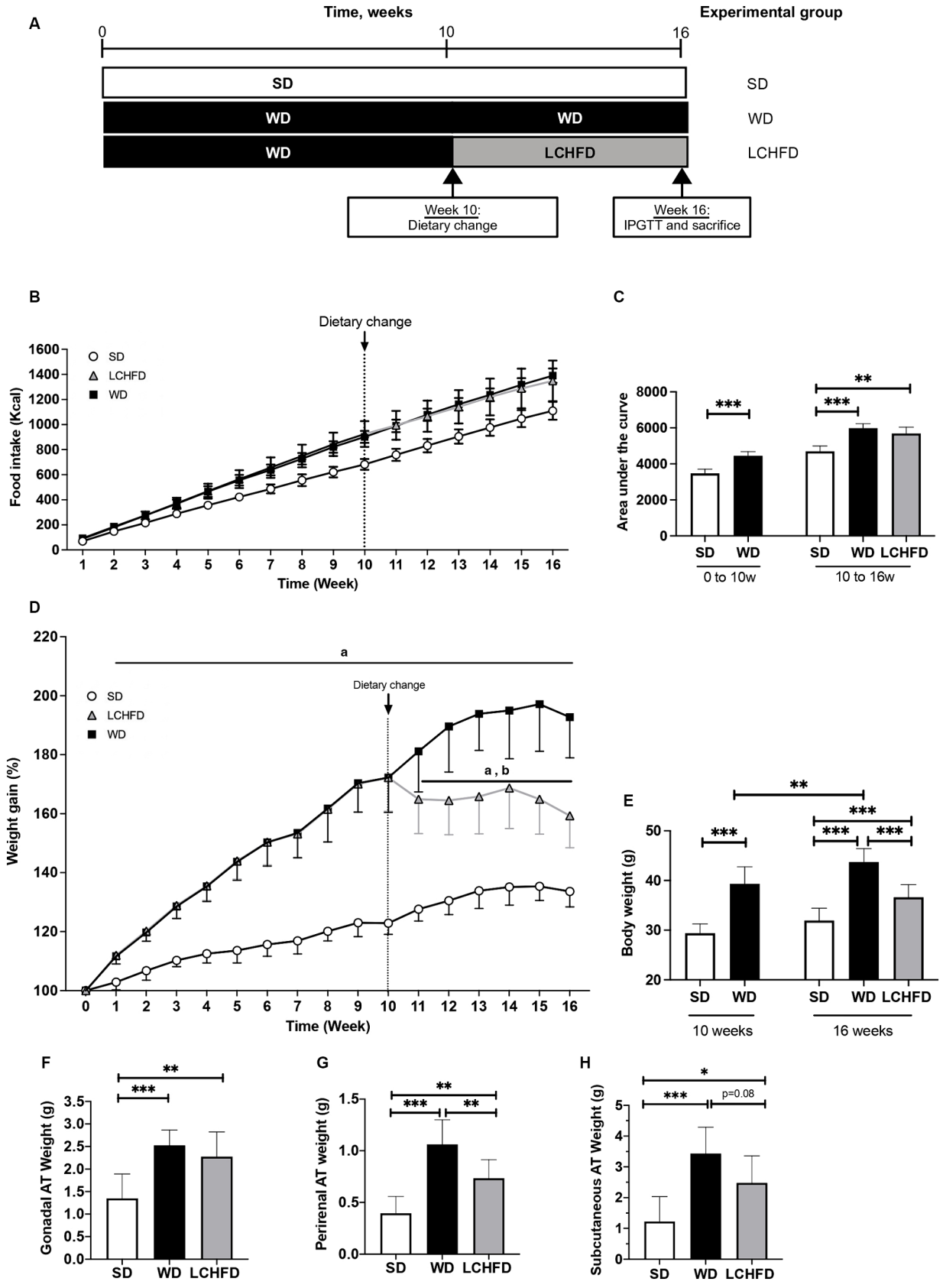

FIGURE 1
Effect of a WD and LCHFD on caloric intake, weight gain and adipose tissue (AT) accumulation. **(A)** Experimental design of the procedure with the SD group (fed a standard diet throughout the study), WD group (fed a Western diet throughout the study) and LCHFD group (10 weeks of a Western diet feeding followed by 6 weeks of low-carbohydrate high-fat diet feeding). **(B)** Food intake in kcal throughout the procedure. Circles represent the SD group, squares represent the WD group, and triangles represent the LCHFD group. White represents SD feeding, black represents WD feeding, and grey

(Continued)

FIGURE 1 (Continued)
represents LCHFD feeding. **(C)** Quantification of the food intake area under the curve from 0 to 10 weeks and from 0 to 16 weeks. Time refers to weeks. **(D)** Weight gain throughout the procedure expressed in % of the initial body weight. a = different from the SD group, b = different from the WD group. Circles represent the SD group, squares represent the WD group, and triangles represent the LCHFD group. White represents SD feeding, black represents WD feeding, and grey represents LCHFD feeding. **(E)** Body weight of the mice expressed in grams at 10 weeks and 16 weeks. Weight in grams of **(F)** gonadal A. **(G)** perirenal AT and **(H)** Subcutaneous AT. Mean ± SD, $n = 8–10$ * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. SD, standard diet; WD, Western diet; LCHFD, low-carbohydrate high-fat; AT, adipose tissue.

by centrifugation (1,000 × g for 10 min at 4°C) and frozen for biochemical analysis. Tissues were harvested, weighed and then either snap-frozen in liquid nitrogen for molecular experiments or cooled in 2-methylbutane immersed in liquid nitrogen for histological staining. All samples were stored at −80°C.

2.3 Experimental procedures

2.3.1 Blood sample analyses

Insulin, triglycerides, and total, HDL and LDL cholesterol plasma levels were determined by the Institut Clinique de la Souris (Strasbourg, France) phenotyping platform using an AU-480 automated laboratory workstation (Beckman Coulter France SAS, Villepinte, France). To assess insulin resistance, the HOMA-IR (homeostasis model assessment of insulin resistance) index was calculated as (fasting serum glucose × fasting serum insulin/22.5) (25).

2.3.2 Histological analyses

Liver tissue sections were made at −20°C on a cryostat microtome (Cryostar NX70, Fisher Scientific, Waltham, MA, United States).

For hematoxylin and eosin staining, 10 µm tissue sections were subjected to staining using a standard protocol. First, cryosections were fixed in acetone for 3 s and dried at 37°C for 1 h. Then, they were stained with Harris' hematoxylin solution for a duration of 2 min, followed by a gentle wash in tap water for 3 min. Next, the sections were bleached in 1% acid alcohol for a brief 2 s period, followed by another wash in tap water for 3 min. Subsequently, counterstaining was performed using an eosin solution for 1 min. Finally, the sections were rinsed in tap water for 3 s, followed by sequential washes in 80% ethanol and 100% ethanol. Finally, the stained sections were mounted with Eukitt medium (Orsatec, Germany). NAS score (NAFLD activity score) was assessed by calculating the score of each NAS component [steatosis (0–3), lobular inflammation (0–3), ballooning (0–2)] (26).

For Oil Red O staining, 10 µm cryosections were initially rehydrated in PBS for 2 min and stained by immersion in Oil Red O solution (Sigma-Aldrich, O0625) for a duration of 3 min. A brief wash in 60% isopropanol for 30 s, followed by a wash in deionized water for 1 min, was applied before counterstaining in Harris' hematoxylin for 3 min. Then, the cryosections were washed in tap water for 2 min and mounted in Aquatex aqueous medium (Sigma-Aldrich, 108,635). Stained slides were scanned using a Zeiss Apotome.2 microscope (CTK Instruments, Carlsbad, CA, United States), and lipid quantification was assessed using Adobe Photoshop (Adobe Systems, San Jose, CA, United States). The results are expressed as the fold change compared to the SD group.

2.3.3 RNA isolation, reverse transcription, and real-time quantitative PCR

First, total liver RNA was isolated with the Kingfisher Duo Prime (Fisher Scientific, Massachusetts, United States) using the MagMAX™ mirVana™ Total RNA Isolation Kit according to the manufacturer's instructions (Applied Biosystems™, California, United States) and then stored at −80°C. The quantity and purity of the RNA were assessed with Qubit™ RNA Broad Range (BR) and Integrity Quality (IQ) assay kits using the Invitrogen Qubit 4 Fluorometer according to the manufacturer's instructions (Invitrogen™, California, United States). cDNA was synthesized from 2 µg of total liver RNA with Maxima™ H Minus cDNA Synthesis Master Mix (Fisher Scientific, Massachusetts, United States). Real-time PCR was performed in triplicate in a total reaction volume of 15 µL using either PowerTrack™ SYBR Green Master Mix (Applied Biosystems, California, United States) or in a total reaction volume of 20 µL with Taqman Fast Advanced Master mix (Applied Biosystems, Massachusetts, United States), following the manufacturer's recommendation. To amplify genes, TaqMan probes were used for the hypoxanthine Phosphoribosyltransferase (Hprt, Mm03024075_m1) and the fatty acid synthase (FASN, Mm00662319_m1), or forward and reverse primers described in Table 1. Real-time PCRs were measured in a QuantStudio 3 Real-Time PCR System (Applied Biosystems™, California, United States) using the following cycle parameters: UNG incubation at 50°C for 10 min, enzyme activation at 95°C for 20 s, 40 cycles of denaturation at 95°C for 1 s followed by annealing/extension at 95°C for 20 s. The relative mRNA levels were normalized to Hprt housekeeping gene levels, which were unaffected by the experiment. We used the ΔΔCt method to normalize cycle threshold values for each gene of interest (27).

2.4 Statistical analysis

All data are shown as the mean ± SD. Normal distribution of data was checked using the Shapiro–Wilk test and outliers were checked using ROUT method. Two-group comparisons were assessed with student's *t*-test, 3-group or more comparisons were made with one-way ANOVA or Kruskal–Wallis tests, followed by Tukey's or Dunns' post hoc test. Correlations were assessed with Pearson or Spearman tests, depending on the result of the normality test. All statistical analyses were performed using GraphPad 8® (GraphPad Software, Inc.). Statistical significance is shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3 Results

3.1 A low-carb high-fat diet stopped weight gain and adipose tissue accumulation following Western diet feeding

Eight-weeks-old C57Bl6/J male mice were fed a WD for 10 weeks. Compared to the SD mice, the WD mice presented a significant

TABLE 1 Primers for liver qPCR.

Gene	Forward primer (5′–3′)	Reverse primer (3′–5′)
Hprt	GTTGGATACAGGCCAGACTTTGTTG	GATTCAACTTGCGCTCATCTTAGGC
Acadl	GAAGATGTCCGATTGCCAGC	AGTTTATGCTGCACCGTCTGT
Acadm	ATGACAAAAGCGGGGAGTACC	CCATACGCCAACTCTTCGGT
Acc1	AGGCGGATATCTGCTGAGAC	CCAGACATGCTGGATCTCAT
Echs1	GCAAAGCAGGCAGGTCTTGT	TAGCTGCCAGTTCTCAGTGG
Hadh	TCGTGAACCGACTCTTGGTG	ATTTCATGCCACCCGTCCAA
Pparα	ACTACGGAGTTCACGCATGTG	TTGTCGTACACCAGCTTCAGC
Scd1	ATCGCCCCTACGACAAGAAC	AACTCAGAAGCCCAAAGCTCA
Srebp1	GGAACTTTTCCTTAACGTGGGC	ATGAGCTGGAGCATGTCTTCG

Hypoxanthine phosphoribosyltransferase (Hprt), acyl-CoA dehydrogenase long chain (Acadl), acyl-CoA dehydrogenase medium chain (Acadm), acetyl-coA carboxylase 1 (Acc1), enoyl coenzyme A hydratase (Echs1), hydroxyacyl-coenzyme A dehydrogenase (Hadh), peroxisome proliferator activated receptor alpha (Pparα), stearoyl-coA desaturase-1 (Scd1) and sterol regulatory element-binding protein 1 (Srebp1).

increase in caloric intake (Figure 1B), displayed by the area under the curve ($p < 0.001$, Figure 1C), and a statistically significant increase in weight gain ($p < 0.001$, Figure 1D). At the end of the 10th week, the WD mice reached a body weight of 39.3 ± 3.4 g, while the SD mice weighed 29.4 ± 1.8 g ($p < 0.001$, Figure 1E). This significant weight gain was associated with gonadal (+42%, $p < 0.05$) and perirenal AT accumulation (+75%, $p < 0.01$, Supplementary Figure S1), compared to the SD mice. After 10 weeks, the WD mice were separated into two groups, one of which continued with the WD and the other of which transitioned to the LCHFD, for 6 weeks. The WD and LCHFD mice continued to consume a higher caloric amount than the SD mice ($p < 0.001$, Figures 1B,C), without a significant difference between the WD and LCHFD groups.

The WD group exhibited continuous weight gain throughout the study, reaching a significant weight gain of +93% at 16 weeks ($p < 0.001$) compared to the SD group (Figure 1D), and a final body weight of 43.7 g ± 1.0, compared to their 10 weeks weight ($p < 0.01$, Figure 1E). This weight gain was accompanied by a significant increase in gonadal adipose tissue, perirenal adipose tissue and subcutaneous adipose tissue ($p < 0.001$, Figures 1F–H) in comparison with the SD group.

Interestingly, when the mice switched to the LCHFD, weight gain stopped (Figures 1D,E). Indeed, after 6 weeks of the LCHFD, the final body weight of the LCHFD mice was significantly lower than that of the WD mice ($p < 0.001$) and similar to those measured at 10 weeks (Figure 1E). LCHFD mice presented a significantly higher gonadal, perirenal ($p < 0.001$, Figures 1F,G) and subcutaneous AT weight ($p < 0.05$, Figure 1H), compared to the SD mice. Interestingly, the LCHFD mice presented a significantly lower perirenal AT weight (−27%, $p < 0.01$) and a tendency toward a lower subcutaneous AT weight (−22%, $p = 0.080$) in comparison with the WD 16w mice.

3.2 A low-carb high-fat diet prevents ongoing Western diet-induced glucose intolerance and dyslipidemia

Despite exhibiting a similar fasting blood glucose level to those of SD mice (Figure 2A), WD mice exhibited exacerbated abnormal glucose tolerance, the area under the curve quantification confirmed that the WD group had consistently higher glycemia throughout the test, compared to the SD group ($p < 0.001$, Figure 2C). Moreover, insulin levels and the HOMA-IR index of the WD mice were significantly increased compared to those of the SD mice (respectively +143%, $p < 0.05$ and + 118%, $p < 0.01$, Figures 2D,E)

In contrast, the fasting blood glucose levels of the LCHFD mice were tended to be lower than those of the WD mice (−24%, $p < 0.0595$, Figure 2A). The glucose tolerance curve of the LCHFD mice was similar to that of the SD mice (Figure 2B), and the area under the curve was significantly different from that of the WD mice ($p < 0.001$, Figure 2C). Moreover, the insulin levels and the HOMA-IR index of the LCHFD mice remained similar to those of the SD mice and were, respectively, 56 and 65% lower than those of the WD 16w mice ($p < 0.05$, Figures 2D,E).

Regarding lipid profile measurements, 16 weeks of a WD did not impact triglycerides levels (Figure 2F) but induced a substantial increase in total cholesterol ($p < 0.05$), HDL ($p < 0.01$), and LDL ($p < 0.05$) levels in comparison with those of the SD mice (Figures 2G–I). Interestingly, mice who switched to a LCHFD showed a decrease of total cholesterol and HDL levels in comparison with the WD mice ($p < 0.05$), and displayed triglycerides, total cholesterol, LDL HDL levels similar to those of the SD mice (Figures 2G–I).

3.3 A low-carb high-fat diet prevents ongoing Western diet-induced hepatic steatosis and *de novo* lipogenesis

As NAFLD is a prevalent comorbidity associated with obesity, we conducted liver analyses. First, we characterized the hepatic effects of 10 weeks of WD feeding. The liver weight of the WD 10w mice was similar to that of the SD mice and histological staining revealed few steatosis spots, without significant hepatic lipid accumulation compared to the SD mice (Supplementary Figure S1).

After 16 weeks, the WD mice developed hepatomegaly with a considerable rise in liver weight compared to the SD (+45%, $p < 0.01$, Figure 3A). Hematoxylin-eosin staining and NAS score revealed NAFLD development, characterized by steatosis and hepatocyte ballooning ($p < 0.01$, Figures 3B,E), along with a significant red lipid droplet accumulation compared to the SD mice (+139%, $p < 0.05$, Figures 3C,D).

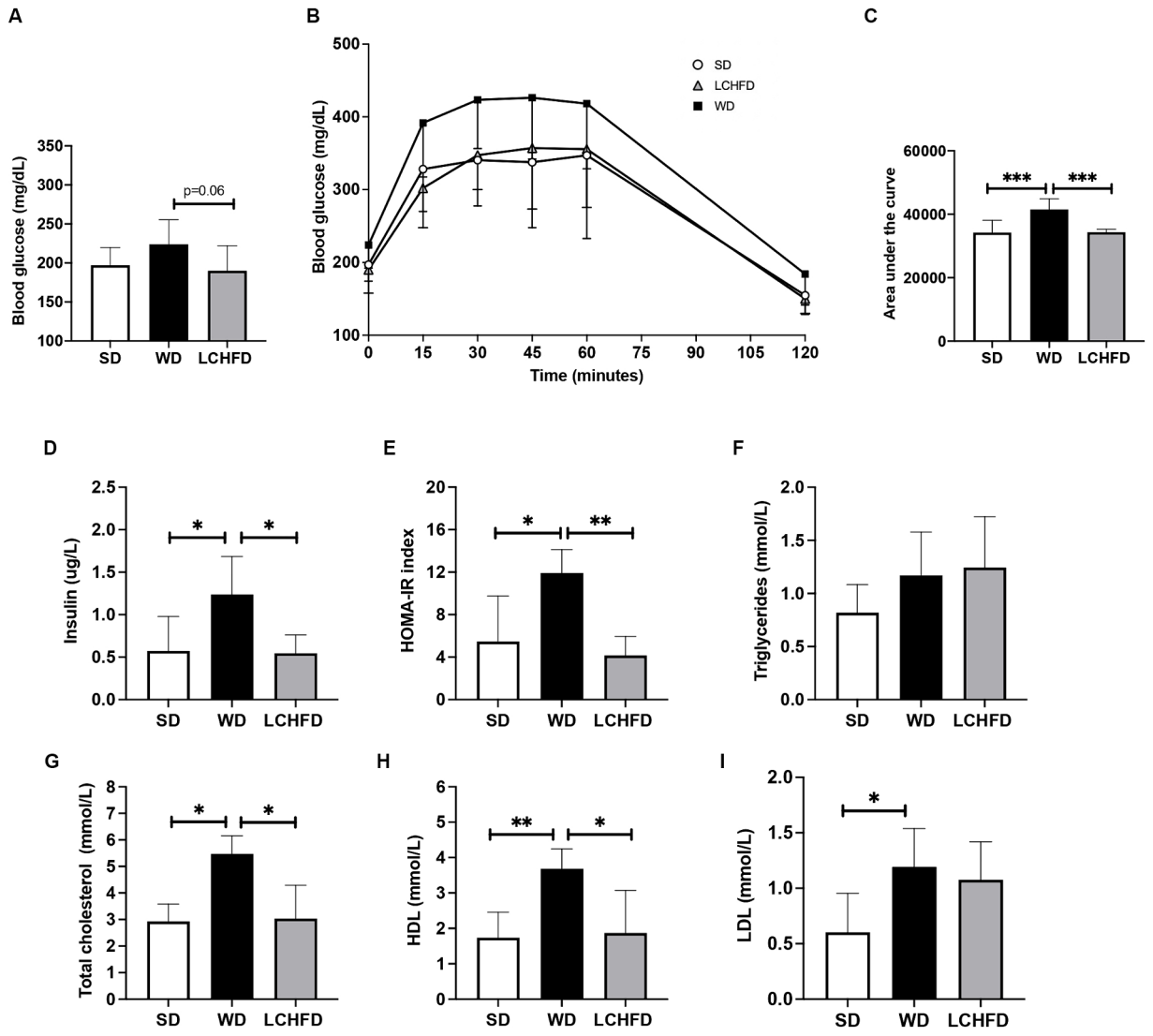

FIGURE 2
Effects of a WD and LCHFD on glucose tolerance, insulin levels and lipid profiles. **(A)** IPGTTs were performed after 10 weeks of diet (WD 10w) and after 16 weeks (SD, WD 16w and LCHFD groups, $n = 8–10$). **(B)** IPGTT corresponding area under the curve. **(C)** Fasting blood glucose measured from the vein tail after 4 h of fasting ($n = 8–10$). Plasma level measurement of **(D)** fasting insulin. **(E)** Triglycerides. **(F)** Total cholesterol. **(G)** High-density lipoprotein (HDL) cholesterol. **(H)** Low-density lipoprotein (LDL) cholesterol. For blood samples, $n = 5–6$. Mean ± SD * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. SD, standard diet; WD, Western diet, LCHFD, low-carbohydrate high-fat.

Interestingly, the LCHFD prevented hepatomegaly, as liver weight in the LCHFD group was comparable to that of the SD group but lower than that of the WD 16w group (Figure 3A, $p < 0.01$). Hepatic histological staining of LCHFD group presented fewer steatosis spots and lower red lipid droplet accumulation compared to WD 16w liver (−100%, $p < 0.0629$, Figures 3B–D). NAS score of LCHFD mice was similar to that of SD mice and lower than the WD group ($p < 0.01$, Figure 3E).

Regarding the hepatic gene expression of the actors involved in fatty acid metabolism, Fasn gene expression of the WD mice was fourfold higher than that of SD mice ($p < 0.05$) but the relative gene expression of Pparα, Acadl, Acadm, Echs1 or Hadh remains similar to the SD mice (Figures 4E,F). Interestingly, the transition to a LCHFD induced a significant decrease in Fasn gene expression in comparison with the WD mice ($p < 0.05$) and a decrease in Scd1 gene expression in comparison with SD mice ($p < 0.05$). Moreover, mice showed a tendency to decrease Acc1 ($p = 0.0866$) and increase Acadm ($p = 0.0965$) gene expression compared to the SD group (Figures 3F,G).

Finally, we measured the relationship between the NAS score and several markers of progression and improvement of the NAFLD (Figure 4) (28–30).

We found that the final body weight ($r^2 = 0.5459$, $p = 0.0003$), the Δ body weight ($r^2 = 0.4009$, $p = 0.0036$), the fat mass ($r^2 = 0.2689$; $p = 0.0229$), the visceral adipose tissue mass ($r^2 = 0.2567$; $p = 0.0269$) and the liver weight ($r^2 = 0.2963$; $p = 0.016$), were correlated with the NAS score. Moreover, the fasting blood glucose tended to correlate with the NAS score ($r^2 = 0.1835$, $p = 0.0673$).

4 Discussion

The effect of dietary macronutrient composition rather than caloric amount on obesity and NAFLD pathophysiology remains a topic of much debate. In this study, we conducted a comparative analysis to assess the metabolic impact of high sugar/carbohydrate

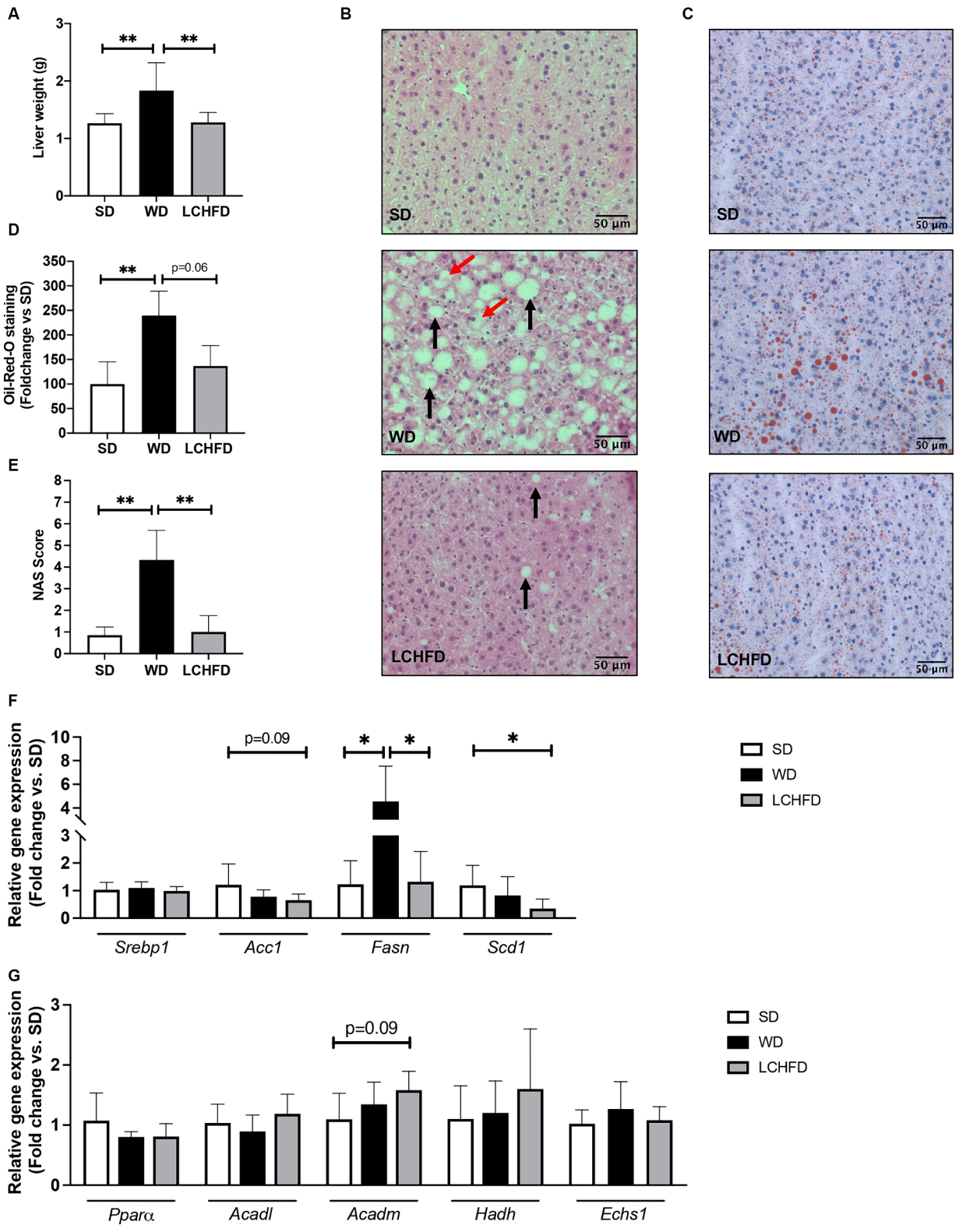

FIGURE 3
Effects of a WD and LCHFD on liver histology and fatty acid metabolism actor's gene expression. **(A)** Liver weight in grams, $n = 8–10$. **(B)** Hematoxylin-eosin staining of liver sections. The black arrow indicates steatosis, and the red arrow indicates hepatocyte balloonization. **(C)** Oil Red O staining of liver sections. Lipids are stained red. **(D)** Oil Red O staining quantification, $n = 4–5$. **(E)** NAFLD activity score (NAS) score, $n = 6–8$. Relative gene expression of **(F)** fatty acid oxidation markers including peroxisome proliferator activated receptor alpha (Pparα), acyl-CoA dehydrogenase long chain (Acadl), acyl-CoA dehydrogenase medium chain (Acadm), hydroxyacyl-coenzyme A dehydrogenase (Hadh) and (H) enoyl coenzyme A hydratase (Echs1). Relative gene expression of markers of fatty acid storage **(G)** including sterol regulatory element-binding protein 1 (Srebp1), fatty acid synthase (Fasn) and stearoyl-coA desaturase-1 (Scd1), $n = 6–8$. Mean ± SD * = $p < 0.05$ and ** = $p < 0.01$. SD, standard diet; WD, Western diet; LCHFD, low-carbohydrate high-fat diet.

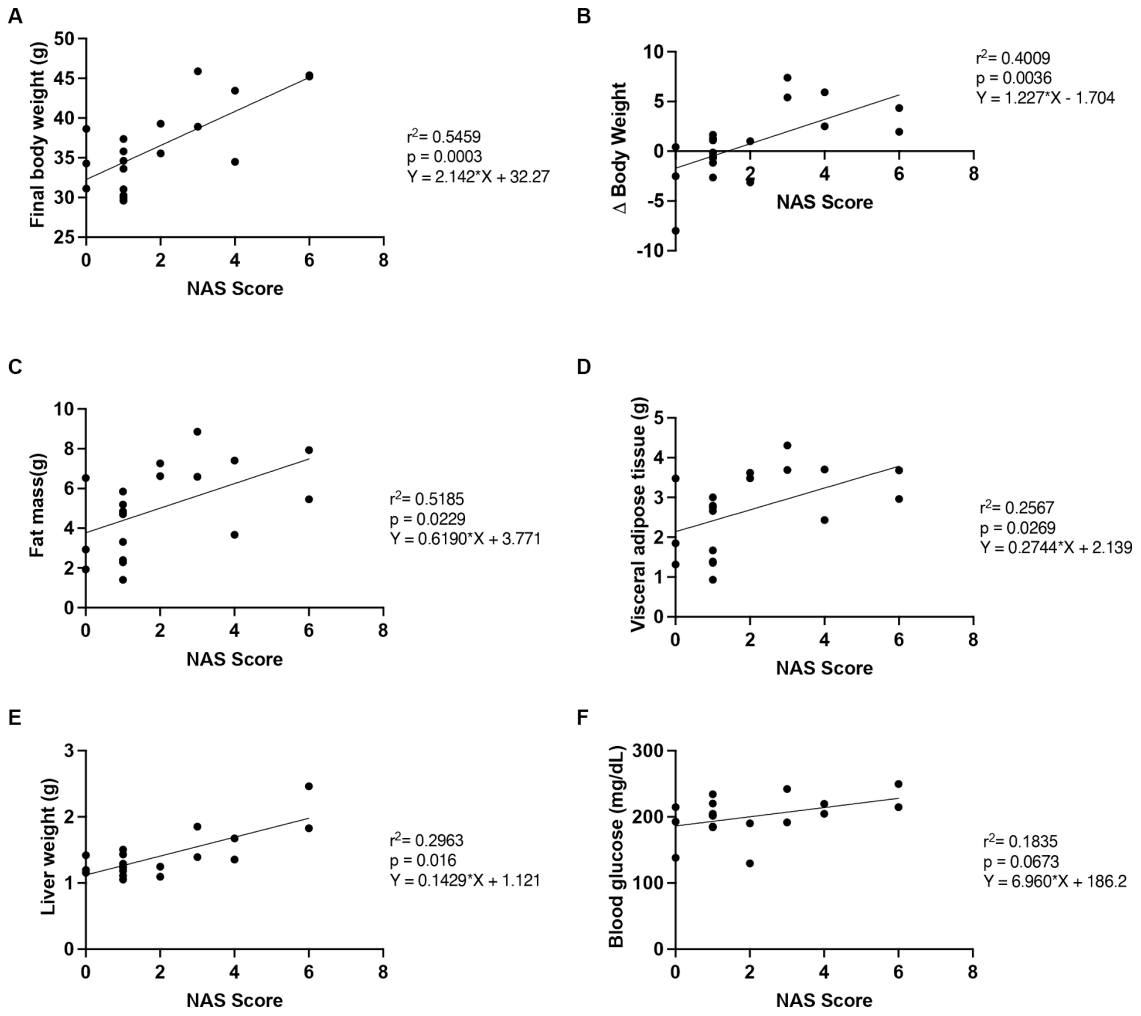

FIGURE 4
Correlation of NAS score with outcome measures. The relationship between NAS score and **(A)** final body weight, **(B)** Δ body weight (between the 10th and 16th weeks), **(C)** fat mass, **(D)** visceral adipose tissue mass, **(E)** liver weight and **(F)** fasting blood glucose, *n* = 19. SD, standard diet; WD, Western diet; LCHFD, low-carbohydrate high-fat diet.

amounts in high-calorie diets by studying the effects of hypercaloric WD and LCHFD on obesity progression and complications development. We reported that the WD induced obesity, glucose intolerance, and NAFLD development, whereas a LCHFD limited weight gain, and maintained a normal glucose regulation, insulin levels and hepatic health, preventing the development of all symptoms. These findings suggest that the detrimental effects of the WD are primarily attributed to the combination of sugar and lipids rather than the hypercaloric nature of the diet.

After 10 weeks of a WD, mice displayed a significant increase in weight gain indicating obesity development, but without hepatic complications. These results are consistent with other studies using diet-induced obesity (22–24). When the WD was continued for an additional 6 weeks, mice showed a progression of obesity, associated with several obesity-related disorders, such as hyperinsulinemia, glucose intolerance, dyslipidemia and NAFLD development. In contrast, switching the high-fat high-sugar WD to a low-carbohydrate high-fat diet mitigated WD-induced weight gain, and prevented the development of dyslipidemia, glucose intolerance and NAFLD in mice. Indeed, the LCHFD mice stopped their weight gain, even though their caloric intake was comparable to that of the WD mice and substantially higher than that of the SD mice. The same observations were also reported previously, in diet-induced obesity models when mice were fed *ad libitum* with a LCHFD (22–24). Moreover, a study demonstrated that healthy mice maintaining a hypercaloric LCHFD showed a decrease in weight compared to the SD mice, although LCHFD mice consumed significantly more calories than their SD counterparts (31). Altogether, our results and those of the literature indicate that excess calories alone cannot explain obesity development, although energy imbalance is conventionally stated to be the main driver of weight gain (32). Therefore, macronutrient repartition seems to be a trigger for obesity development, particularly when carbohydrates and sugars are consumed conjointly with lipids.

Interestingly, we found a significant increase of the HDL levels in the WD, compared with both SD and KD mice, while increased

HDL levels are commonly associated with a reduction of the cardiovascular risks (33). The increased HDL cholesterol levels have been previously described in C57Bl/6 mice as well as human upon WD feeding (34, 35). This phenomenon may be an adaptive response to the greater need to transport the high lipids content, suggesting that the association between HDL levels and metabolic risk is more complicated than originally proposed, and as such, increased HDL-C levels should be interpreted with care (34). Other studies suggest that LDL and total cholesterol levels should be the gold standard metabolic risk markers for cardiovascular disease, instead of using plasma HDL levels as an indicator of cardiovascular health (36, 37). Therefore, the significant increase of HDL and total cholesterol levels in the WD mice indicates an increase of cardiometabolic risk while LCHFD prevented the development of hypercholesterolemia.

The deleterious effect of carbohydrates has been described in the carbohydrate-insulin model of obesity development, which postulates that the accumulation of adipose tissue is linked to the high secretion of insulin that occurs in response to the consumption of a high-carbohydrate diet (14, 38). Indeed, insulin is an anabolic hormone that promotes the storage of fatty acids in adipose tissue as well as in the liver (39). In our model, the WD mice, which had a significant increase in weight gain, showed hyperinsulinemia and glucose intolerance, while the LCHFD mice stopped the weight gain, had normal glucose regulation, and exhibited fasting insulin values similar to control mice, suggesting that the insulin rise could be the main driver of fat mass accumulation. Our observations are consistent with previous studies on diet-induced obese mice, where the authors showed that a LCHFD promoted fat mass decrease, glucose regulation improvement and insulin decrease (22–24). Moreover, a study conducted on 218 diabetic and overweight patients showed a beneficial effect of a hypercaloric LCHFD on weight loss and glucose regulation, including a significant decrease in insulin levels (18). Therefore, our results and the literature suggest that the beneficial effects of a LCHFD on weight gain are directly due to the decrease in insulin secretion, in response to carbohydrate restriction.

In the liver, the WD and LCHFD mice exhibited opposite metabolic adaptations. Indeed, the WD mice showed a massive accumulation of triglycerides while the LCHFD mice showed a healthy liver, without excessive triglyceride accumulation, steatosis, or ballooning. Interestingly, the correlation study revealed that the development of NAFLD is associated with the body weight, adiposity and liver weight. Weight gain and visceral obesity are important risk factors for the onset of NAFLD (40), so the reduction of weight gain and visceral fat accumulation in LCHFD mice is involved in the protective effect of the LCHFD on NAFLD development. At molecular levels, the two diets also exhibited opposite regulation. WD mice showed a significant increase in Fasn gene expression, which plays a major role in *de novo* lipogenesis, and consequently to NAFLD (41). Conversely, the LCHFD mice showed a hepatic Fasn gene expression similar to the SD mice, a decrease of Scd1 and a tendency to decrease Acc1 gene expression. Interestingly, the control of *de novo* lipogenesis is primarily transcriptional because insulin activates the endoplasmic reticulum membrane-bound transcription factor sterol regulatory element binding protein 1 (SREBP1), which translocates to the nucleus and upregulates the genes involved in the fatty acid biosynthetic pathway and triglycerides synthesis pathway, including Acc1, Fasn and Scd1 (42–44). Although we did not find changes in SREBP1 expression, the modification of its downstream targets suggests the involvement of its pathway in the response to the dietary changes. We hypothesized that the opposite *de novo* lipogenesis gene expression in both the WD and LCHFD mice may be a consequence of a distinct hormonal response due to the macronutrient composition of the diet. The significant increase of insulin induced by the WD could lead to an upregulation of Fasn gene expression and thus, to the increase of hepatic lipid accumulation, while maintaining normal insulin levels does not promote the activation of *de novo* lipogenesis and triglyceride synthesis, protecting the liver from NAFLD development (45, 46). We also focus on the β-oxidation, but the diets did not seem to influence the actors involved in this pathway. However, PPARα is a transcription factor normally activated by fatty acids, inducing the transcription of a certain number of target genes, including beta-oxidation enzymes (47). It would seem that this pathway is not involved in the opposite metabolic effects observed between the two diets. Therefore, the beneficial effects of LCHFD on obesity and NAFLD seem to be explained by the reduction in insulin secretion, which would limit both weight gain due to the accumulation of lipids in adipose tissue and *de novo* lipogenesis activation, but they not involve an increase of fatty oxidation pathway.

However, our study has some limitations. Firstly, our study design was exclusively intended to investigate the effects of macronutrient changes on insulin regulation and the development of NAFLD. However, the dysregulation of visceral adipose tissue is also involved in the development of obesity-related complications (48). Given the favorable impact of LCHFD on weight gain, there is a compelling interest in extending this study to characterize its effects on visceral adipose tissue, particularly at the molecular level. Furthermore, we did not explore the influence of dietary changes on total energy expenditure, despite it can affect weight gain. Future studies should focus on investigating the impact of LCHFD on metabolic adaptations, using the approach of metabolic cage, for example. Finally, like the other studies that focus on the metabolic effects of LCHFD on obesity, our research only involved male subjects. Considering the hormonal differences between males and females, as well as the sexual dimorphism in response to a WD, future studies should incorporate both sexes to comprehensively determine the effects of LCHFD, including its impact on females.

In this study, we therefore demonstrated that two diets with the same caloric amount, but a different macronutrient composition exhibited opposite metabolic responses. On the one hand, the obesogenic WD induces hyperinsulinemia, resulting in an increase in *de novo* lipogenesis activation in the liver. On the other hand, the LCHFD maintains insulin levels and *de novo* lipogenesis Fasn gene expression similar to the control animals, protecting the liver against NAFLD development (Figure 5). Regarding the clinical implication of our study, we emphasize the detrimental effects of high sugar/carbohydrate intake in high-calorie diets and propose that nutritional management be reconsidered to enhance obesity management in patients. Indeed, considering the major impact of high sugar levels in any diet rather than aiming to simply reduce

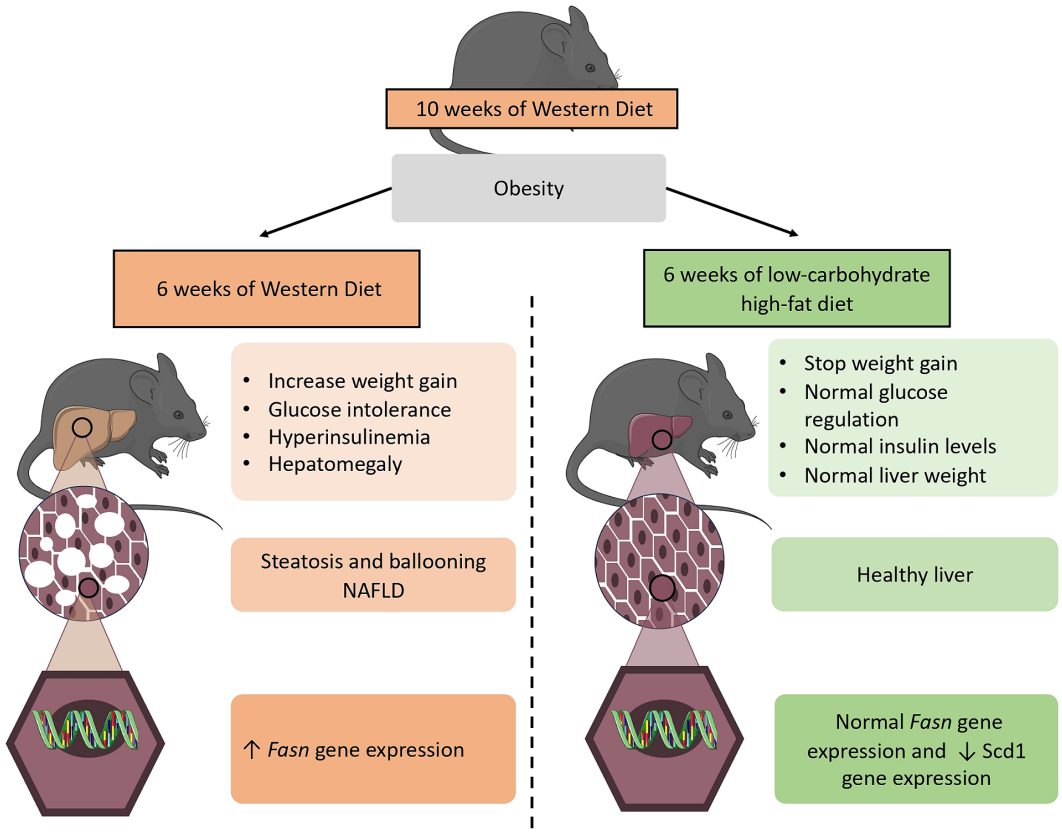

FIGURE 5
Diagram showing the 6 weeks dietary effects of both a WD and LCHFD on the diet-induced obesity mouse model. The 10 weeks consumption of a high-fat high-sugar WD is responsible for obesity development. When the WD is maintained for 6 additional weeks, weight gain continues to rise, insulin levels are elevated, and mice develop NAFLD, which is associated with an increase in *de novo* lipogenesis Fasn gene expression in the liver. In contrast, a low-carbohydrate high-fat LCHFD stop weight gain and protect from glucose intolerance and NAFLD development. The figure was partly generated using Servier Medical Art (Servier, Creative Commons Attribution 3.0 unported license).

caloric intake may help to understand the following consequences on insulin levels and, ultimately, weight changes and hepatic complications in humans. We demonstrated the beneficial effects of the low-carbohydrate high-fat diet on obesity and NAFLD development, and we believe that healthcare professionals should consider this approach to reduce weight gain and prevent the onset of obesity-related complications such as glucose intolerance and NAFLD.

Data availability statement

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

Ethics statement

The animal study was approved by Comité régional d'éthique en matière d'expérimentation animale de Strasbourg (CREMEAS), Strasbourg, France. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AC: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. AB: Investigation, Writing – review & editing. JM: Investigation, Writing – review & editing. A-LC: Investigation, Methodology, Writing – review & editing. NN: Investigation, Writing – review & editing. DD: Investigation, Methodology, Writing – review & editing. AP: Investigation, Methodology, Writing – review & editing. BG: Validation, Writing – review & editing. JZ: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1366883/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Weight of **(A)** gonadal AT, **(B)** perirenal AT, **(C)** subcutaneous AT and **(D)** liver. n=8-9. **(E)** Hematoxylin-eosin staining of liver sections. The black arrow indicates steatosis. **(F)** Oil Red O staining quantification. n=4-5. **(G)** Oil Red O staining of liver sections. Lipids are stained red. Mean ± SD *=p<0.05 and **=p<0.01. SD: standard diet group; WD 10w: animal fed a western diet for 10 weeks; AT: adipose tissue.

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Exploring the impacts of ketogenic diet on reversible hepatic steatosis: initial analysis in male mice

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Metabolic dysfunction-associated fatty liver disease (MAFLD) is the most common chronic liver disease. Ketogenic diet (KD), a diet with very low intake in carbohydrates, gained popularity as a weight-loss approach. However, in mice models, it has been reported that an excess exposition of dietary fat induces hepatic insulin resistance and steatosis. However, data published is inconsistent. Herein, we investigated in a mouse model, the metabolic effects of KD and its contribution to the pathogenesis of NALFD. Mice were exposed to KD or CHOW diet for 12 weeks while a third group was exposed to KD for also 12 weeks and then switched to CHOW diet for 4 weeks to determine if we can rescue the phenotype. We evaluated the effects of diet treatments on fat distribution, glucose, and insulin homeostasis as well as hepatic steatosis. Mice fed with KD developed glucose intolerance but not insulin resistance accompanied by an increase of inflammation. KD-fed mice showed an increase of fat accumulation in white adipose tissue and liver. This effect could be explained by an increase in fat uptake by the liver with no changes of catabolism leading to MAFLD. Interestingly, we were able to rescue the phenotype by switching KD-fed mice for 4 weeks on a CHOW diet. Our studies demonstrate that even if mice develop hepatic steatosis and glucose intolerance after 12 weeks of KD, they do not develop insulin resistance and more importantly, the phenotype can be reversed by switching the mice from a KD to a CHOW.

KEYWORDS

ketogenic diet, hepatic steatosis, inflammation, glucose intolerance, insulin resistance, rescue

1 Introduction

Metabolic disorders (MD) are a group of conditions that affect metabolism homeostasis a central process for conversion of food into energy and for maintenance of tissue integrity. This syndrome is often associated with insulin resistance in response to elevated dietary fats (1). One of the most important contributors to metabolic syndrome (MS) is obesity. One approach to treat obesity and associated metabolic disorders are dietary interventions. Such as ketogenic diet (KD) (2, 3). This diet is typically very low in carbohydrates (less than 5% of daily calories) and very high in lipids (more than 80%) (4). For decades, the KD has been also used to treat epilepsy, especially in children with seizures (5, 6). This diet creates a metabolic state called ketosis, characterized by an increased level of plasmatic ketone bodies (7).

Due to the presence of a large proportion of fat in KD, it has been reported that this diet could participate in the development of hepatic steatosis also called metabolic dysfunction-associated fatty liver disease (MAFLD). MAFLD development is indeed highly correlated with

a high dietary fat ingestion (8–10). This leads to the increase of fatty acid anabolism in liver driven by the activation of the master regulator of *de novo* lipogenesis and fatty acid uptake, sterol regulatory element-binding protein-1 (SREBP-1) (11). In humans, this ectopic accumulation of fat observed in MAFLD is also associated with hypertriglyceridemia, low levels of high-density lipoprotein (HDL) – cholesterol, glucose intolerance, insulin resistance leading to the development of type 2 diabetes (T2D) (12–14). Hyperglycemia and hyperlipidemia also disrupt the insulin signaling pathway activating stress and inflammation pathways (15). In this case, protein kinase B (Akt) and p70S6 Kinase (P70S6K) phosphorylation are decreased and circulating inflammatory cytokines increase developing a nonalcoholic steatohepatitis (NASH) (16–19). At this stage of the disease, symptoms can be reversible, but they become irreversible when cirrhosis appears (20).

Recently, Grandl et al. (21) showed that when compared to a high fat diet (HFD), mice fed with KD for 3 days, containing 90% kcal from fat and 0.3% kcal from carbohydrate, developed hepatic insulin resistance as well as induce glucose resistance (21). More recently, Long et al. (22) showed that a low carbohydrate, high fat diet (79.1% kcal form fat and 3.8% kcal from carbohydrate) induce hepatic fibrosis and NASH associated with high inflammation and severe hepatic steatosis (22). However, other studies showed opposite effects of KD on metabolic features. When comparing athletes fed a regular western diet to athletes fed with KD, the latter enhanced ketosis, demonstrating a decrease in body fat and an increase of lean mass. This is associated with a decrease in plasmatic inflammatory cytokines levels (such as IL-6, IL-1β, and TNFα) (23). Moreover, two human studies showed that obese patients, with or without T2D on KD for 12 weeks presented an amelioration of their metabolic profiles reflected by decreased plasmatic glycated-hemoglobinA1c, blood and hepatic triglycerides, hydrolysis by mitochondrion, low-density lipoprotein (LDL)-cholesterol concentration and fasting blood glucose and insulin levels while decreased (24, 25).

Ketogenic diets are used in a variety of cases, notably for their benefits in aging or epilepsy. In previous studies, several sources of fat were used, such as cocoa butter (26), vegetable oils (27) or lard (28, 29). Lard contained mainly saturated fat responsible for adverse metabolic effects (30). In mice, most of the studies have evaluated the effect of obesogenic diets by feeding the animals for 8 to 16 weeks, in order to observe significant metabolic changes (31–34). In addition, the ketogenic diets previously used contained various amount of carbohydrates (21–25). In the present study, we evaluated the effect of dietary saturated fats contained in lard and the total absence of carbohydrate in KD (84,5% fat from Lard, 0% carbohydrates) on mice's metabolic profiles in order to evaluate the impact of KD as well as the absence of sugar. Animals were fed with the KD for 12 weeks and part of the mice were then switched on standard diet (CHOW) for an additional 4 weeks, general metabolic features were analyzed.

2 Materials and methods

2.1 Animals

C57BL/6 male mice were maintained at 23°C, 12 h dark-12 h light cycle. CHOW diet (D11112201) and ketogenic diet (KD, D16062902) were obtained from OpenSource Diet and ResearchDiet Inc., respectively. As summarized in the Supplementary Table S1, KD contains 5.5% fat (kcal) from soybean oil and 84.5 from lard. Carbohydrates represent less than 0.1%. 3 weeks old mice were fed *ad libitum* for 12 weeks with either CHOW diet or KD. Food intake was measured by weighing diet each day for CHOW and KD group and converted into calories. Part of the KD-fed mice were then switched to the regular CHOW diet for 4 more weeks ($n = 3$ per group). Mice were anesthetized in 2% isoflurane chamber and then euthanized in CO2 chamber at 12 weeks for two groups and at 17 weeks for the third group after 6 h fasting. Blood samples and liver tissues were collected. Liver tissues were fixed in 4% paraformaldehyde or flash frozen in liquid nitrogen. All experiments followed adherence to the care and use of laboratory animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Quebec in Montreal.

2.2 Biochemical analysis

Plasmatic levels of insulin were determined using an Ultra-Sensitive Mouse Insulin ELISA Kit (CrystalChem) while plasmatic levels of ketone bodies were measured using the β-Hydroxybutyrate LiquiColor Assay (Stanbio). Liver triglycerides (TG) were determined using a triglyceride colorimetric assay kit (CaymanChemical). Glucose tolerance (GTT) and insulin tolerance (ITT) tests were assessed after 6 h diurnal fasting. Blood glucose concentration was measured from 2 µL of tail vein blood using an Accu-Check Aviva glucometer (Roche). For GTT, glucose level was evaluated at time: 0, 15, 30, 60, and 90 min after the intraperitoneal D-(+)-Glucose injection (1 g/kg body weight; Sigma-Aldrich, cat #G8270). For ITT, the glucose level was evaluated at time: 0, 15, 30, 45, and 60 min after the intraperitoneal injection of human recombinant insulin (0.5 U/kg body weight; Humulin R, Lilly).

2.3 Histology

Liver microtome sections (8 µm) were stained with RedOil and hematoxylin as previously described (35). Samples were visualized under white light using a Nikon Eclipse Ti microscope equipped with a Scion CFW-1612C color camera. Lipid droplets sizes and areas were quantified using the Particle Analysis function of the ImageJ software. The Particle Analysis function has been set to consider LD with a sphericity coefficient of 0.55–1.0 and a size range of 0.07–2,000 µm^2.

2.4 Western blotting

Total proteins were extracted from liver samples as previously described (36). Denatured proteins were loaded on SDS-PAGE, and immunoblot analyses were carried out using the following primary antibodies: Akt (1:1000), P70S6K (1:1000), α-tubulin (1:1000), SREBP-1 (1:1000), Acetyl-CoA Coenzyme A (ACC) (1:1000), and Carnitine PalmytoylTransferase-1 (CPT1) (1:1000). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Abcam, cat. #ab6721) or anti-mouse IgG (Cell Signaling technology, cat. #7076) were used as secondary antibodies (1:1000). Bands intensities were quantified by the Analyze Gels function of the Image J software.

2.5 Quantitative retrotranscriptase-polymerase chain reaction

Total RNA was extracted from liver and adipose tissues using TRIzol Reagent (Life Technologies, cat. #15596-018; Carlsbad, United States) following the manufacturer's instructions. One μg of RNA was reverse transcribed into cDNA using the ProtoScript II Reverse Transcriptase kit (New England Biolab). Quantitative PCR was performed on 50 ng cDNA using the following specific primers: Stearoyl-CoA desaturase 1 (SCD1) Fwd-5'-TCATACTGGTTCCCTC CTGC-3'/Rev-5'-TGCCTTGTAAGTTCTGTGGC-3'; Cluster of Differentiation 36 (CD36/FAT) Fwd-5'-GAATTAGAACCGGGC CACGTAGAAA-3'/Rev-5'-ACAGCTCCAGCAATGAGCCCAC-3'; Peroxisome Proliferator Activated Receptor alpha (PPARα) Fwd-5'-GGAAGACCACTCGCATTC-3'/Rev-5'-GTAATCAGCAACCATT GGGTCA-3′, Interferon beta (IFN-β) Fwd-5'-ATAGTCTCA TTCCACCCAGTGC-3′/Rev-5'-AGAGTTACACTGCCTTTGCC-3′; Interleukin-4 (IL-4) Fwd-5'-GGAGATGGATGTGCCAAACG-3'/ Rev-5'-CTTGGAAGCCCTACAGACGAG-3′ and Hypoxanthine Phosphoribosyl-Transferase (HPRT) Fwd-5'-TCAGTCAAC GGGGGACATAAA-3/Rev-5'-GGGGCTGTACTGCTTAACCAG-3′. Reactions were performed using a Light Cycler 480 thermocycler (Roche, cat. #050152278001). Gene expression was normalized to Hprt1 expression and calculated using the comparative dCt method.

2.6 Statistical analysis

All data are presented as the mean +/− standard error of the mean. Independent replicas of each data point (*n*) are identified in figure legends. The Prism 9 software was used to performed Student's *t*-tests or one way ANOVA when the test was appropriate. A one-tailed unpaired test was applied. A p-value <0.05 was regarded as statistically significant.

3 Results

3.1 Effect of KD on metabolic profile

To evaluate the effect of KD on metabolic profile, we fed 3 weeks old C57BL/6 mice (just after weaning) with either standard diet (CHOW) or KD for 12 weeks. Mice consumed the same amount of calories and no change in body weight was observed between the two groups (Figure 1A). As expected, compared to chow diet, KD increased the plasmatic level of β-hydroxybutyrate (Figure 1A). KD-fed mice showed a higher fasting plasma glucose and insulin levels (Figure 1A), suggesting impairment in glucose homeostasis. However, KD-fed mice were still responsive to insulin as shown by ITT (Figure 1B), suggesting the absence of insulin resistance. To evaluate hepatic insulin resistance, we measured the phosphorylation state of AKT and P70S6K. As shown, in Figure 1C, the levels of phosphorylation of the two proteins, known to be impaired by insulin resistance, are similar between the two group of mice. Our data suggests that mice fed during 12 weeks with our KD, develop glucose intolerance but not insulin resistance.

3.2 Effect of KD on fat storage

To evaluate the effect of KD on fat storage, we analyzed white adipose tissues and livers, in mice fed with either CHOW diet or KD. Compared to the CHOW-fed mice, KD-fed mice showed an increased mass of fat in both subcutaneous and visceral white adipose tissues (Figure 2A). In KD-fed mice compared to the control group, we observed a 3 folds increase in hepatic lipid droplets sizes. The LD area represented a higher percentage of total liver tissue and consequently, a larger affected total hepatic area (Figure 2B) demonstrating ectopic TG accumulation, the first step of hepatic steatosis development (Figure 2A). High consumption for 12 weeks of KD, mainly composed of saturated fat, leads to increase adiposity and hepatic steatosis.

3.3 Effect of KD on hepatic lipid metabolism

The development of hepatic steatosis often arises from an imbalance between lipid catabolism and lipid anabolism (37). Therefore, we analyzed the effects of KD treatment on the key players of theses pathways. Surprisingly, lipogenesis is decreased in the liver of mice fed with KD compared to the chow group, as seen by a lower phosphorylation state of ACC and an important down regulation of SCD1 mRNA expression (Figure 3A). However, no differences in the expression of the mature form of the SREBP-1 was observed. The level of expression of CPT1 was also not affected by KD suggesting that in our experimental conditions, the β-oxidation is not modulated. On the other hand, the mRNA expression of CD36, the main receptor implicated in hepatic lipid uptake was strongly increased in the KD group versus the CHOW group. An increased in PPARα mRNA expression was also observed. Compellingly, this transcription factor is known to be essential in the ketogenesis process (38) (Figure 3A). Our data suggest that the development of hepatic steatosis in mice fed with KD is mainly due to an increase in exogenous lipids uptake and not to *de novo* lipogenesis. Depending on the nature of the dietary fatty acids, an increase in hepatic inflammation can be observed (30). We have shown that KD-fed mice exhibit a slight increase in concentration of hepatic pro-inflammatory cytokine TNF-α compared to the CHOW group, associated with a decrease in the expression of the anti-inflammatory cytokines IFN-β and IL-4 (Figure 3B). Our data suggest that the liver of mice fed for 12 weeks with KD present a pro-inflammatory profile seen in MAFLD (39).

3.4 Restauration of metabolic profile after 4 weeks on CHOW diet

Despite presenting some deleterious metabolic features, mice fed with KD for 12 weeks do not appear to develop insulin resistance. Therefore, we evaluated the effect of switching the mice for 4 weeks on a regular CHOW diet after 12 weeks of KD, and if this change could restore a healthier metabolic phenotype. As expected, after 4 weeks on chow diet, the circulating level of ketone bodies was substantially decreased, restoring the levels observed in mice not fed with KD. As seen by the GTT, mice were no longer glucose intolerant. Similar

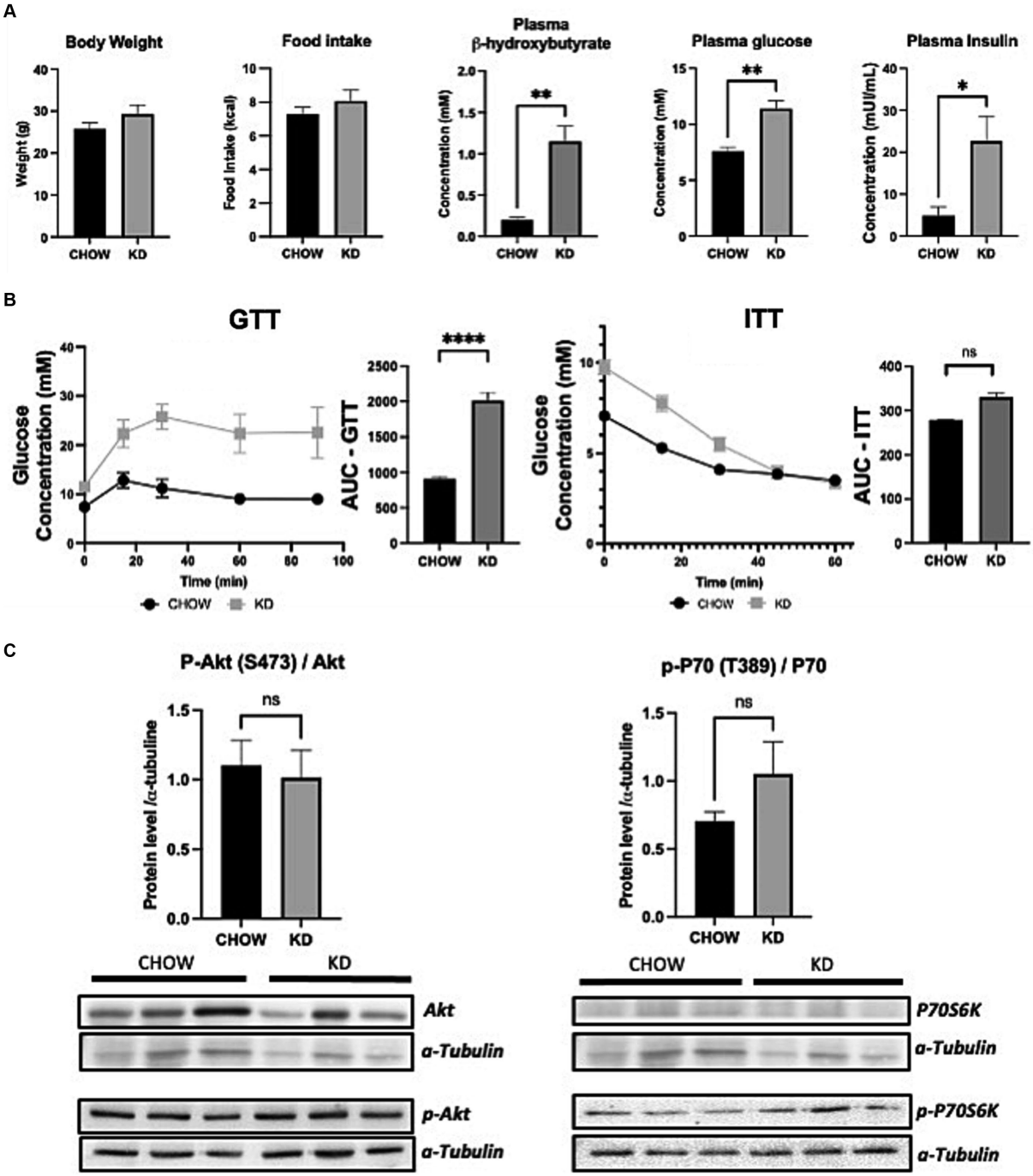

FIGURE 1
Metabolic profile and insulin sensitivity of mice fed *ad libitum* for 12 weeks with either CHOW or ketogenic diets (KD). **(A)** Evaluation of body weight, daily food intake plasma β-hydroxybutyrate and fasting glucose and insulin. **(B)** GTT (glucose tolerance test) was measured at week 9 and ITT (insulin tolerance test) was measured at week 10 after the beginning of the diet. AUC represents the calculated area under the curve. **(C)** Total and phosphorylated Akt and P70S6K levels measured in mice livers. Representative Western blots are depicted. α-tubulin was used as control ($n = 3$). Results are presented as mean +/− SEM ($n = 3$). ns, non-significant, *p-value <0.05, **p-value <0.01, and ****p-value <0.0001.

observation was made when we performed ITT (Figure 4A). Moreover, mice have shown a better response of insulin tolerance test compared to the control group (Figure 4A). Switching mice from the KD to CHOW diet for 4 weeks showed that the visceral adipose tissue mass is strongly decreased reaching a similar level to the one observed in CHOW-fed mice. Despite being decreased, the subcutaneous fat mass is not statistically different than the one measured in mice fed only with KD (Figure 4B). The change in diet also totally resolved the hepatic steatosis, TG accumulation and restored a normal inflammatory profile (Figures 4B,C). These results suggest that the deleterious effect of KD on metabolic profile can be rapidly restored by switching the animals to a regular diet for only 4 weeks.

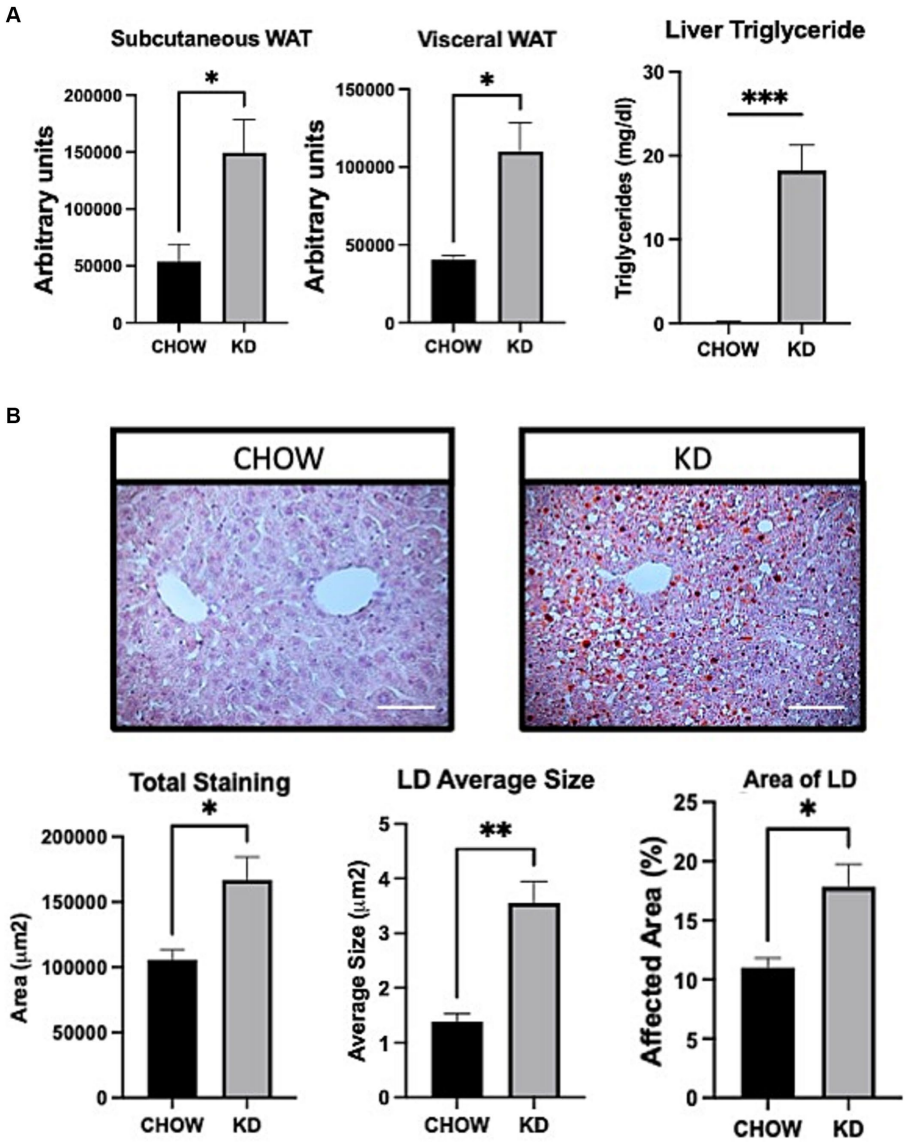

FIGURE 2
Adipose tissue and liver sections analysis in mice fed *ad libitum* for 12 weeks with either CHOW diet or Ketogenic Diet. **(A)** Representative images of white adipose tissue (WAT) distribution. Red arrows indicate subcutaneous WAT while blue arrows show visceral WAT. Scale Bar = 20 mm (Pictures in Supplementary Data). Quantifications were performed using the Particle Analysis function of ImageJ. Liver triglyceride levels. **(B)** Representative images of RedOil and eosin/hematoxyl staining of hepatic tissues sections. Scale bar = 70 µm. Quantification of total, average size area and percentage area of lipid droplets in liver sections were performed using ImageJ. Data are the mean +/− SEM. ($n = 3$). ns, non-significant, *p-value <0.05 and **p-value <0.01.

4 Discussion

As of today, there is still controversy surrounding the KD diet, particularly regarding its effects on glucose and insulin sensitivities, on weight loss and on MS development. In the present study, we evaluated the effect of 12 weeks of KD on fat mass distribution, glucose and insulin tolerance, hepatic steatosis, lipid metabolism and inflammatory profile in mice. Furthermore, we analyzed the same metabolic features after switching mice from a 12 weeks KD to chow diet for an additional 4 weeks.

As expected, mice fed with KD for 12 weeks in total absence of sugars showed an increase in the plasma concentration of ketone bodies, notably β-hydroxybutyrate (βOHB), a marker of ketosis (7). In response to exogenous fatty acid overload, PPARα is activated inducing the expression of several of its targeted genes involved in ketogenesis, such as CPT1, hydroxymethylglutaryl-CoA synthase 2 (HMGCS 2) or uncoupled protein 2 (UCP2) (40). In our study, exogenous fatty acid overload is also induced by our KD. Additionally, we observed an increase in the expression of PPARα but also of one of its target gene CPT1 enabling the mice to create ketone bodies (40).

As showed in other studies using our KD in absence of any sugar (21, 22), an elevation of fasting plasma glucose and insulin levels was observed. This suggests a development of insulin resistance (41). However, performing GTT and ITT, we showed that our KD-mice are glucose intolerant but remained insulin sensitive. Glucose intolerance is characterized by elevated fasting glucose (IFG) and impaired

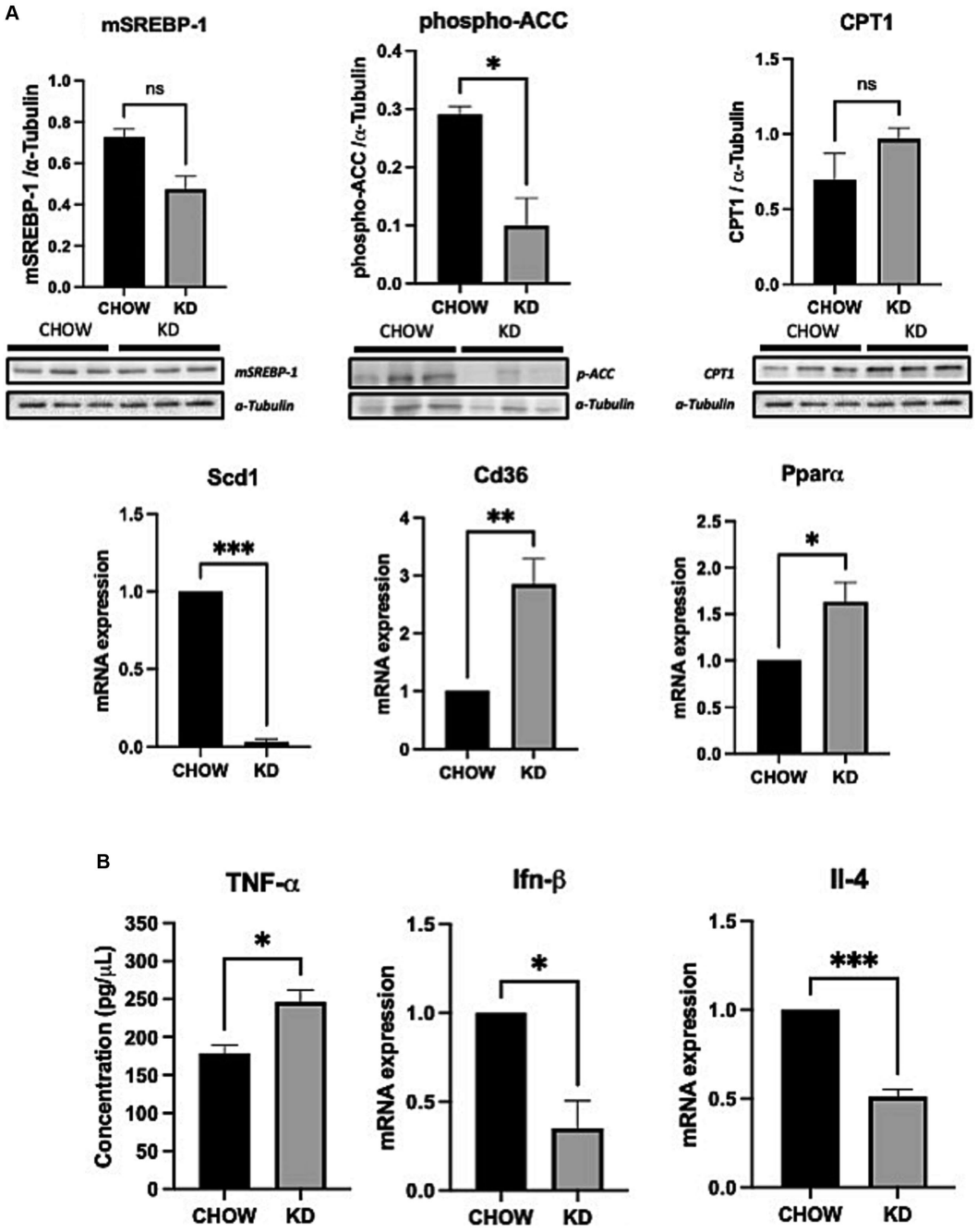

FIGURE 3
Hepatic expression of lipid metabolic genes and inflammatory cytokines in liver of mice fed *ad libitum* for 12 weeks with either CHOW diet or Ketogenic Diet. **(A)** Protein expressions of SREBP-1, phospho-ACC, CPT1 and mRNA expression of Scd1, Pparα and Cd36 are presented. α-TUBULINE and Hprt1 were used, respectively, as control. **(B)** Protein and mRNA expression of TNF-α (evaluated by ELISA) and Ifn-β and Il-4 are presented. Hprt1 was used as control for qPCR. Data are shown as mean +/− SEM. $n = 3$. Student's t-test: *p-value <0.05, **p-value <0.01, ***p-value <0.001, and ****p-value <0.0001.

glucose management upon glucose challenge, also name impaired glucose tolerance (IGT) (42). Patients with IFG and/or IGT are often defined as "Prediabetic" (43). Glucose intolerance represents the first step in the development of insulin resistance as observed in mice fed, for 12 weeks, with an obesogenic diet (60% lard, 25% carbohydrates) (44). Surprisingly, in another study, severe insulin resistance has been observed following exposure to a low-sugar diet (90% fat and 0.3% carbohydrate) within only 3 days (21). In another study, authors showed that 12 weeks of KD (79.1% fat and 3.8% carbohydrate) induce insulin resistance associated with impaired hepatic insulin signaling (22). In contrast, humans' studies showed that KD (with less than 5% carbohydrates) improve glucose and insulin sensitivities suggesting differences in response to dietary sugars between mice and human (23–25). Moreover, ketone bodies are also known to reduce circulating glucose and insulin levels, thus improving insulin sensitivity (45). Our results showed that 12 weeks of KD also have deleterious effects,

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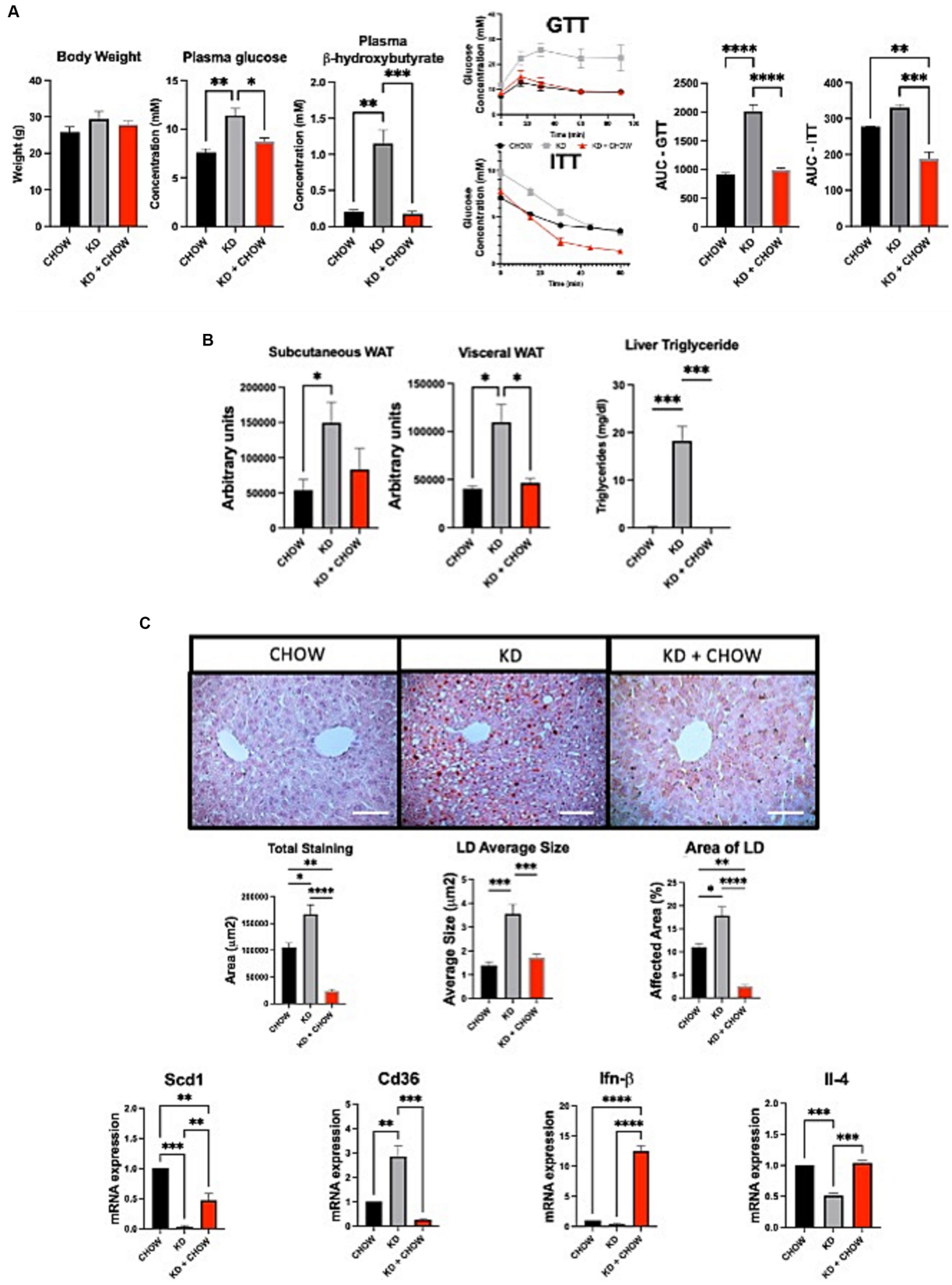

FIGURE 4
Effect of 4 weeks of CHOW diet after 12 weeks of Ketogenic Diet. **(A)** Evaluation of body weight, plasma β-hydroxybutyrate and fasting glucose, GTT was measured at week 3 and ITT was measured at week 4 after the switch of the diet. AUC represents the calculated area under the curve. **(B)** Representative images of WAT distribution. Red arrows indicate subcutaneous WAT while blue arrows show visceral WAT. Scale Bar = 20 mm (Pictures in Supplementary Data). Quantification was performed using the Particle Analysis function of ImageJ. Liver triglyceride levels.

(Continued)

FIGURE 4 (Continued)

(C) Representative images of RedOil and eosin/hematoxyl staining of hepatic tissues sections. Scale bar = 70 μm. Quantification of total, average size area and percentage area of lipid droplets in liver sections were performed using ImageJ. mRNA expression of Scd1, Cd36, Ifn-β, and Il-4 are presented. Hprt1 were used as control. Data are shown as mean +/− SEM. $n = 3$. Student's t-test: *p-value <0.05, **p-value <0.01, ***p-value <0.001, and ****p-value <0.0001.

although with less severity than the effects observed in other mice studies (21, 22). This can be explained by the total absence of carbohydrate in our experimental conditions suggesting that even a low level of sugar in the diet can induce insulin resistance at least in mice.

In an *ad libitum* context, our KD-fed mice showed an accumulation of both subcutaneous and visceral white adipose tissues, as well as hepatic TG accumulation. Our diet is composed by 40% saturated fatty acids (SFA), known to increase LDL-cholesterol levels (46). SFA is also known to play a crucial role in the development of low-grade inflammation and metabolic diseases such as NASH (30, 46). Dietary palmitate, the most common SFA (around 30% of fat-part of our KD), is known to increase the level of the plasmatic pro-inflammatory cytokine TNF-α and to decrease the levels of hepatic anti-inflammatory cytokines IFN-β and IL-4, as seen in our study (46, 47). IFNβ may play multiple roles as well pro-and anti-inflammatory (48). However, a study performed mice fed with a high fat diet, showed that IFNβ overexpression has been observed to attenuate adipose tissue inflammation, adipose tissue expansion and body weight gain. The authors argue that IFNβ expression helps the high fat diet-fed mice to attenuate production of pro-inflammatory cytokines (such as Tnf-α, Il-1β, and Il-6). In addition, in this study, the authors also showed that IFNβ improves insulin sensitivity and glucose homeostasis (49). As expected, the presence of high concentration of palmitate in our KD, considerably activated the hepatic expression of CD36. High expression of this fatty acid translocase in liver is strongly associated with MAFLD development and elevated hepatic intracellular oxidative stress (46, 50). CD36 also participates in the inflammatory response via the activation of the c-Jun N-terminal kinase pathway (51). *De novo* lipogenesis is also increased during the development of MAFLD, notably through the activation of the transcription factor SREPB-1 (52). In our study, we did not observe induction in the expression of the mature form of SREBP1 (mSREBP1c). On the contrary, an abolition of SCD1 expression and a strong inhibition of ACC phosphorylation was observed. This suggests that 12 weeks of treatment with KD does not induce hepatic lipogenesis and may inhibit it. This could be partially explained by the total absence of carbohydrates in our KD, since hepatocytes derive their acetyl-CoA, the main substrate for hepatic lipogenesis, essentially from carbohydrate catabolism (53). Moreover, a correlation between low SCD1 expression and high ketone body level has been observed in Nuclear Factor erythroid 2-Related Factor 2-KnockOut (Nrf2-KO) mice. The transcription factor Nrf2 activate SCD1 and SREBP1 in response to a diet-induced obesity (54). Interestingly, Nrf2-KO mice develop ketosis. Nrf2 is a transcription factor targeting genes involved in lipid uptake (such as CD36) and lipogenesis (such as SCD1 and SREBP) and its deletion increase the expression of genes involved in lipid oxidation (such as PPARα and CPT1) (54). Ketone bodies production and VLDL secretion is observed in Nrf2-KO mice (55). However, the authors have not identified the molecular mechanisms underlying the link between SCD1 and ketone bodies production (55). In our study, we observed a strong decrease in SCD1 expression without any effect on mSREBP1c expression suggesting a mechanism independent of SREBP-1c that need to be identified. Medium-chain triglycerides (MCT) are also known for their ability to be rapidly converted into ketone bodies. In mice fed an MCT-enriched diet for a year, glucose homeostasis is altered an associated with decreased hepatic SCD1 expression by an unidentified mechanism (56, 57). Taken together, our data suggests that the development of hepatic steatosis observed in our KD-fed mice for 12 weeks is probably due to an increase in SFA uptake and not to an activation of *de novo* lipogenesis.

It is well known that inflammation is involved in the progression from MAFLD to NASH (58). As predicted, our KD-fed mice showed an increase in the expression of the pro-inflammatory marker TNF-α associated with a decrease in the expressions of the anti-inflammatory markers IL-4 and IFN-β. Several studies performed on mice, both wild-type and mice with T2D, demonstrate the harmful impact of KDs in promoting inflammation and participating in the development of hepatic steatosis, notably due to the large quantity of SFA present in the diets from lard (59). On the other hand, several studies in humans show opposite effects. In obese subjects with hepatic steatosis, 14 days of KD [76% fat (mix of lard, butter, and corn oil), 4% carbohydrate] improved the inflammatory profile by reducing the cytokines TNF-α and IL-6 (59). Moreover, induction of ketogenesis or treatment with βOHB attenuates TNF-α-induced apoptosis in human intestinal cell lines, demonstrating a protective effect of ketone body production (60). However, it is well known that high dietary SFA, as in our experimental KD, play an important role in the development of chronic inflammation and is correlated with increased secretion of TNF-α (30) and a decrease in anti-inflammatory cytokines (61–64).

Apart from these negative effects, KDs also offer interesting possibilities. In the second phase of our research, our mice fed KD for 12 weeks were switched to a chow diet. Within only 4 weeks, the mice showed a decrease in visceral adipose tissue mass and an improvement in the hepatic lipid storage compared to the KD group. As expected, the ketosis disappears but, interestingly, most of the metabolic deleterious effects are reversed. The mice showed an improvement in insulin sensitivity, exceeding the results obtained in the control group, fed with chow diet only. Our results showed that even if the zero-sugar KD is used in these experiments, some deleterious effects (mild compared to those observed in other previous studies), this can be reversed by a return to a more normal diet.

Several recent studies performed in both humans and rodents fed intermittent-fasting and/or ketogenic diet also demonstrated an improvement in weight loss, glucose tolerance, insulin sensitivity, chronic inflammation as well as a better MS score (65–67). In another study, mice were fed with an obesogenic (60% lard, 20% carbohydrate) diet for 8 weeks, followed by 4 weeks on either a KD diet (90% cocoa butter fat containing 60% of SFA) or a chow diet (68). Interestingly

and in agreement with our observations, the KD diet reversed the development of hepatic steatosis induced by the obesogenic diet, though, to a lower degree than with what is observed with a standard diet (68). The KD diet may present reversible mild deleterious effects in the development of MAFLD for a healthy individual exclusively fed on KD. KD, however, has therapeutical beneficial effects to alleviate the deleterious effects of an obesogenic diet (60% fat, 20% carbohydrates) (69). In fact, switching mice from the obesogenic diet to KD for 8 weeks normalized the glycemic index and significantly reduced the body fat (69).

In conclusion, a KD, in absence of any sugar, can be beneficial for the treatment of diseases such as MAFLD and NASH but also can be used for periodic treatments of seizures, as conveyed in our study. The absence of sugar distinctly decreases the impact on insulin resistance allowing a restauration of healthier metabolic profiles after only 4 weeks on a regular diet. Despite the benefit effects of KD, precautions need to be taken concerning the nature of fats present in the diet. Alternative strategies must be evaluated such as the replacement of SFA by either unsaturated fatty acids or MCT to avoid development of MAFLD and systemic inflammation.

Data availability statement

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

Ethics statement

The animal study was approved by Comité institutionnel de protection des animaux (CIPA) de l'UQAM. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GR: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. AC: Methodology. CM: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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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

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1290540/full#supplementary-material

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Association between blood chromium and hepatic steatosis assessed by liver ultrasound transient elastography: National Health and Nutrition Examination Survey 2017–2020

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Background: Hepatic steatosis is a significant pathological feature of fatty liver disease (FLD) which is widely spread with no effective treatment available. Previous studies suggest that chromium (Cr) intake reduces lipid deposition in the liver in animals. However, the connection between blood Cr and hepatic steatosis among humans remains inconclusive.

Methods: Using the data from the National Health and Nutrition Examination Survey (NHANES) 2017–2020, we performed a cross-sectional analysis, including 4,926 participants. The controlled attenuation parameter (CAP) measured by the vibration controlled transient elastography (VCTE) was used to evaluate the degree of liver steatosis. Weighted univariate regression, multivariate linear regression, smooth fitting curves and subgroup analysis were used. In addition, we carried out trend tests, multiple interpolations, and interaction analyses to conduct sensitivity analyses.

Results: After adjusting with various covariables, multivariate linear regression analysis demonstrated a significant negative correlation between blood Cr and CAP [β (95% CI) = −5.62 (−11.02, −0.21)]. The negative correlation between blood Cr and CAP was more significant in the males, 50–59 years, overweight, hypercholesterolemia, HDL-C ≥ 65 mg/dL, HbA1c (5.70–6.10 %), HOMA-IR (0.12–2.76), total bilirubin (0.30–0.40 mg/dL), ever alcohol consumption subjects. Of note, the relationships between blood Cr and CAP followed a U-shaped curve in the smokers and non-smokers, with blood Cr thresholds of 0.48, 0.69 μg/L, respectively.

Conclusions: There is an independently negative correlation between blood Cr and hepatic steatosis in American. Our study provides clinical researchers with a new insight into the prospective prevention of hepatic steatosis.

KEYWORDS

blood chromium, hepatic steatosis, fatty liver disease, controlled attenuated parameter, liver ultrasound transient elastography, NHANES

1 Introduction

Fatty liver disease (FLD) is a prevalent and serious chronic liver disease worldwide, which potentially leads to steatohepatitis, fibrosis, cirrhosis and even death (1). Hepatic steatosis, defined as excessive storage of intrahepatic lipids, is the basis of FLD (2). The severity of hepatic steatosis can be evaluated by the controlled attenuation parameters (CAP) value using the vibration controlled transient elastography (VCTE) which has been proven to be a cost-effective, safe, and rapid non-invasive liver disease assessment tool (3). Given the lack of effective treatments for FLD, it is crucial to prevent the development of hepatic steatosis. Hepatic steatosis is closely related to disorders of glucolipid metabolism (4). Hence, improving the disorder of glucose and lipid metabolism is an important strategy for alleviating the severity of hepatic steatosis.

Organic chromium (Cr) is a trace dietary mineral, which participates in carbohydrate and lipid metabolism (5). Drugs reducing the concentration of blood Cr, such as doxepin and clozapine, were proven to worsen hepatic lipid deposition in obse rats, which indicate the potential role of blood Cr and hepatic steatosis (6, 7). A recent study observed an elevated blood and hepatic Cr as well as an ameliorated hepatic steatosis in BALB/c mice after Cr trichloride gavage (8). However, the relationship between blood Cr and hepatic steatosis in humans is still unknown.

With the worsen environmental pollution, inorganic Cr has increased in soil, which is widely recognized as a human carcinogen and an environmental pollutant (9). Seeing the contradictory effects of organic and inorganic Cr on human health, the relationship between blood Cr and various disease are controversial. The 2017–2020 US National Health and Nutrition Examination Survey (NHANES) assessed hepatic steatosis by VCTE and the level of blood Cr by inductively coupled plasma mass spectrometry (ICP-MS) in the U.S. population, which provided an opportunity to examine the importance of Cr and the relationship between blood Cr and hepatic steatosis.

2 Materials and methods

2.1 Study population and design

Of 15,560 NHANES 2017–2020 participants who were included in the survey, 4,926 were eventually included in this study. This study established strict exclusion criteria, and 10,634 subjects were excluded based on the following criteria: (a) participants missing blood Cr data ($n = 9{,}913$); (b) participants missing and ineligible transient elastography data ($n = 359$); (c) participants partial exam transient elastography ($n = 362$; Figure 1).

2.2 Study variables

2.2.1 Dependent variables: the controlled attenuation parameters

NHANES 2017–2020 used VCTE to estimate the degree of hepatic steatosis by measuring CAP. VCTE was performed by FibroScan model 502 V2 Touch (Echosens, Paris, France) equipped with a medium (M) or large (XL) probe. Only patients who satisfied the following conditions were considered to complete the assessment of hepatic steatosis: (a) fasting time $\geq$3 h; (b) a liver stiffness interquartile (IQR)/median <30%; (c) complete stiffness measures $\geq$10 times. CAP $\geq$285 dB/m was defined as hepatic steatosis (3).

2.2.2 Independent variable: blood chromium levels

During the 2017–2020 US NHANES survey, blood samples were collected from participants aged 40+ years old at Mobile Examination Centers (MEC). These blood samples were then analyzed for blood Cr concentrations using ICP-MS. According to the *Centers for Disease Control and Prevention (CDC) Laboratory Procedure Manual*, the measurable range concentration of Cr was from 0.41 to 5,000 μg/L. An imputed fill value, which was calculated as the lower limit of detection (LLOD) divided by the square root of 2 [LLOD/sqrt [2]], was applied to analyze with results below LLOD. In addition, we identified individuals with blood Cr levels below 0.7 μg/L as Cr deficiency (10).

2.2.3 Covariates

The socio-demographic (age, gender, race, education level, and family income-to-poverty ratio), anthropometry [liver stiffness measurement (LSM), body mass index (BMI), weight], biochemical indicators [alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, urine albumin, total calcium, triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), blood cobalt, hs-CRP, HbA1c, insulin, HOMA-IR, HOMA-IS, and eGFR], health-related behaviors (smoking status, alcohol consumption) and comorbidities (diabetes, hypertension, hepatitis B, hepatitis C, and autoimmune hepatitis) data were used.

2.3 Statistical analysis

All continuous variables were statistically presented as mean ± SD (normal distribution) or median (quartile; skew distribution), and were compared by two sample *t*-test (normal distribution) or chi-square test (skew distribution) among groups, including age, BMI, weight, TG, TC, LDL-C, HDL-C, insulin, HbA1c, HOMA-IR, HOMA-IS, hs-CRP, ALT, AST, GGT, ALP, total bilirubin, blood Cr, blood cobalt, total calcium, urine albumin, eGFR, LSM, and CAP. Similarly, categorical variables were described using percentage or frequency, and compared by chi-square test among groups, including gender, race/ethnicity, education, family income-to-poverty threshold ratio (FITR), alcohol consumption, smoking status, hypertension, diabetes, hepatitis B, hepatitis C, and autoimmune hepatitis. Univariate and multivariate linear regression were used to explore the association between blood Cr and CAP. Three models were built in the multivariate test. Model I had no variables adjusted; Model II included age, gender, and race; Model III adjusted with all covariates mentioned above. Trend tests and subgroup analyses were also performed. The

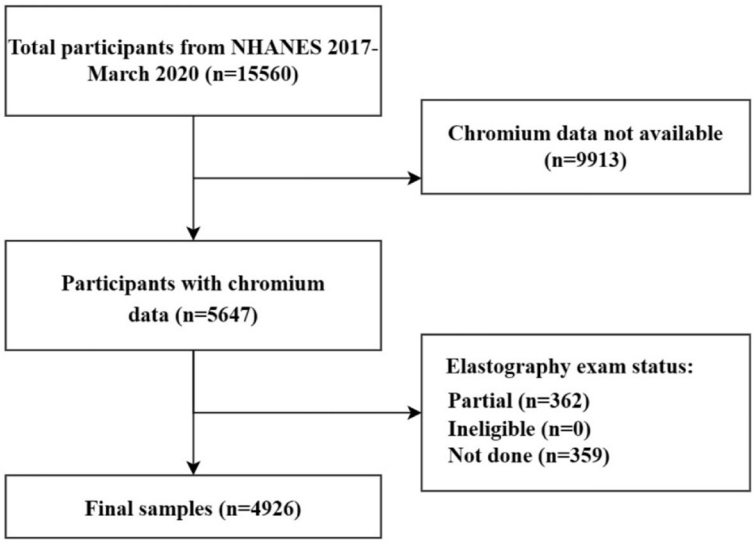

FIGURE 1
Flowchart of participant selection. NHANES, National Health and Nutrition Examination Survey.

smooth curves through the analysis of generalized additive models (GAMs) were done to explore whether the non-linear relationship existed or not. Based on the piecewise regression model, we carried out a log-likelihood ratio test to examine the relationship and inflection point. Simultaneously, we used multiple imputations to fill miss continuous covariates, and dummy variables to present the categorical variables. Statistical significance was defined as having $p < 0.05$ (bilateral). We used the NHANES sample weights and accounted for unequal selection probabilities and non-response by applying standard methods. The statistical analyses were conducted by R version 4.2.0 (http://www.R-project.org) and EmpowerStats version 4.1 (http://www.empowerstat.com).

3 Results

3.1 Study population characteristics

A total of 4,926 participants were included in this study [Male: 2,448 (49.70%), Female: 2,478 (50.30%)]. The mean age of participants was 59.79 ± 11.63 years. The mean of CAP and the median (quartile) of blood Cr were 272.66 ± 60.21 dB/m, and 0.29 (0.29–0.29) μg/L, respectively. Significant statistical differences existed in CAP and blood Cr when participants were categorized by gender ($p < 0.05$; Supplementary Table 1). According to the cut-off point of CAP at 285 dB/m, the clinical characteristics of participants were demonstrated in Table 1. There were 2,064 in the hepatic steatosis group [Male: 1,124 (54.46%); Female: 940 (45.54%)], and 2,862 in the non-hepatic steatosis group [Male: 1,324 (46.26%); Female: 1,538 (53.74%)]. There was a significant statistical difference in blood Cr levels between the two groups ($p < 0.05$). There were also significance difference in gender, ethnicity, LSM, weight, BMI, ALT, AST, GGT, ALP, urine albumin, TG, HDL-C, blood cobalt, hs-CRP, HbA1c, HOMA-IS, eGFR, smoking status, diabetes, hypertension, hepatitis B, and hepatitis C between the groups ($p < 0.05$), and we take these as covariates in the following study.

As shown in Figures 2A, B and Supplementary Table 2, Cr deficiency was widespread in Americans aged 40+ [Cr deficiency vs. normal: 4,615 (93.69%) vs. 311(6.31%)]. Besides, males with blood Cr deficiency had higher CAP compared to those with normal blood Cr levels ($p < 0.05$; Figure 2C). As shown in Figure 2D, Hispanic population and non-Hispanic White population with blood Cr deficiency had the higher level of CAP ($p < 0.05$).

3.2 Associations between other covariates and hepatic steatosis

To find the covariables which affected the degree of hepatic steatosis, we used the Spearman's correlation to analyze the between-group variation and other hepatic steatosis related covariables. As represented in Figure 3, CAP levels were not only correlated with blood Cr, but also with gender, ethnicity, LSM, weight, BMI, TG, HDL-C, HbA1c, HOMA-IS, ALT, AST, GGT, ALP, blood cobalt, urine albumin, eGFR, hs-CRP, diabetes, hypertension, hepatitis C, and hepatitis B ($p < 0.05$).

Next, univariate regression analysis was used to observe the degree of association between CAP and the above related covariates in the US population. As shown in Table 2, blood Cr had a significant negative correlation with the CAP levels [β (95% CI) = −5.19 (−8.93, −1.46)] ($p < 0.01$). Compared with males, females were less likely to develop hepatic steatosis [β (95% CI) = −12.20 (−15.54, −8.85)] ($p < 0.01$). Among different ethnicity, with Hispanic as the reference, non-Hispanic black had a lower degree of hepatic steatosis [β (95% CI) = −21.88 (−26.75,

TABLE 1 Baseline characteristics of the study population based on the controlled attenuated parameter (CAP).

Characteristic	Total ($n = 4,926$)	Non-hepatic steatosis (CAP < 285 dB/m, $n = 2,862$)	Hepatic steatosis (CAP ≥ 285 dB/m, $n = 2,064$)	*p*-value
Socio-demographic characteristics				
Gender				<0.01
Man	2,448 (49.70%)	1,324 (46.26%)	1,124 (54.46%)	
Woman	2,478 (50.30%)	1,538 (53.74%)	940 (45.54%)	
Age (years)	59.79 ± 11.63	60.05 ± 11.97	59.43 ± 11.14	0.116
Ethnicity				<0.01
Hispanic	1,047 (21.25%)	523 (18.27%)	524 (25.39%)	
Non-Hispanic White	1,787 (36.28%)	997 (34.84%)	790 (38.28%)	
Non-Hispanic Black	1,278 (25.94%)	838 (29.28%)	440 (21.32%)	
Non-Hispanic Asian	597 (12.12%)	373 (13.03%)	224 (10.85%)	
Other race	217 (4.41%)	131 (4.58%)	86 (4.17%)	
Education				0.73
Less than high school	983 (19.96%)	568 (19.85%)	415 (20.11%)	
High school	1,174 (23.83%)	673 (23.52%)	501 (24.27%)	
More than high school	2,760 (56.03%)	1,617 (56.50%)	1,143 (55.38%)	
Not recorded	9 (0.18%)	4 (0.14%)	5 (0.24%)	
FITR (%)				0.73
<1.0	712 (14.45%)	414 (14.47%)	298 (14.44%)	
1.0 to <2.0	1,103 (22.39%)	635 (22.19%)	468 (22.67%)	
2.0 to <3.0	677 (13.74%)	401 (14.01%)	276 (13.37%)	
3.0 to <5.0	877 (17.80%)	491 (17.16%)	386 (18.70%)	
≥5	901 (18.29%)	534 (18.66%)	367 (17.78%)	
Not recorded	656 (13.32%)	387 (13.52%)	269 (13.03%)	
Physical examinations				
LSM (kPa)	5.10 (4.20–6.40)	4.80 (4.00–5.80)	5.80 (4.60–7.30)	<0.01
CAP (dB/m)	272.66 ± 60.21	231.24 ± 37.04	330.09 ± 32.74	<0.01
Weight (kg)	82.88 ± 21.01	75.75 ± 17.59	92.72 ± 21.38	<0.01
BMI (kg/m^2)	29.93 ± 6.78	27.57 ± 5.72	33.19 ± 6.77	<0.01
Biochemical indicators				
ALT (U/L)	18.00 (13.00–26.00)	16.00 (12.00–23.00)	21.00 (15.00–30.00)	<0.01
AST (U/L)	19.00 (16.00–24.00)	19.00 (16.00–23.00)	20.00 (16.00–25.00)	<0.01
GGT (IU/L)	22.00 (15.25–34.00)	20.00 (14.00–30.00)	26.00 (19.00–41.00)	<0.01
ALP (IU/L)	80.13 ± 25.72	78.32 ± 25.42	82.61 ± 25.93	<0.01
Total bilirubin (mg/dL)	0.40 (0.30–0.60)	0.40 (0.30–0.60)	0.40 (0.30–0.60)	0.21
Urine albumin (ug/mL)	9.40 (4.70–20.55)	8.60 (4.30–19.00)	10.50 (5.38–23.83)	<0.01
Total calcium (mg/dL)	9.28 ± 0.39	9.27 ± 0.39	9.28 ± 0.39	0.13
TG (mg/dL)	97.00 (67.00–139.00)	83.00 (60.00–118.00)	117.00 (85.00–164.00)	<0.01
TC (mg/dL)	190.07 ± 42.21	190.87 ± 42.02	188.97 ± 42.47	0.12
LDL-C (mg/dL)	111.42 ± 36.97	112.39 ± 36.56	110.05 ± 37.51	0.13
HDL-C (mg/dL)	54.09 ± 16.41	57.78 ± 16.86	49.01 ± 14.30	<0.01

(Continued)

TABLE 1 (Continued)

Characteristic	Total ($n = 4,926$)	Non-hepatic steatosis (CAP < 285 dB/m, $n = 2,862$)	Hepatic steatosis (CAP ≥ 285 dB/m, $n = 2,064$)	*p*-value
Blood cobalt (ug/L)	0.14 (0.11–0.18)	0.14 (0.11–0.19)	0.14 (0.10–0.17)	<0.01
Blood chromium (μg/L)	0.29 (0.29–0.29)	0.29 (0.29–0.29)	0.29 (0.29–0.29)	<0.01
hs-CRP (mg/L)	2.04 (0.91–4.44)	1.60 (0.75–3.55)	2.85 (1.29–5.75)	<0.01
HbA1c (%)	6.04 ± 1.18	5.82 ± 0.96	6.36 ± 1.37	<0.01
Insulin (μU/mL)	10.11 (6.30–16.39)	7.84 (5.15–11.84)	14.92 (9.48–22.63)	0.55
HOMA-IR	2.76 (1.66–4.88)	2.06 (1.32–3.31)	4.44 (2.68–7.16)	0.35
HOMA-IS	0.11 (0.07–0.18)	0.09 (0.06–0.14)	0.16 (0.10–0.24)	<0.05
eGFR (ml/min/1.73 m^2)	79.87 ± 31.84	78.33 ± 31.73	82.01 ± 31.88	<0.01
Lifestyle factors				
Alcohol consumption				0.28
Never	1,337 (27.14%)	777 (27.15%)	560 (27.13%)	
≤3 times/month	2,028 (41.17%)	1,147 (40.08%)	881 (42.68%)	
Once a week	306 (6.21%)	178 (6.22%)	128 (6.20%)	
≥2 times/week	583 (11.84%)	352 (12.30%)	231 (11.19%)	
Not recorded	672 (13.64%)	408 (14.26%)	264 (12.79%)	
Smoking status				<0.01
Yes	829 (16.83%)	521 (18.20%)	308 (14.92%)	
No	1,377 (27.95%)	740 (25.86%)	637 (30.86%)	
Not recorded	2,720 (55.22%)	1,601 (55.94%)	1,119 (54.22%)	
Comorbidities				
Diabetes				<0.01
Normal	1,944 (39.46%)	1,375 (48.04%)	569 (27.57%)	
Prediabetes	1,767 (35.87%)	1,013 (35.39%)	754 (36.53%)	
Diabetes	1,215 (24.67%)	474 (16.56%)	741 (35.90%)	
Hypertension				<0.01
Normal	2,513 (51.02%)	1,597 (55.80%)	916 (44.38%)	
Hypertension	2,406 (48.84%)	1,261 (44.06%)	1,145 (55.47%)	
Not recorded	7 (0.14%)	4 (0.14%)	3 (0.15%)	
Hepatitis B				<0.01
Normal	4,403 (89.38%)	2,515 (87.88%)	1,888 (91.47%)	
Hepatitis B	521 (10.58%)	346 (12.09%)	175 (8.48%)	
Not recorded	2 (0.04%)	1 (0.03%)	1 (0.05%)	
Hepatitis C				<0.01
Normal	4,742 (96.26%)	2,732 (95.46%)	2,010 (97.38%)	
Hepatitis C	183 (3.71%)	130 (4.54%)	53 (2.57%)	
Not recorded	1 (0.02%)	0 (0.00%)	1 (0.05%)	
Autoimmune hepatitis				0.94
Autoimmune hepatitis	14 (0.28%)	8 (0.28%)	6 (0.29%)	
Not recorded	4,912 (99.72%)	2,854 (99.72%)	2,058 (99.71%)	

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; HbA1c, glycated hemoglobin; hs-CRP, high-sensitivity C-reactive protein; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; BMI, body mass index; LSM, liver stiffness measure; CAP, controlled attenuation parameter; FITR, Family income-to-poverty threshold ratio.

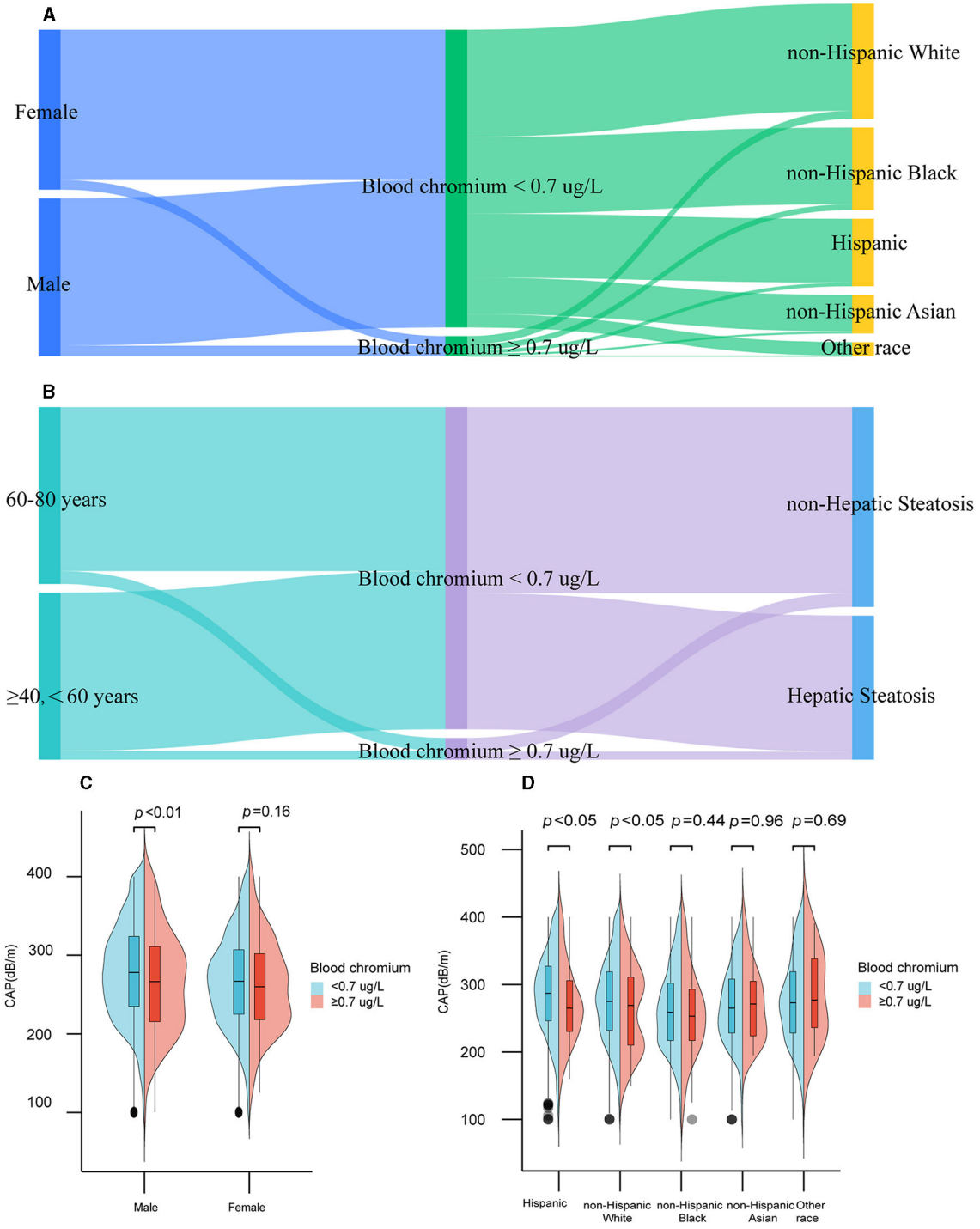

FIGURE 2
Distribution of blood chromium by gender, ethnicity, age, and hepatic steatosis among participants in the United States, 2017–2020. Distribution of blood chromium by gender, ethnicity **(A)**, age, and hepatic steatosis **(B)**. CAP at different gender **(C)** and ethnicity **(D)** among blood chromium deficiency or normal participants.

−17.00)] ($p < 0.01$). Hepatic steatosis had positive correlations with LSM, weight, BMI, ALT, AST, GGT, ALP, TG, hs-CRP, HbA1c, insulin, HOMA-IR, HOMA-IS, eGFR, smoking status, diabetes, and hypertension. Conversely, hepatic steatosis showed negative correlations with age, HDL-C, hepatitis C, and hepatitis B ($p < 0.05$).

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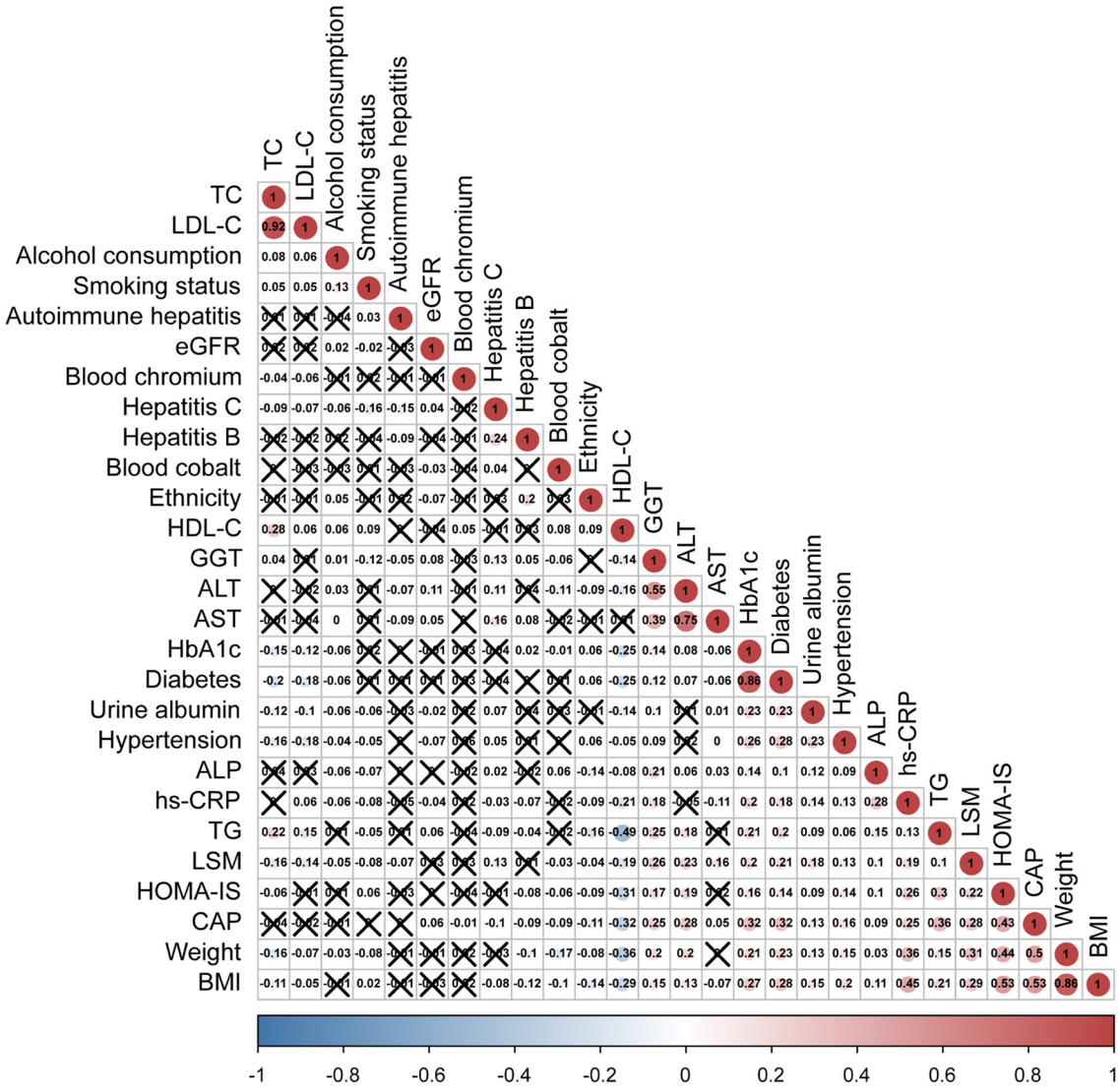

FIGURE 3
Spearman's rank correlation coefficient plot. With the decrease of Spearman's *r*, the blue in the figure deepens, which means the negative correlation is stronger; with the increase of Spearman's *r*, the red in the figure deepens, which means that the positive correlation is stronger. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; HbA1c, glycated hemoglobin; hs-CRP, high-sensitivity C-reactive protein; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; BMI, body mass index; LSM, liver stiffness measure; CAP, controlled attenuation parameter.

3.3 Weighted multivariate linear regression between blood chromium (Cr) and the controlled attenuation parameter (CAP)

Weighted multivariate linear regression analysis was conducted to evaluate the relationship between blood Cr and CAP in participants. Based on the results of the correlation analysis and the clinical significance, we adjusted the model with various covariates (age, gender, ethnicity, education, family income to-poverty threshold ratio, alcohol consumption, smoking status, BMI, weight, TG, TC, LDL-C, HDL-C, insulin, HbA1c, HOMA-IR, HOMA-IS, hs-CRP, ALT, AST, GGT, ALP, total bilirubin, blood cobalt, total calcium, urine albumin, eGFR, hypertension, diabetes, hepatitis B, hepatitis C, autoimmune hepatitis, and LSM). As demonstrated in Table 3, blood Cr had a negative correlation with the value of CAP. The negative correlation between blood Cr and CAP was present in the unadjusted model [β (95% CI) = −5.19 (−8.93, −1.46)], model II [β (95% CI) = −4.83 (−8.52, −1.14)], and model III [β (95% CI) = −5.62 (−11.02, −0.21)] ($p < 0.05$). In addition, to ensure the stability of the results, blood Cr was categorized into three classes according to clinical significance, and trend test was carried out in this study. With blood Cr < 0.41 g/L as reference, model I–III showed a negative correlation between blood Cr level and CAP (all p for trend < 0.05), which indicated that there was a stable and negative correlation between blood Cr level and the CAP. After the interpolation, the negative correlation also existed (Supplementary Table 3).

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TABLE 2 Univariate regression analysis for the controlled attenuation parameter (CAP).

Covariates	β (95% CI) p-value	Covariates	β (95% CI) p-value
Gender		**Hepatitis C**	
Man	Reference	Normal	Reference
Woman	−12.20 (−15.54, −8.85) <0.01	Hepatitis C	−19.93 (−28.80, −11.05) <0.01
Ethnicity		Not recorded	61.61 (−56.18, 179.41) 0.31
Hispanic	Reference	LSM (kPa)	1.93 (1.58, 2.28) <0.01
Non–Hispanic White	−9.05 (−13.60, −4.49) <0.01	Weight (kg)	1.39 (1.32, 1.46) <0.01
Non–Hispanic Black	−21.88 (−26.75, −17.00) <0.01	BMI (kg/m^2)	4.37 (4.16, 4.59) <0.01
Non–Hispanic Asian	−15.39 (−21.39, −9.38) <0.01	Blood chromium (μg/L)	−5.19 (−8.93, −1.46) <0.01
Other race	−7.80 (−16.53, 0.93) 0.08	ALT (U/L)	0.55 (0.46, 0.64) <0.01
Diabetes		AST (U/L)	0.16 (0.05, 0.27) <0.05
Normal	Reference	GGT (IU/L)	0.13 (0.09, 0.16) <0.01
Prediabetes	19.11 (15.40, 22.82) <0.01	ALP (IU/L)	0.19 (0.12, 0.25) <0.01
Diabetes	45.26 (41.13, 49.38) <0.01	Urine albumin (ug/mL)	−0.00 (−0.01, 0.00) 0.10
Hypertension		TG (mg/dL)	0.13 (0.11, 0.16) <0.01
Normal	Reference	HDL–C (mg/dL)	−1.11 (−1.21, −1.01) <0.01
Hypertension	16.64 (13.30, 19.97) <0.01	Blood cobalt (ug/L)	−1.70 (−3.96, 0.56) 0.14
Not recorded	25.50 (−18.74, 69.75) 0.26	HbA1c (%)	12.89 (11.51, 14.27) <0.01
Hepatitis B		hs–CRP(mg/L)	0.53 (0.34, 0.72) <0.01
Normal	Reference	HOMA–IS	41.94 (32.88, 50.99) <0.01
Hepatitis B	−12.88 (−18.33, −7.42) <0.01	eGFR	0.12 (0.06, 0.17) <0.01
Not recorded	−21.53 (−104.82, 61.76) 0.61		

LDL–C, low–density lipoprotein cholesterol; HDL–C, high–density lipoprotein cholesterol; TC, total Cholesterol; TG, triglyceride; HbA1c, glycated hemoglobin; hs–CRP, high–sensitivity C-reactive protein; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; BMI, body mass index; LSM, liver stiffness measure; CAP, controlled attenuation parameter.

3.4 Subgroup analysis

Next, we conducted subgroup analysis by dividing participants into groups by gender, age, BMI, TC, HDL-C, HbA1c, HOMA-IR, total bilirubin, alcohol consumption, and smoking status, respectively. As shown in Figure 4, it was noted that blood Cr had a negative correlation with CAP in the male [β (95% CI) = −9.35 (−17.76, −0.94)], age quartile 2 (50–59 years) [β (95% CI) = −12.72 (−25.05, −0.40)], overweight [β (95% CI) = −6.42 (−12.43, −0.42)], hypercholesterolemia [β (95% CI) = −13.43 (−26.00, −0.86)], HDL-C $\geq$ 65 mg/dL [β (95% CI) = −16.39 (−31.02, −1.76)], HbA1c quartile 3 (5.70–6.10%) [β (95% CI) = −13.97 (−26.63, −1.30)], HOMA-IR dichotomous 1 (0.12–2.76) [β (95% CI) = −11.38 (−19.68, −3.09)], total bilirubin tertile 2 (0.30–0.40 mg/dL) [β (95% CI) = −9.54 (−18.06, −1.03)] and ever alcohol consumption [β (95% CI) = −8.55 (−15.98, −1.13)] ($p < 0.05$). Furthermore, the negative correlation between blood Cr and CAP was also significant in ($p < 0.05$). The smoking status didn't affect the presence of the relationship between CAP and blood Cr ($p > 0.05$). Moreover, we conducted an interaction test, which found that negative correlation between blood Cr and CAP was significantly modified by HOMA-IR (p for interaction < 0.05).

3.5 Dose-response relationship between blood chromium and the controlled attenuation parameter (CAP)

The smooth curve fitting diagrams were drawn to visually estimate the dose-response relationship between blood Cr and CAP by GAMs. As shown in Figures 5A, B, the relationship between the blood Cr and CAP was linear after adjusting with all covariates [β (95% CI) = −5.62 (−11.02, −0.21)].

Due to alcohol consumption and smoking being common unhealthy lifestyle habits, we subsequently investigated whether alcohol consumption and smoking status would affect the dose relationship between blood Cr and CAP. After dividing the participants according to alcohol consumption, we observed that blood Cr showed a negative linear relationship with CAP in both alcohol consumption and non-alcohol consumption group (Figure 5C). Different with the results above, while dividing the participants with smoking status, the relationship between blood Cr and CAP showed U-shape curves in both somking and non-smoking groups. In smoking participants with blood Cr above 0.48 μg/L and non-smoking participants with blood Cr below 0.69 μg/L, the blood Cr showed a negative correlation with CAP ($p < 0.05$); while in smoking participants with blood Cr below 0.48 μg/L and non-smoking participants with blood Cr above 0.69 μg/L, the negative relationships between blood Cr and CAP were disturbed (p for log likelihood ratio test < 0.05; Figure 5D and Table 4).

4 Discussion

Our results suggest that blood Cr level is associated with hepatic steatosis in Americans. FLD is a wide spread chronic liver disease,

TABLE 3 Multivariate linear regression between blood chromium and the controlled attenuation parameter (CAP).

	Model I		Model II		Model III	
	β (95% CI)	*p*-value	β (95% CI)	*p*-value	β (95% CI)	*p*-value
Blood chromium	−5.19 (−8.93, −1.46)	<0.01	−4.83 (−8.52, −1.14)	<0.05	−5.62 (−11.02, −0.21)	<0.05
Blood chromium categories						
< 0.41 μg/L	Reference		Reference		Reference	
≥ 0.41, < 0.7 μg/L	−4.78(−10.11, 0.55)	0.08	−3.81(−9.09, 1.46)	0.16	0.41(−5.86, 6.68)	0.90
≥ 0.7 μg/L	−10.05 (−16.98, −3.11)	<0.01	−9.32 (−16.18, −2.46)	<0.01	−10.12 (−19.29, −0.96)	<0.05
p for trend	<0.01		<0.01		<0.05	

Model I: no covariates were adjusted.
Model II: age, gender, and race/ethnicity were adjusted.
Model III: age, gender, race/ethnicity, education, family income-to-poverty threshold ratio, alcohol consumption, smoking status, BMI, weight, TG, TC, LDL-C, HDL-C, insulin, HbA1c, HOMA-IR, HOMA-IS, hs-CRP, ALT, AST, GGT, ALP, total bilirubin, blood cobalt, total calcium, urine albumin, eGFR, hypertension, diabetes, hepatitis B, hepatitis C, autoimmune hepatitis, and LSM were adjusted.

and at the same time, one of the leading causes of death (11). In this study, we found that an elevation of 1 μg/L in blood Cr is corresponded with a decrease in CAP by −5.62 (−11.02, −0.21) dB/m, suggesting that the effect of intervening blood Cr may be a potential therapeutic strategy of FLD.

To our current knowledge, this research is the first to present the association between blood Cr level and hepatic steatosis in a large population. In earlier studies, researchers found that patients provided with an oral dose of 400 μg of Cr picolinate (CrPic) for 3 months showed the same degree of hepatic steatosis as the placebo group (12). However, dietary Cr intake does not reflect Cr absorption, and blood Cr level is a better indicator of Cr metabolism and distribution in the body, which was not investigated in this study (13). Furthermore, this study was also limited by its small sample size. In line with our results, another randomized controlled clinical trial reported that 1,000 μg CrPic daily intake for 24 weeks resulted in an increase in blood Cr level as well as a significant reduction in hepatic lipid deposition among type 2 diabetes mellitus (T2DM) insulin sensitivity responders (14). These findings remind us that insulin sensitivity may play a role in the interactivities between Cr and hepatic steatosis.

The underlying mechanisms of the negative association between blood Cr and hepatic steatosis may involves the alterations in insulin sensitivity, immunity, oxidative stress, gluco-lipid metabolism and gut microbiota by Cr. Impaired insulin sensitivity disrupts the balance of glucose and lipid metabolism, increasing the risk of hepatic steatosis (15), and trivalent Cr, as a co-factor to activate the insulin receptor, improves insulin action in insulin-sensitive tissues, such as adipose, skeletal muscle, liver tissue, etc. (16, 17). In adipose tissue, Cr supplementation upregulates peroxisome proliferator-activated receptor-γ (PPARγ) expression in T2DM rats (18). The activation of PPARγ pathway reduces the free fatty acid (FFA)-induced damage and restoring insulin sensitivity in peripheral tissues (19). In skeletal muscle, Cr increases the activity of Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) and upregulates the gene expression of insulin receptor (IR), glucose transporter 4 (GLUT4), and uncoupling protein-3 (UCP-3), improving insulin sensitivity and promoting the glucose transport (20, 21). In liver, Cr supplementation inhibited the expression of insulin degrading enzyme (IDE) in liver of KKAy mice and HepG2 cells, promoting insulin signal transduction (22). Moreover, Cr supplementation increased the p-IRS-1, GLUT2, and GLUT4 expression in the liver of various animal models of metabolic disorders, such as NAFLD, obesity, T2DM and metabolic syndrome, and thus lowering the hepatic TG levels of these models (23–27).

Cr has also been proven to regulate immune response and alleviate liver inflammation in animal models of obesity, metabolic syndrome, T2DM, and FLD. Cr supplementation decreased the expression of liver nuclear factor kappa-B (NF-κB) p65, and lowered plasma levels of C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), and intercellular cell adhesion molecule-1 (ICAM-1) in animal models of obesity, metabolic syndrome and T2DM. Furthermore, Cr supplementation downregulates CD68 and myelo-peroxidase (MPO) protein expression, reduces levels of pro-inflammatory cytokines in serum, and increases the secretion of the anti-inflammatory cytokine in hepatic steatosis animal models (8, 23, 24, 27, 28).

Cr also plays a positive role in affecting oxidative stress. *In vitro* experiments, Cr supplementation effectively protects LO2 cells from $H_2O_2^-$ induced oxidative damage, maintaining mitochondrial integrity and improving cell viability (29). In NAFLD KK/HlJ mice, Cr supplementation was found to reduce hepatic malondialdehyde (MDA) levels, increase liver Cu/Zn-SOD, catalase (CAT), and glutathione peroxidase (GPx) levels, and inhibit oxidative stress (24). In aged diabetic Zucker fatty rats and Spontaneously hypertensive rats, supplementation of Cr compounds reduced the hepatic and renal lipid peroxidation and DNA fragmentation (30, 31).

Cr affects energy metabolism and suppresses hepatic lipid deposition by modulating glucolipid metabolism-related enzymes. Cr supplementation inhibits the activities of α-amylase and α-glucosidase, exerting a beneficial effect on postprandial blood glucose (28, 29). In animal models of metabolic syndrome and T2DM, Cr supplementation inhibited hepatic gluconeogenesis by decreasing mRNA expression of fructose 1,6-bisphosphatase

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Subgroup	β(95%CI)	p value	p for interaction
Gender			0.27
Male	-9.35 (-17.76, -0.94)	<0.05	
Female	-2.83 (-9.96, 4.30)	0.44	
Age			0.67
Quartile1 (40-49 yaers)	-6.55 (-25.84, 12.73)	0.51	
Quartile2 (50-59 years)	-12.72 (-25.05, -0.40)	<0.05	
Quartile3 (60-68 years)	-4.60 (-16.38, 7.18)	0.44	
Quartile4 (69-80 years)	-2.46 (-19.14, 14.23)	0.77	
Body-mass index			0.6
< 25 kg/m2	-0.91 (-14.64, 12.82)	0.9	
25 kg/m2 or more	-6.42 (-12.43, -0.41)	<0.05	
TC			0.17
< 200 mg/dL	-4.20 (-10.30, 1.89)	0.18	
≥ 200 mg/dL	-13.43 (-26.00, -0.86)	<0.05	
HDL-C			0.74
< 65 mg/dL	-4.93 (-13.11, 3.24)	0.24	
≥ 65 mg/dL	-16.39 (-31.02, -1.76)	<0.05	
HbA1c			0.35
Quartile1 (2.80-5.30%)	-14.40 (-35.54, 6.74)	0.18	
Quartile2 (5.40-5.60%)	-9.68 (-23.53, 4.18)	0.17	
Quartile3 (5.70-6.10%)	-13.97 (-26.63, -1.30)	<0.05	
Quartile4 (6.20-15.20%)	-0.19 (-12.50, 12.12)	0.98	
HOMA-IR			<0.05
Dichotomous 1 (0.12-2.76)	-10.77 (-19.06, -2.47)	<0.05	
Dichotomous 2 (2.76-239.80)	1.18 (-9.55, 11.91)	0.83	
Total Bilirubin			0.2
Tertile 1 (0.10-0.20 mg/dL)	12.50 (-17.68, 42.68)	0.42	
Tertile 2 (0.30-0.40 mg/dL)	-9.54 (-18.06, -1.03)	<0.05	
Tertile 3 (0.50-2.90 mg/dL)	-1.70 (-11.69, 8.30)	0.74	
Alcohol consumption			0.36
Never	-0.79 (-13.43, 11.86)	0.9	
Ever	-8.55 (-15.98, -1.13)	<0.05	
Smoking status			0.88
Yes	-8.50 (-21.51, 4.51)	0.2	
No	-7.57 (-24.89, 9.75)	0.39	

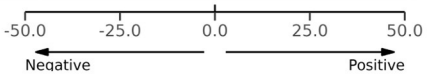

FIGURE 4
Association between blood chromium and the controlled attenuation parameter (CAP) in prespecified and exploratory subgroups.

(FBPase), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) and promoted hepatic glycogen synthesis by upregulating glucokinase (Gk) mRNA expression in the liver (32–34). In addition, Cr reduced the expression of CD36 in liver tissue and SMMC-7721 cells, thus decreasing the uptake of exogenous fatty acids, while increasing the levels of liver type fatty acid binding protein (L-FABP) mRNA in liver tissue, promoting fatty acid transport across cell membranes and into mitochondria, which enhanced hepatic fatty acid utilization (8, 33–36). In animal models of NAFLD and metabolic syndrome, Cr supplementation downregulated the expression of sterol regulatory element-binding protein-1 (SREBP-1) mRNA, which is a key transcription factors involved in the synthesis of cholesterol and fatty acids (28, 33, 37). In animal models of NAFLD and non-alcoholic fatty liver hepatitis (NASH), Cr downregulated the mRNA expression of diacylglycerolacyltransferase (DGAT)-1, DGAT-2, and fatty acid synthase (FASN), while suppressed protein expression of perilipin-2 (PLIN-2), consequently lessening the storage of TG in liver tissue (28). Furthermore, Cr enhances cholesterol metabolism. In Kunming mice with metabolic syndrome, Cr supplementation promoted cholesterol transport and metabolism by upregulating the mRNA expression of apolipoprotein E (ApoE) and low-density lipoprotein receptor (LDLR) and suppressed cholesterol synthesis by upregulating cytochrome P450 7A1 (CYP7A1) mRNA expression (32, 33). Finally, recent studies suggest that Cr supplementation significantly alteres the gut microbiome in animal models of T2DM and metabolic syndrome, leading to the improvement of glucose and lipid metabolism and the

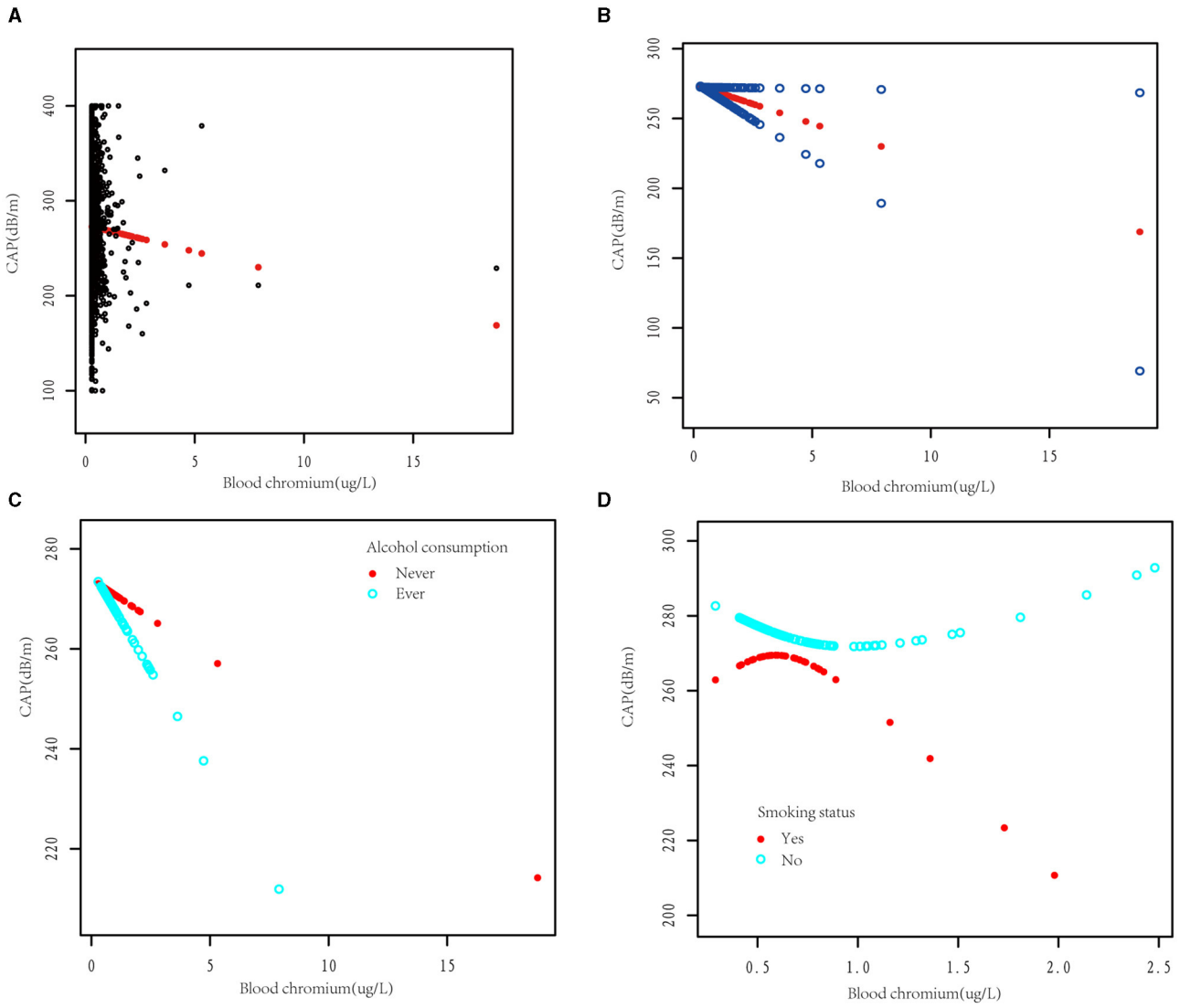

FIGURE 5
The dose-response association between blood chromium and the controlled attenuation parameter (CAP). **(A)** Each black point represents a sample subject. **(B)** The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. The dose-response relationship between blood chromium and CAP was stratified by alcohol consumption **(C)** and smoking status **(D)**. Adjusted for age, gender, race/ethnicity, education, family income-to-poverty threshold ratio, alcohol consumption, smoking status, BMI, weight, TG, TC, LDL-C, HDL-C, insulin, HbA1c, HOMA-IR, HOMA-IS, hs-CRP, ALT, AST, GGT, ALP, total bilirubin, blood cobalt, total calcium, urine albumin, eGFR, hypertension, diabetes, hepatitis B, hepatitis C, autoimmune hepatitis, and LSM except the stratification variable.

inhibition of liver lipid accumulation by the "gut-liver axis" (32–34).

By dividing the participants according to smoking status, the correlation between blood Cr and CAP were disturbed in smoking participants. This phenomenon may be related to Cr deposition in the lung caused by smoking. Cr not only distributes in blood, but also deposites in lung, and the pulmonary Cr concentration increases with the elevation of the smoking time (38, 39). Noteworthily, smoking primarily induces ectopic deposition of lipids in the liver through the induction of inflammation, oxidative stress, gut microbiota dysbiosis, disturbances in glucose and lipid metabolism and insulin resistance (40, 41). Additionally, the pulmonary Cr may alleviate the toxic effects of nicotine on liver by multiple ways (24, 28, 34, 42). However, the specific mechanism by which nicotine affects the interactivities between blood Cr and liver steatosis is still unclear. The impact of blood Cr on hepatic steatosis in smokers requires further investigation.

Nevertheless, there are several limitations in our research. Firstly, because the gold standard for diagnosing hepatic steatosis—liver biopsy data weren't available, we evaluated the hepatic steatosis using CAP from liver ultrasound transient elastography. Secondly, there are still knowledge gaps and lack of international consensus on how to define Cr deficiency and toxicity. Referring to the definitive reference for clinical chemistry, we defined Cr deficiency as blood Cr below 0.7 μg/L (10). Thirdly, it is difficult to avoid recall bias caused by self-reported data obtained from questionnaires. Fourthly, NHANES database lacked the information of genetic variations, complete medical history and

TABLE 4 Threshold effect analysis of blood chromium on the controlled attenuation parameter (CAP) stratified by smoking status using two-piecewise linear regression model.

	Smoker	Non-smoker
	Adjusted β (95% CI)	Adjusted β (95% CI)
	p-value	*p*-value
Inflection point	0.48	0.69
<Inflection point	86.35 (−14.85, 187.55) 0.10	−42.74 (−79.63, −5.85) <0.05
>Inflection point	−55.26 (−104.36, −6.15) <0.05	18.88 (−11.11, 48.86) 0.22
p for log likelihood ratio test	<0.05	<0.05

Adjusted for age, gender, race/ethnicity, education, family income-to-poverty threshold ratio, alcohol consumption, smoking status, BMI, weight, TG, TC, LDL-C, HDL-C, insulin, HbA1c, HOMA-IR, HOMA-IS, hs-CRP, ALT, AST, GGT, ALP, total bilirubin, blood cobalt, total calcium, urine albumin, eGFR, hypertension, diabetes, hepatitis B, hepatitis C, autoimmune hepatitis, and LSM except for the stratification variable.

dietary intake, thus we cannot rule out the bias caused by the aforementioned confounding factors. Fifthly, the NHANES only examined blood Cr levels in individuals aged 40 and above, and there is a lack of blood Cr investigation in individuals below the age of 40, particularly in children and pregnant women. A clinical study reported that blood Cr levels relieved the degree of insulin resistance in obese children, and improved other characteristic pathogenic events, such as inflammation, oxidative stress, abnormal glucose metabolism and dyslipidemia (43). For maternal rat, chronic maternal Cr restriction in rats increased offspring body adiposity by programming their epigenome (44). Studies have shown that Cr enhances insulin resistance and ameliorates obesity in kids, pregnant women and their offspring. However, there is a lack of research on the association between blood Cr levels and hepatic steatosis in children and pregnant women, and further studies are required. Finally, as a cross-sectional study, the causality and temporal order between blood Cr and hepatic steatosis could not be clarified. Hence, prospective cohort studies with sufficient observation periods and sample sizes are desired in the future.

5 Conclusions

Our study demonstrates that Cr deficiency is widespread in the American population, and there is an independent negative correlation between blood Cr level and hepatic steatosis. These results suggest that elevating blood Cr by diary supplement or other manners may be a potential therapeutic strategy of FLD.

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

This study was reviewed and approved by NCHS Ethics Review Board, and informed consent was obtained from all participants involved in the study.

Author contributions

YXi: Writing—original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. RZ: Writing—original draft, Methodology, Data curation. ZW: Writing—original draft, Investigation. YXu: Writing—original draft, Investigation. YC: Writing—original draft, Investigation. LS: Writing—original draft, Investigation. ZZ: Writing—original draft, Investigation. PX: Writing—original draft, Investigation. GZ: Writing—original draft, Investigation. WS: Writing—review & editing, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1307519/full#supplementary-material

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Diagnostic indicators and lifestyle interventions of metabolic-associated fatty liver disease

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MAFLD has become a major global health problem and is the leading cause of liver disease worldwide. The disease progresses from a simple fatty liver to gradual fibrosis, which progresses to cirrhosis and even hepatocellular liver cancer. However, the methods currently used for diagnosis are invasive and do not facilitate clinical assessment of the condition. As a result, research on markers for the diagnosis of MAFLD is increasing. In addition, there are no clinical medications for the treatment of MAFLD, and lifestyle interventions remain effective in the prevention and treatment of MAFLD. In this review, we attempt to make a summary of the emerging diagnostic indicators and effective lifestyle interventions for MAFLD and to provide new insights into the diagnosis and treatment of MAFLD.

KEYWORDS

metabolic dysfunction-associated fatty liver disease, biomarker, lifestyle interventions, diet, activity

1 Introduction

In 2020, the hepatology community introduced the novel term "metabolic dysfunction-associated fatty liver disease" (MAFLD) to replace "non-alcoholic fatty liver disease" (NAFLD) (1). Recently, this term was again replaced by "metabolic dysfunction-associated steatotic liver disease" (MASLD), along with a revised definition of metabolic dysfunction (2). MAFLD is a common chronic liver disease worldwide. The high prevalence of MAFLD has been noted in several reports (3–5). Overall prevalence is statistically increasing steadily and is projected to reach 55.7% by 2040 (6). MAFLD is a type of liver injury characterized by increased fatty deposits in hepatocytes with varying histological severity and the potential to progress to fibrosis, cirrhosis, and hepatocellular liver cancer. Fibrosis stage was the only independent predictor of long-term prognosis in MAFLD. Liver biopsy remains the gold standard for the diagnosis of liver fibrosis, but because it is an invasive test that is difficult to use frequently in the clinic, it has become important to find a noninvasive index for the effective assessment of MAFLD. In addition, there are no approved medications for the treatment of MAFLD, and lifestyle interventions continue to be the most recognized method of preventing and treating MAFLD.

The purpose of this review is to summarize current diagnostic indicators and effective lifestyle intervention methods for MAFLD.

2 Diagnostic indicators

2.1 Liver fat content

Magnetic Resonance Proton Density Fat Fraction (MRI-PDFF) has the ability to quantify hepatic fat over the entire dynamic range. It is a more accurate quantitative imaging biomarker that can be used to evaluate liver fat content. In a meta-analysis ($n = 6$ studies in 635 patients with biopsy-proven NAFLD), the summary AUROC values of MRI-PDFF for detecting steatosis S0 and S1–S3 (≥5%), S0–S1 and S2–S3 (≥33%), S0–S2 and S3 (≥66%) were 0.98, 0.91, and 0.90, respectively. Pooled sensitivity and specificity were 93 and 94%, 74 and 90%, and 74 and 87%, respectively (7). However, due to its high cost, it is not recommended for routine clinical use.

Controlled attenuation parameter (CAP) is the algorithm available on the FibroScan system (Echosens, Paris, France) for quantification of the liver fat content. This is a convenient and readily available modality that has been widely used to assess liver fat content. However, several covariates such as NAFLD, diabetes mellitus, and BMI affect CAP values. Currently, guidelines consider values above 275 dB/m to have high sensitivity and PPV (>90%) in NAFLD, although there is no uniform cut-off value for CAP (8). One study summarized that in healthy adolescent boys/girls, the upper limit of normal for CAP is 258.9/243.1 dB/m (12–13.9 years old), 251.9/266.3 dB/m (14–15.9 years old), 247.0/265.2 dB/m (16–17.9 years old), and 249.3/246.0 dB/m (18–19.9 years old) (9). Recent studies have analyzed that in children with NAFLD, using MRI-PDFF grading as a criterion for steatosis, the optimal cutoffs for CAP in the subgroups of S0 vs. S1–S3 (≥6.4%), S0–S1 vs. S2–S3 (≥17.4%), and S0–S2 vs. S3 (≥22.1%) were 265 dB/m, 299 dB/m, and 303 dB/m, respectively (10).

Recently, a study constructed a new CAP-based scoring scale, CBST.CBST = −14.27962 + 0.05431 × CAP −0.14266 × BMI + 0.01715 × AST. Confirmed that CBST scores are more accurate than CAP itself. The optimal CBST cutoff values for MRI-PDFF diagnosis of ≥20%, ≥10%, and ≥5% were −0.5345 (sensitivity = 72.1%, specificity = 70.8%, PPV = 53.7%, NPV = 84.4%), −1.7404 (sensitivity = 88.7%, specificity = 76.0%, PPV = 91.2%, NPV = 70.4%) and −1.9959 (sensitivity = 86.4%, specificity = 92.9%, PPV = 99.4%, NPV = 35.1%) (11). This would be a practical diagnostic basis if more studies were available to verify its accuracy.

2.2 Fibrosis score

2.2.1 Fibrosis-4 score, the aspartate aminotransferase-to-platelet ratio index

The FIB-4 and APRI is a score that uses routine laboratory parameters to assess liver fibrosis, and the formula is FIB-4 score = age(year) × AST(U/L)/[PLT(109/L) × ALT1/2(U/L)] APRI = [AST(U/L)/(AST(upper limit of normalcy)(U/L)]/ PLT(109/L) × 100). It has been shown that it can be used to assess the degree of hepatic fibrosis in chronic hepatitis B patients. The sensitivity and specificity of the APRI score > 0.342 to differentiate between patients with "no and mild fibrosis" and those with "severe fibrosis" were 63 and 64%. Respectively, and the sensitivity of the FIB-4 score > 0.70 to differentiate between patients with "no and mild fibrosis" and those with "severe fibrosis" was 71%, with a sensitivity of 71% (12). It has been noted that despite the high diagnostic accuracy of FIB-4 and APRI for advanced fibrosis in patients with NAFLD, their ability to diagnose hepatic fibrosis in its early stages remains controversial (13). Furthermore, it was shown that the APRI (AUROC range 0.73–0.80) and FIB-4 (AUROC range 0.66–0.82) outperformed the NAFLD fibrosis score (NFS) (AUROC range 0.63–0.75) in predicting liver fibrosis in MAFLD (14). In a meta-analysis of more than 40,000 participants, it was noted that when used to predict the severity of liver fibrosis in patients with MASLD, FIB-4 had good predictive diagnostic accuracy for any fibrosis, and FIB-4 had good diagnostic accuracy for cirrhosis, and could be used to assess the prognosis of MASLD (15).

2.2.2 MAFLD screening score, fatty liver index, hepatic steatosis index

Detection of hepatic steatosis HS is a mandatory criterion for the diagnosis of MAFLD, and the development of a non-invasive means of rapid and standardized detection of HS has become very important. Han et al. retrospectively evaluated the performance of FLI and HSI with respect to the prediction of MAFLD diagnosed by computed tomography (CT), and FLI (AUROC, 0.793) and HSI (HSI 0.784) were feasible in the prediction of MAFLD (16). In a large cross-sectional survey in China including 135,436 patients, the FLI AUROC used to predict MAFLD for male and female patients was 0.870 and 0.923 (17). A Mexican population-based study developed a score, MAFLD-S, for the prediction of MAFLD with an AUC of 0.852, 95% CI = 0.828–0.877, an optimal cutoff of 0.548, a sensitivity of 78.8%, and a specificity of 82.8% (18). In a cross-sectional study, assessment of MAFLD-S, FLI, and HSI is a valid tool to accurately predict MAFLD in patients with inflammatory bowel disease IBD (19). Valuable tool for FLI prediction of MAFLD noted in Japanese population-based survey (20). In a cross-sectional study based on adults in Xinjiang, FLI, and HSI had good screening for MAFLD in both men and women, with FLI having the best screening ability (21). FLI and HSI have excellent discriminatory ability in predicting MAFLD in the general population, both in the general population and in individuals at metabolic risk, FLI and HIS will be effective tools for predicting and screening for MAFLD (22).

2.2.3 TyG-body to mass index, TyG-waist circumference

A cross-sectional study of people aged 25–75 years showed that TyG-WC, TyG-BMI were the best predictors of MAFLD, independent of age, sex, obesity or diabetes status (23). Some studies have shown that TyG-BMI has good diagnostic efficacy in identifying people at risk for MAFLD in western China. In contrast, TyG-WC had the best diagnostic performance for identifying MAFLD risk in the US population. These findings suggest the need to select the most appropriate predictive model based on regional and racial differences (24). A Korean population-based study suggests that TyG-WC, TyG-BMI are positively correlated with MAFLD risk and can be used clinically to rapidly identify patients at risk of MAFLD (25).

2.3 Serum biomarker of MAFLD

2.3.1 Chitinase-3-like protein 1

Chitinase-3-like protein 1 (CHI3L1, YKL-40 protein) is a glycoprotein that is abundantly expressed in liver tissues and is mainly

involved in inflammation and tissue remodeling (26). It has been shown that CHI3L1 is significantly elevated in chronic liver diseases such as HBV-Related Liver Diseases, HCV-Related Liver Diseases, and hepatocellular carcinoma, and is associated with the degree of fibrosis (27–31). Studies have shown that CHI3L1 is an indicator used to assess fibrosis, and Xiaoting Huang el confirmed that serum CHI3L1 is a viable indicator for measuring liver fibrosis. The AUC for serum CHI3L1 was 0.812, $p < 0.000$. The Youden index was highest at a serum CHI3L1 level of 83.36 ng/mL, with a sensitivity of 88.2% and a specificity of 66.4% (32). Yanqiang Liao el demonstrated that CHI3L1 detected by chemiluminescent immunoassay (CLIA) has good diagnostic value for HBV-associated hepatocellular carcinoma (27). It has been shown that CHI3L1 is a good biomarker in MAFLD and assesses the risk of severe liver fibrosis. The AUC for CHI3L1 in the diagnosis of significant liver fibrosis was 0.716 (95% CI, 0.596, 0.836), with a corresponding optimal cutoff value of 125.315 ng/mL (33). High levels of serum CHI3L1 in patients with type 2 diabetes may indicate liver fibrosis in patients with MAFLD, CHI3L1 showed the area under the ROC curve (AUC) for detection of significant fibrosis (0.749, 95% CI, 0.668–0.829, $p < 0.001$), and the value of 94.89 ng/mL (sensitivity:54.4%, specificity:87.6%, $p < 0.001$) was the best cutoff point to predict significant liver fibrosis in T2DM-MAFLD patients (34).

2.3.2 Leukocyte cell-derived chemotaxin-2

LECT2 is a chemokine (35), it is mainly produced by the liver. It has been shown that LECT2 attenuates fatty changes and insulin resistance in the liver (36). Whereas insulin resistance is a key factor in the development of MAFLD, LECT2 may be involved in the development of MAFLD. It has been shown that LECT2 levels were 31.2 (20.9, 41.5) ng/mL in the group of people with NAFLD compared to those without NAFLD, which was significantly higher than in the group without NAFLD (37). It is proposed that LECT2 is highly expressed in both hepatic tissue and serum in biliary atresia (BA), suggesting that LECT2 can be used as a biomarker for BA (38). LECT2 levels are increased in patients with metabolic syndrome (MetS) and correlate with severity, suggesting that LECT2 can be used as a biomarker for MetS (39). Currently, there are experiments showing an increase in serum LECT2 concentration in children with NAFLD, which showed a diagnosis of NAFLD at a concentration of 3.76 ng/mL with a sensitivity of 90.5% and a specificity of 54.8%, suggesting that LECT2 can be used as a diagnostic biomarker for MAFLD in children (40), which has not been studied in adults with MAFLD.

2.3.3 Cathepsin D

Cathepsin D (CTSD) is a lysosomal enzyme involved in inflammatory responses and lipid metabolism, and previous data have shown that hepatic inflammation is associated with hepatic CTSD activity and expression (41, 42). In addition, plasma CTSD concentrations were associated with different stages of NAFLD, Significantly elevated in metabolic dysfunction-associated steatohepatitis (NASH) (43). A recent study analyzed RNA sequencing of MASLD and found higher expression of CTSD in severe MASLD compared to controls and mild disease, and validated the diagnostic role of serum CTSD in MASLD, showing that CTSD contributes to the accuracy of FIB-4 in diagnosing MASLD. The AUC of serum CTSD in predicting NASH fibrosis was 0.731 with a cutoff value of 10624.5 pg./mL (specificity 47.1%, sensitivity 93.3%) (44).

2.3.4 Plasminogen activator inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) is the most important inhibitors of the plasminogen/plasmin system (45). PAI-1 plays an important role in hepatic fibrogenesis (46). It has been shown that elevated plasma levels of PAI-1 correlate with the degree of hepatic steatosis (47). The study found that the PAI-1 gene was significantly down-regulated in NAFLD by bioinformatic analysis of the GEO database. Although bias may be induced by the small sample size, it suggests that PAI-1 has the potential to serve as a diagnostic marker for NAFLD (48). Recent studies have found that as liver pathology progresses toward nonalcoholic steatohepatitis (NASH), there is a corresponding increase in the level of mRNA expression of Serpine1, as well as the protein level of PAI-1. Use of Serum PAI-1 Levels as a noninvasive biomarker to identify NASH-associated fibrosis (49). However, no experiments have been performed to assess its diagnostic value in MAFLD.

3 Lifestyle interventions of MAFLD

3.1 Healthy diet

3.1.1 Daily dietary patterns

Moderate fiber intake is associated with lower odds of MAFLD compared to low fiber intake (50). Healthy low-carbohydrate and low-fat diets are protective against MAFLD, while unhealthy low-fat diets have deleterious effects on MAFLD (51). A study based on a Korean population showed that a dairy-rich dietary pattern was associated with a lower risk of MASLD, The cumulative incidence of MASLD was also significantly lower when adhering to a dietary pattern rich in dairy products (52). βHB, one of the ketone, has been reported to have inhibitory effects on adipocyte lipolysis, liver fat accumulation and inflammatory responses, suggesting a possible protective effect against MAFLD (53). The ketogenic diet (KD) is a diet that is very low in carbohydrate intake, and KD can be beneficial in treating diseases such as MAFLD and NASH. Sugar deficiency markedly reduces the effect on insulin resistance, and despite the benefits of KD, precautions need to be taken regarding the nature of dietary fats, and saturated fatty acids can be replaced with polyunsaturated fatty acids (54). One study tried to assess the effects of 8 weeks of a very low-calorie ketogenic diet (VLCKD) on MASLD, and found that it significantly reduced liver stiffness, and substantially reduced WC, fat mass, systolic and diastolic blood pressure, and BMI, and effectively reduced fasting glucose, insulin, insulin resistance (measured by HOMAIR), triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, and GT. This suggests that VLCKD improves insulin sensitivity and results in elevated levels of vitamin D. VLCKD treatment also reduces low-grade inflammation (55) (Figure 1).

The Mediterranean diet is currently the more recommended diet for the prevention of MAFLD (56). The Mediterranean diet includes plenty of vegetables, fruits, legumes, nuts and olive oil, as well as small to moderate consumption of red meat and wine, which has anti-inflammatory, antioxidant and antifibrotic components. Adherence to the Mediterranean diet, especially higher fruit intake, is associated with lower severity of MAFLD (57). Additional studies have found that the Mediterranean diet DM and the Mexican regional diet RMD are equally effective in improving MASLD symptoms, particularly

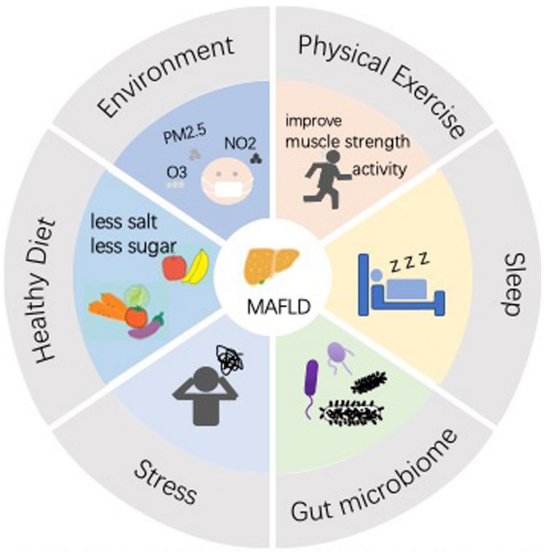

FIGURE 1
Lifestyle interventions are now a widely recognized method of prevention and treatment for MAFLD. An effective lifestyle includes a proper diet (less salt, less sugar, good quality carbohydrates, unsaturated fatty acids, vitamin D, etc.), and good habits (exercise, adequate and good quality sleep, good mood, improved gut microbiology, reduced air pollution, etc.).

steatosis, highlighting the feasibility of using regionally adapted diets to treat fatty liver. DM implementation is not feasible in all countries. Therefore, adapting the diet to the ingredients available in that country is a better option, as balanced implementation can improve the health of patients with fatty liver disease (58).

In China, three regional dietary patterns have been evaluated for their association with MAFLD: the Sichuan Basin pattern, characterized by high intakes of fish/seafood, poultry, fresh fruits and vegetables; the Yunnan-Guizhou Plateau pattern, characterized by high intakes of animal oils and salt; and the Qinghai-Tibet Plateau pattern, characterized by high intakes of coarse grains, wheat products, tubers and tea. The results showed that both the Yunnan-Guizhou Plateau dietary pattern and the Tibetan Plateau dietary pattern were positively associated with MAFLD. Studies point to an undesirable role of salt, animal oils and high carbohydrates in the progression of MAFLD, with animal oils rich in saturated fatty acids (SFA) being positively associated with MAFLD (59).

In addition, a vegetarian diet is now considered a healthy diet, and a vegetarian diet means replacing meat and fish with soy and refined carbohydrates with whole grains. Studies have shown that vegetarian diets may be associated with less pronounced liver fibrosis and that vegetarians have lower NAFLD fibrosis scores, which could mean lower cardiovascular-related mortality in the future (60). However, there has been no extensive research on its relationship with MAFLD progression, incidence or prevalence (61).

Therefore, a dietary pattern with less salt, less sugar and more vegetables and fruits has a favorable effect on the prevention of MAFLD.

3.1.2 Dietary supplement

In addition to daily dietary changes that are beneficial in preventing MAFLD, increasing or decreasing the intake of some dietary supplements may also be beneficial in reducing the incidence of MAFLD. Studies have shown that high dietary selenium intake increases the risk of MAFLD by modulating insulin biosynthesis and secretion dysregulation as well as stimulating glucagon secretion, insulin resistance and dyslipidemia (62). Reducing the ratio of Se and vitamin E, or not supplementing them at all, may reduce the prevalence of MAFLD (63). Patients with MAFLD consume more vitamin E in their daily diet than healthy controls, so increased vitamin E may be positively associated with MAFLD (64). Thus suggesting that to prevent MAFLD, we need to reduce se and vitamin E intake.

Patients with MAFLD consume more polyunsaturated fatty acids PUFA and iron in their daily diets than healthy controls Increased intake of these substances may be positively associated with MAFLD (64). According to an analysis of NHANES 2017–2020 data, high copper intake and moderate iron intake are associated with low odds of MAFLD (65). Therefore, we believe that there is also a relationship between copper and iron intake, and the occurrence of MAFLD. A significant association between vitamin D insufficiency and increased incidence of MAFLD suggests the potential of vitamin D as an anti-adipogenic and anti-fibrotic agent (66). A randomized controlled trial of vitamin D supplementation on serum levels of VDR, fibrotic factors and fibrotic microRNA (MiR) levels in patients with MASLD revealed for the first time significant reductions in some hepatic fibrotic factors, hepatic aminotransferases, and the corresponding changes in some fibrotic-associated MiR and some metabolic factors (67). suggests that vitamin D supplementation is beneficial in reducing the likelihood of MASLD fibrosis.

MAFLD is primarily caused by fat accumulation, and oral glutamine supplementation leads to insulin resistance in fat cells, which reduces fat (68). Glutamine was shown to contribute to attenuating the severity of hepatic lipid injury in mice exposed to high-fat diet (HFD) induced MAFLD by ameliorating changes in serum lipids, hepatic lipid metabolism, and oxidative stress (69).

Silymarin is a flavonoid compound derived from milk thistle seed that has been used for many years, as a Chinese herbal treatment for liver diseases (70). Silymarin can not only promote the production of glutathione to enhance the ability to resist oxidative stress (71), but also can inhibit the expression of NF- κ B, reduce TNF- α, IFN- γ, IL-2, and IL-4, and reduce the inflammatory response (72). In addition, silymarin can improved glucose tolerance and insulin tolerance in NAFLD patients, and the colonizing of altered microbiota from silymarin and polyherbal extract treated mice directly ameliorated NAFLD (73).

Insulin resistance is a common pathological feature in MAFLD patients, and artichoke has been found to have positive amelioration on insulin resistance. Previous studies have shown that water extract from artichoke ameliorates high-fat diet-induced non-alcoholic fatty liver disease in rats (74). Deng's study (75) demonstrated that artichoke water extract (AWE) reduced the expression of acid enol phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), and inhibited insulin resistance to improve glucose metabolism. Meanwhile, AWE also inhibited the endoplasmic reticulum stress to protect HepG2 cells. This is thought to be the resulting from the regulation of IRS 1/PI3K/AKT/FoxO 1 and GSK-3 β signaling.

Bergamot is a citrus fruit with extensive biological activity which has anti-proliferative, pro-apoptotic, anti-inflammatory and

anti-oxidation effects to make positive influence on MAFLD (76). Bergamot extract can reduce blood glucose levels in type 2 diabetic mice, prevent hyperglycemia caused by leptin receptor disruption, and regulate glucose homeostasis by enhancing insulin sensitivity. In this study, Liu et al. (77) also observed reduced TC, TG, and LDL-C levels, which effectively controlled blood lipids and protected the liver. In NAFLD, bergamot extracts can decrease the hepatic steatosis (78).

P. tenuifolia seed oil (PWSO) therapeutic diet (17% lard and 14% PWSO diet) inactivates SREBP1 and SREBP2, which are involved in lipogenesis, to attenuate hepatic lipid accumulation and reduce inflammatory responses induced through the NF-κB signaling pathway. Studies have shown that PWSO can be a relatively effective dietary supplement to inhibit the onset and progression of MAFLD (79).

We believe that moderate dietary supplements are beneficial for the prevention of MAFLD (Table 1).

3.1.3 Drinks

Green tea is generally recognized as a healthful drink (80). Tea consumption (≥1 cup/day) is not associated with the prevalence of newly diagnosed NAFLD among the general Chinese adult population, and further research may be needed to examine the association between higher frequency tea consumption and MASLD, says a study based on the Tianjin population (81). A 2020 study suggested that green tea reduced liver enzyme levels in participants with non-alcoholic fatty liver disease (NAFLD), but liver enzymes were significantly increased in healthy subjects (82). This suggests to us that drinking more green tea may be effective in reducing the prevalence of MAFLD.

It has also been suggested that caffeine attenuates liver fat and stiffness in patients with diabetes and NAFLD (83). Coffee is associated with NAFLD severity in type 2 diabetics (84). For overweight/obese patients with MASLD and T2D, coffee consumption may have potential benefits (85). It has also been shown that excessive soft drink consumption is still significantly associated with MASLD (86).

Therefore, appropriately increasing the consumption of green tea and coffee and decreasing the consumption of soft drinks in daily life may help prevent MAFLD.

3.2 Living habits

3.2.1 Physical exercise

According to data from the 2003–2006 U.S. population, longer physical activity (PA) is associated with a lower risk of cardiovascular disease-related death in patients with NAFLD (87). A study based on the 2017–2018 U.S. population shows that physical activity is strongly associated with a lower risk of obese and non-obese MAFLD (88). Active physical activity PA and adequate weekday sleep duration are both inversely associated with the risk of MASLD, and combining them can further reduce the risk of MASLD (89). Increased physical activity improves the strength of the muscles in the body. Whereas muscle strength may play a critical role in the incidence and progression of NAFLD/MAFLD, interventions to improve muscle strength in the management of NAFLD/MAFLD may be helpful (90). One study showed that adherence to an overall healthy lifestyle of non-smoking, non-alcohol use, physical activity, and healthy diet was associated with a 19% reduction in adjusted MAFLD risk (91).

It has been suggested that concentrating on physical activity 1 or 2 days per week (WW model) versus an average weekly schedule of physical activity (RA model) are both associated with lower DXA measures of fat mass (both in the abdominal region and whole body), BMI, and waist circumference. The WW model may also be applicable to the prevention of NAFLD (92).

3.2.2 Sleep

Short sleep duration and poor sleep quality are significantly associated with increased risk of NAFLD, according to a Korean population-based study (93). A study of 2,172 people in Japan found that the prevalence of NAFLD declined progressively with increasing sleep duration, with the lowest prevalence in the subgroup with 6 to ≤7 h of sleep and the highest in the groups with ≤6 and >8 h of sleep (94). A study of 708 non-diabetic adolescents found that sleep deprivation was associated with the presence of NAFLD in the younger population (95). Patients with sleep-disordered SD have higher risk of NAFLD in a Taiwanese population-based study (96). In addition, poor sleep patterns were associated with a high risk of MAFLD and severe fibrosis, and sleep difficulties, snoring, excessive daytime sleepiness, and sleep apnea symptoms were positively associated with the odds of MAFLD when specific factors of sleep patterns were examined in isolation (97). Good sleep habits, therefore, help reduce the prevalence of MAFLD.

3.2.3 Stress

The stress-eating relationship is mediated by the release of cortisol from the hypothalamic pituitary adrenal (HPA) axis. In a survey studying the relationship between occupational stress and NAFLD among Chinese police officers, it was found that the higher the stress, the higher the risk of developing NAFLD (98). Chronic stress linked to obesity, stress management important in treating NAFLD (99). Studies based on the Korean population have shown direct and indirect associations between psychological factors and NAFLD, depending on individual susceptibility (100). And in another study, it was also demonstrated that higher perceived stress was associated with increased prevalence of NAFLD (101). All of these studies have shown that the greater the psychological stress the greater the risk of developing NAFLD. Another recent Mendelian randomized analysis of studies points out that depression increases the prevalence of NAFLD (102). Suggesting the impact of the psychological illness on MAFLD, attention needs to be paid to managing stress and proper relaxation and stress relief in life.

3.2.4 Gut microorganisms

Study proves gut microbes linked to NAFLD development (103). Study finds that gut microbial diversity declines in NAFLD, and modulating gut microbiota health may help overcome NAFLD (104). In addition, probiotic yogurt significantly improves metabolic disorders through modulating intestinal microflora and lipid metabolism and effectively regulating the occurrence and development of MAFLD (105). By improving the flora of the gut microbiota, it helps reduce the risk of developing MAFLD.

3.3 Environment

It has been suggested that long-term exposure to $PM_{2.5}$ significantly increases the risk of metabolic disorders, which can increase lipid accumulation and loss of liver function (106). Ambient $PM_{2.5}$ stems from

TABLE 1 Effect of dietary supplements in MAFLD.

Author (reference)	Supplement	Experimental model	Concentrations of supplement	Findings
Niu et al. (64)	Polyunsaturated fatty acids (PUFAs); vitamin E; Iron	MAFLD patient	The percentages of individuals consuming PUFAs for >11% of their total energy intake, >14 mg/day of vitamin E, and >12 mg of iron	• Risk of MAFLD↑
Guo and Yu (63)	γ-tocopherol; α-tocopherol; Se	MAFLD patient	serum concentrations of γ-tocopherol (1.43 ± 0.52 μmol/L), α-tocopherol (3.38 ± 0.31 μmol/L) and Se (0.89 ± 0.13 μmol/L)	• Risk of MAFLD↑
Hou et al. (65)	Copper; Iron	MAFLD patient	Copper intake > 0.53 mg/1,000 kcal, Iron intake 5.19–7.57 mg/1,000 kcal	• Risk of MAFLD↓
Lee et al. (66)	Vitamin D	MAFLD patient	Serum vitamin D levels of 14.07 ± 3.55 ng/mL (men); Serum vitamin D levels of 12.57 ± 3.98 ng/mL (women)	• Risk of MAFLD↑
Ebrahimpour-Koujan et al. (67)	Vitamin D	MASLD patient	4,000 IU/d vitamin D (12 weeks)	• ALT, AST, FBS, and LDL-C levels↓ • Serum 25(OH) vitamin D, VDR, and HDL-C↑ • MiR-21 and MiR-122 gene expressions↓
Abboud et al. (68)	Oral glutamine	Wistar rats on a high-fat diet (HFD); Overweight (BMI ≥ 25 kg/m2) and obese (BMI ≥ 30 kg/m2) humans	Glutamine (0.4 g in 1 mL) 3 days a week for 4 weeks (rats); a total of 30 g of Gln per day, lasted for 14 days (humans); a dose of 0.4 g/kg of glutamine in humans and 2.4 g/kg in rats	• Waist Circumference and Circulating LPS↓ • Weight↓ • Glucose incorporation in adipose tissue↓ • Not increase insulin-induced Akt phosphorylation
Zhang et al. (69)	Glutamine	High-fat diet (HFD)-induced MAFLD C57BL/6 mouse model	HFD concomitant with 4% glutamine treatment for 24 weeks	• Lipid catabolism↑ • lipid accumulation↓ • Glutamine-based treatments alone cannot reverse serum lipid dysregulation
Sozen et al. (71)	Silymarin	Adult female Wistar Albino rats	100 mg/kg/ day oral SIL for 14 days, once in 24 h	• Hepato-cyte degeneration and multinuclear giant cell formation↑ • Prevented DNA damage • Oxidative stress in tissues ↓
Wang et al. (73)	Silymarin	High-Fat Diet-Induced NAFLD in Mice	HFD supplemented with a medium or high dose of silymarin (0.101 g, 0.202 g)	• Glucose tolerance and insulin tolerance↑ • Pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-17 (IL-17) in the liver↓ • altered microbiota from silymarin and polyherbal extract treated mice directly ameliorated NAFLD

(Continued)

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TABLE 1 (Continued)

Author (reference)	Supplement	Experimental model	Concentrations of supplement	Findings
Deng et al. (74)	Water extract of artichoke	Rats were fed a high-fat diet (HFD) for 8 weeks to induce NAFLD	Treated with WEA at three doses (0.4, 0.8, and 1.6 g/kg body weight, BW) for 8 weeks	• Blood lipid metabolism and liver function↑ • Hepatic oxidative stress↓ • mRNA expression of inflammatory genes↓ • Akt phosphorylation at Ser473 in the liver↑
Deng et al. (75)	Water extract of artichoke	Palmitic acid (PA)-induced IR model in HepG2 cells	AWE (containing 1.2% chlorogenic acid, 4.8% cynarin)	• PA-induced lipotoxicity and IR in HepG2 cells↓
Liu et al. (77)	Soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) from bergamot	Diabetic mice	Diabetic mice treated with SDF 800 mg/kg BW/day or IDF 800 mg/kg BW/day	• Blood glucose levels↓ • Homeostatic index of insulin resistance ↓
Maurotti et al. (78)	Bergamot and artichoke	NAFLD patient	Containing *Cynara cardunculus* extract and bergamot polyphenol fraction 300 mg/d	• Hepatic steatosis↓ • Peripheral vascular endothelial function in adults↑
Xin et al. (79)	*P. tenuifolia* seed oil	Rats were fed a high-fat diet (HFD, 31% lard oil diet)	PWSO treatment diet (17% lard oil and 14% PWSO diet) for 8 weeks	• Hepatic levels of TC and TG↓ • IL-6 levels and TNF-α level↓

a complex interaction of multiple emissions and chemical reactions; it is a mixture of various chemical components such as elemental carbon, organic carbon, sulfate (SO_4^{2-}), nitrate (NO_3^-), and ammonium (NH_4^+), and chronic exposure to $PM_{2.5}$ and its five major chemical components may increase MAFLD risk. Of these, nitrate may have the greatest impact on MAFLD (107). In addition, in a study analyzing the correlation between cirrhosis and air pollution in patients with NAFLD, based on the UK population, it was noted that long-term exposure to air pollution was associated with the risk of NAFLD and cirrhosis in the UK population (108). There was a dose-dependent relationship between different fibrosis stages and PM2.5 levels ($PM_{2.5}$ levels in patients with fibrosis stages 0, 1–2, and 3–4:27.9, 28.4, and 29.3 μg/m3, respectively; trend $p < 0.001$). Exposure to PM2.5 is associated with advanced liver fibrosis in patients with MAFLD (109). Recently, a cross-sectional study based on Taiwanese and Hong Kong populations analyzed airborne concentrations of nitrogen dioxide (NO_2) and ozone (O_3) and fine particulate matter ($PM_{2.5}$) in relation to advanced fibrosis in NAFLD. The results showed that higher ambient $PM_{2.5}$ and NO_2 were associated with higher odds of NAFLD and advanced fibrosis, and that, in addition, lowering $PM_{2.5}$ and NO_2 concentrations may be an effective method of preventing NAFLD, and that further research is necessary on O_3 (110). From this, we concluded that reducing air pollution or actively reducing exposure to $PM_{2.5}$ can effectively prevent MAFLD.

4 Conclusion and outlook

MAFLD is a liver disease with high prevalence, and currently used to diagnose MAFLD is still mainly diagnosed by imaging. This review summarizes the commonly used imaging markers (MRI-PDFF, CAP) and new evaluation markers based on the original markers (FIB-4, APRI, FLI, HSI, MAFLD-S, TyG-WC, TyG-BMI, CBST). The more studied serological markers (CHI3L1, LECT2, CTSD) are also summarized, and some serological markers in combination with imaging indices help to improve the accuracy. Combined serologic and imaging diagnosis may become an effective method for clinical diagnosis of MAFLD.

However, nowadays, indicators are generally devoted to reflecting the degree of hepatic steatosis, the degree of fibrosis, or predicting MAFLD in other metabolic disorders, and there is a lack of research on specific indicators for MAFLD, which might be useful to look for specific diagnostic indicators at the molecular level.

This review summarizes the effective methods currently used for the prevention and treatment of MAFLD in terms of diet, lifestyle habits and living environment. Dietary supplements are well suited to help in the clinic, as daily diet and lifestyle habits require a great deal of adherence from the patients themselves, and the living environment is difficult to change. Current research, based on cell and animal experiments, suggests that supplements are useful in reducing liver fat accumulation, anti-inflammatory, and antioxidant.

Current treatments targeting obesity, insulin resistance, and cardiovascular aspects are effective but lack specificity for MAFLD. This may be due to the fact that the pathomechanism of MAFLD is very complex and requires further research. It is therefore important to understand the pathogenesis of MAFLD, which may help to find drugs in the future that can both improve metabolic disorders and reduce hepatic inflammation and fibrosis, as well as provide new guidelines for finding early specific diagnostic indicators.

Author contributions

TC: Writing – original draft. XQ: Writing – original draft. JJ: Writing – review & editing. BH: Writing – review & editing.

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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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Fruit and vegetable intake and the risk of non-alcoholic fatty liver disease: a meta-analysis of observational studies

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Purpose: This systematic review and meta-analysis of clinical observational studies aims to clarify the correlation between the intake levels of fruits and vegetables and non-alcoholic fatty liver disease (NAFLD).

Materials and methods: PubMed, Embase, Web of Science, and the Cochrane Library were searched for studies on the association between vegetable or fruit intake with the risk of NAFLD from the foundation of each database up until September 2023. The relative risk (OR) and the 95% confidence interval (CI) were pooled for both the highest and lowest consumption levels of vegetables and fruits to explore their association with the incidence of NAFLD.

Results: The meta-analysis encompassed 11 studies with a total of 493,682 patients. A higher consumption of vegetables (OR = 0.78, 95% CI = 0.67–0.91) and fruits (OR = 0.88, 95% CI = 0.83–0.93) was found to have a negative correlation with the risk of NAFLD, denoting an inverse association. This correlation, however, varied among different ethnic groups and gender.

Conclusions: Our results indicate that increased consumption of vegetables and fruits is associated with a reduced likelihood of developing NAFLD.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/#searchadvanced, identifier: CRD42023460430.

KEYWORDS

vegetable, fruit, non-alcoholic fatty liver disease, diet, meta-analysis

1 Introduction

NAFLD, predominantly caused by metabolic syndrome, is closely associated with obesity, insulin resistance and hyperlipidemia (1). Over the past four decades, the incidence of NAFLD has steadily increased (2, 3), currently affecting 25% of the global adult population (3). The disease prevalence is about 30% in Asia (4), the US, and South America, 24% in Europe, and 13% in Africa (2, 5–7), making it the most widespread chronic liver disease worldwide (2). An estimated 20% of non-alcoholic steatohepatitis (NASH) patients progress to cirrhosis, and the risk of hepatocellular carcinoma in NASH patients surged 7.7·times between 2002 and 2016, from 2.1 to 16.2% (8). In the US, it is expected that NASH medical expenses per patient will jump from $3,636 to $6,968 between 2020 and 2039 (9). Similarly, in Japan, the annual healthcare cost for NASH ranged from

322,206 to 340,399 yen per patient between 2011 and 2017 (10), imposing a substantial economic burden. Therefore, attention must be focused on early disease detection in primary healthcare settings.

Obesity, over nutrition, a high-calorie diet, and a sedentary lifestyle contribute to the accumulation of liver fat (11, 12), and are crucial risk factors for NAFLD (13). The regulatory mechanism of NAFLD is connected with metabolism, heredity, intestinal microorganisms, and other factors (11, 14, 15). At present, the management of NAFLD is centered around reducing insulin resistance and limiting oxidative stress (13). Treatment strategies are founded on lifestyle management, such as modifying diet and increasing physical activity, with the intent of controlling weight and managing risk factors pertinent to metabolic syndrome (16, 17). Consequently, adhering to a balanced diet and maintaining a healthy lifestyle have become pivotal in treating and delaying the progression of this disease (18, 19).

Fruits and vegetables are plant-based foods, rich in dietary fiber, which can help maintain the balance of intestinal flora, reduce inflammation, and decrease fat accumulation in the liver. Moreover, fruits and vegetables are abundant in antioxidants that neutralize free radicals and diminish oxidative stress damage to the liver. The antioxidants (20) and anti-inflammatory compounds (21) in fruits and vegetables enhance insulin sensitivity, accelerate beta-oxidation, and inhibit new fat production (22). As a result, it has been hypothesized that an intake of fruits and vegetables correlates with a lower prevalence of NAFLD (23). However, the relationship between fruit and vegetable intake and NAFLD risk remains a subject of debate. Several observational studies suggest that higher dietary vegetable intake is associated with a lower NAFLD risk (24, 25). Yet, some research indicates that there is no such relationship (23, 26). A similar controversy exists in regard to the correlation between fruit intake and the risk of NAFLD (26–31). Even though the role of vegetable and fruit intake in NAFLD has drawn considerable public attention, no meta-analyses have demonstrated a correlation between vegetable and fruit consumption and NAFLD risk. Therefore, we undertook this meta-analysis to summarize the results of observational studies regarding the association between vegetable and fruit consumption and the risk of NAFLD.

2 Materials and methods

This systematic review and meta-analysis statement followed Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), and the protocol was registered with **PROSPERO (ID: CRD42023460430)**.

2.1 Study strategy

The researchers scoured Pubmed, Embase, Web of Science, and the Cochrane Library for studies on the correlation between vegetable or fruit consumption with the risk of NAFLD from the inception of these databases to September 2023. Key search terms included non-alcoholic Fatty Liver Disease, fruit*, vegetable*, among others. Detailed search strategies are presented in the Supplementary material. The Endnote software (X20 version) was utilized to eliminate duplicate documents fetched from each database, and the remaining potentially eligible documents were manually screened (Appendix 1).

Abbreviations: NAFLD, non-alcoholic fatty liver disease; CI, confidence interval; OR, relative risk; NOS, Newcastle-Ottawa Scale; FAO, fatty acid beta-oxidation.

2.2 Inclusion and exclusion criteria

Two researchers checked the titles and abstracts to select studies that met the inclusion and exclusion criteria. The full texts of these studies were examined to choose eligible studies. In instances where consensus on eligibility could not be reached, a third reviewer was engaged for discussion.

Inclusion criteria were as follows: (1) studies assessing the correlation between varying levels of vegetable and fruit consumption, and the risk of NAFLD; (2) studies that provide relative risk, odds ratio, hazard ratio, and their corresponding 95% confidence intervals; (3) observational studies such as cross-sectional studies, case-control studies, and cohort studies.

Papers were ineligible for inclusion using the following criteria: (1) duplicate papers; (2) irrelevance to the subject matter (irrelevant disease and observation indicators); (3) meta-analysis, reviews, letters, conference abstracts, case reports, guidelines, etc.; (4) animal experiments.

2.3 Data extraction

Two reviewers extracted basic information from the articles finally included. This information comprised the first author, publication year, country, study type, sample size, age of study population, sex ratio, follow-up time, disease diagnosis method, intake assessment method, model adjustment factors, and the relative risk RR (OR, HR) associated with the highest and lowest fruit and/or vegetable intake along with their corresponding 95% confidence intervals. Where possible, the maximally adjusted RR, OR, or HR ratio and 95% CI were extracted. Any disagreements during the review process, if any, were resolved by discussion or, if necessary, consultation with a third reviewer.

2.4 Quality assessment

The assessment of bias and quality of the included studies was performed independently by two reviewers, with discrepancies resolved by a third reviewer. The quality assessment adhered to our published protocols. The quality of case-control studies was evaluated using the Newcastle-Ottawa Scale (NOS) and categorized into high quality (score 7–9), medium quality (score 4–6), and low quality (score 0–3). The quality of cross-sectional studies was assessed using the AHRQ scale from the U.S. Agency for Healthcare Research and Quality, and these cross-sectional studies were assessed as low quality

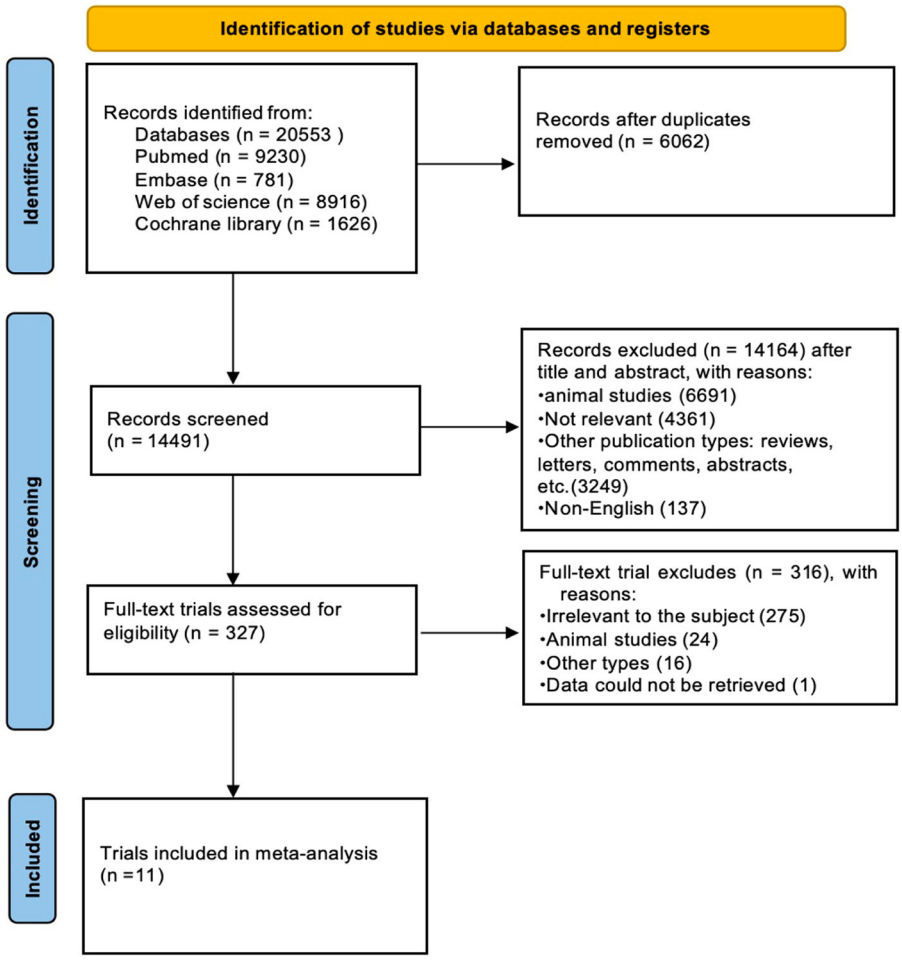

FIGURE 1
Flow diagram of study selection process.

(score 0–3), medium quality (score 4–7), or high quality (score 8–11).

2.5 Statistical analysis

In this study, Stata (version 15.0) was utilized to gather and summarize the OR and its corresponding 95% confidence interval, and to develop a forest map. Heterogeneity was evaluated by the *Q*-test and *I*-square test. A random effects model was employed when I-squared was ≥50% and $p < 0.1$; under other circumstances, a fixed effects model was used. In the presence of high heterogeneity, we conducted subgroup (study type, continent, and intake assessment questionnaire) and stratified analyses to explore heterogeneity sources. Sensitivity analysis was performed by observing the results' stability after sequentially eliminating each article. The potential risk of publication bias was assessed by examining funnel plots. When dealing with ≧10 articles, publication bias was evaluated using Egger's test and Begg's test. If publication bias was present, further evaluation was conducted using the "trim-and-fill" method. A bilateral $P < 0.05$ indicates a notable distinction.

3 Results

3.1 Literature search and selection

Papers related to the association between vegetable and/or fruit intake and the risk of NAFLD were searched from Pubmed (9,230 articles), Embase (781 articles), Web of Science (8,916 articles), and Cochrane Library (1,626 articles) from the inception of these databases until September 2023. After utilizing EndNote(X20) for automatic duplication removal, 14,491 related publications remained. Following a manual check for duplicates, 327 articles were left. These were then screened by their titles and abstracts according to inclusion and exclusion criteria. After a full-text review, only 11 papers were included. The literature screening process is illustrated in Figure 1.

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3.2 Study characteristics

The 11 included studies comprised six cohort studies and five case-control studies. Of the chosen studies, eight were conducted in Asian countries, specifically China (four studies), Iran (two studies), South Korea (one study), and Japan (one study). Additionally, two studies were conducted in Europe (one in Italy and one in the United Kingdom), and one study was conducted in North America (the United States). There was a total of 493,682 participants, consisting of 221,779 males and 271,901 females, whose ages ranged from 18 to 79 years. All included studies were deemed to be of high quality considering their AHRQ and NOS scores (Tables 1, 2). In the included studies, three intake assessment questionnaires were used, including Food Frequency Questionnaire (FFQ) used in nine studies, BDHQ in one study, and Food Diary in one study. The attributes of the included studies are detailed in Table 3.

3.3 Results of the meta-analyses

3.3.1 Vegetable intake

Eleven studies involving 493,682 participants reported the association between vegetable intake and NAFLD risk. A random-effects model was used for data analysis ($I^2 = 77.7\%$, $p < 0.001$). The results found that higher vegetable intake was linked to a reduced risk of NAFLD (OR = 0.78, 95% Cl: 0.67–0.91, $p = 0.001$; Figure 2A).

Due to the greater heterogeneity in the analysis of total vegetable intake, subgroup analyses were conducted according to sex, study type, continent, and intake assessment questionnaire. The results revealed that increased levels of vegetable intake were associated with a reduced risk of NAFLD for both males (OR = 0.83, 95% CI: 0.73–0.96, $p = 0.011$), and females (OR = 0.71, 95% CI: 0.59–0.85, $p < 0.001$; Figures 2B, C). Regarding study types, higher levels of vegetable intake were connected with lower prevalence of NAFLD in cross-sectional studies (OR = 0.78, 95% CI: 0.70–0.86), $p < 0.001$. However, in case-control studies (OR = 0.79, 95% CI: 0.60–1.05, $p = 0.107$), no such association was observed (Supplementary Figure 1A). In terms of the geographic location of the study population, increased vegetable intake was inversely correlated with the risk of NAFLD in the Asian population (OR = 0.68, 95% CI: 0.58–0.82, $p < 0.001$). However, no correlation was found between vegetable intake and the risk of NAFLD in the European (OR = 1.10, 95%CI: 0.77–1.56, $p = 0.6$) and North American population (OR = 0.99, 95% CI: 0.89–1.11, $p = 0.86$; Supplementary Figure 1B). Regarding the intake assessment questionnaires, FFQ (OR = 0.80, 95% CI: 0.68–0.93, $p = 0.005$) and Food Diary (OR = 0.34, 95% CI: 0.15–0.77, $p = 0.009$) indicated that a higher vegetable intake was associated with decreased NAFLD risk. However, results from the BDHQ (OR = 0.83, 95% CI: 0.57–1.21, $p = 0.332$) showed the relationship between the two was not statistically significant (Supplementary Figure 1C).

When an adjustment factor was present in more than two studies, we conducted a single-factor heterogeneity analysis. Upon adjusting for age, gender, smoking and alcohol consumption status, physical activity, energy intake, BMI, economic income

TABLE 1 Quality assessment of six case–control studies.

References	Selection				Comparability	Exposure			Overall score
	Is the case definition adequate?	Representativeness of the cases	Selection of controls	Definition of Controls	Comparability of cohorts on the basis of the design or analysis	Ascertainment of exposure	Same method of ascertainment for cases and controls	Non-response rate	
Emamat et al. (24)	1	1	1	1	2	-	1	-	7
Giraldi et al. (32)	1	1	1	1	2	-	1	-	7
Noureddin et al. (29)	1	1	1	1	2	-	1	1	8
Tutunchi et al. (30)	1	1	1	1	2	-	1	1	8
Guo et al. (26)	1	1	1	1	2	-	1	1	8

TABLE 2 Quality assessment of six cross-sectional studies.

	Chan et al. (27)	Liu et al. (28)	Tajima et al. (23)	Kim and Shin (25)	Li et al. (32)	Du et al. (31)
Define the source of information (survey, record review)	1	1	1	1	1	1
List inclusion and exclusion criteria for exposed and unexposed subjects (cases and controls) or refer to previous publications.	1	1	1	1	1	1
Indicate time period used for identifying patients.	1	1	1	1	1	1
Indicate whether or not subjects were consecutive if not population-based.	1	1	1	1	1	1
Indicate if evaluators of subjective components of study were masked to other aspects of the status of the participants.	1	1	1	1	1	1
Describe any assessments undertaken for quality assurance purposes (e.g., test/retest of primary outcome measurements).	1	1	1	1	1	1
Explain any patient exclusions from analysis.	0	0	0	0	0	0
Describe how confounding was assessed and/or controlled.	1	1	1	1	1	1
If applicable, explain how missing data were handled in the analysis.	1	1	1	1	1	1
Summarize patient response rates and completeness of data collection.	1	1	1	1	1	1
Clarify what follow-up, if any, was expected and the percentage of patients for which incomplete data or follow-up was obtained.	1	1	1	1	1	1
Total score	10	10	10	10	10	10

level, education level, and family history of diseases (hypertension, hyperlipidemia, cardiovascular disease, diabetes), we found that elevated levels of vegetable intake remained associated with a lower NAFLD risk (Supplementary Figures 2A–J). Yet, when we adjusted for hypertension, hyperlipidemia, diabetes, coffee intake, soft drinks, vegetable and fruit intake, and waist circumference, this association was not observed (Supplementary Figures 3A–G).

3.3.2 Fruit intake

Nine studies, involving 465,792 participants, reported the association between fruit intake and NAFLD risk. A fixed-effects model was used (I^2 = 46.9%, p = 0.058). The analysis revealed that higher levels of fruit intake were associated with a lower risk of NAFLD (OR = 0.88, 95% CI: 0.83–0.93, $p < 0.001$; Figure 3A).

Due to the heterogeneity of total fruit intake results, we performed a subgroup analysis based on sex, study type, continent, and intake assessment questionnaire. The fruit intake level was found to be associated with the prevalence of NAFLD in females (OR = 0.78, 95% CI: 0.63–0.95, p = 0.016), but not in males (OR = 0.84, 95% CI: 0.7–1.00; Figures 3B, C). Cross-sectional (OR = 0.84, 95% CI: 0.75, 0.93, p = 0.001) and case-control (OR = 0.90, 95% CI: 0.84, 0.97, p = 0.006) studies showed that higher levels of fruit intake were associated with lower NAFLD risk (Supplementary Figure 4A). In Asian (OR = 0.83, 95% CI: 0.75, 0.92, p = 0.001) and European (OR = 0.90, 95% CI: 0.82, 0.99, p = 0.03) populations, higher levels of fruit intake were associated with a lower risk of NAFLD, while in the North American population (OR = 0.91, 95% CI: 0.81, 1.02, p = 0.109), fruit intake was not associated with lower risk of NAFLD (Supplementary Figure 4B). FFQ (or = 0.89, 95% CI: 0.83, 0.94, $p < 0.001$) showed that a higher level of fruit intake was related to a lower level of NAFLD risk, while those of BDHQ (or = 0.73, 95% CI: 0.50, 1.07, p = 0.105) and Food Diary (or = 0.54, 95% CI: 0.19, 1.55, p = 0.251) showed that fruit intake had no significant impact on the risk of NAFLD (Supplementary Figure 4C).

Following adjustment for variables such as alcohol consumption, BMI, education level, energy intake, and physical activity, we observed that a high intake of fruits was still significantly associated with a reduced risk of NAFLD (Supplementary Figures 5A–E). However, further adjustments for factors such as age, gender, smoking status, coffee consumption, soft drink intake, history of diabetes, family history of diseases (hypertension, hyperlipidemia, cardiovascular disease, and diabetes), and waist circumference did not reveal any substantial correlation between fruit consumption and a lower risk of NAFLD (Supplementary Figures 6A–H).

3.4 Sensitivity analyses and publication bias

A sensitivity analysis was conducted to evaluate the stability of the results regarding the intake of vegetables or fruits and

TABLE 3 Study characteristics of the association between fruit and vegetable intake levels and the incidence of NAFLD were evaluated.

References	Country	Research type	Total number of participants	Baseline age (years)	Gender (male/female)	Follow-up period (years)	Methods of disease diagnosis	Quality of study
Chan et al. (27)	China	Cross-sectional study	797	36.2–60.3	332/465	/	Measurement of intrahepatic triglyceride content (IHTG) by 1H-MRS	Good
Liu et al. (28)	China	Cross-sectional study	1,639	18.55 ± 1.48	880/759	/	B-ultrasonic examination	Good
Tajima et al. (23)	Japan	Cross-sectional study	2,444	40–69	977/1,467	/	Abdominal ultrasonography	Good
Emamat et al. (24)	Iran	Case-control study	999	43.26 ± 14.03	430/569	/	Controlled attenuation parameter (CAP) score in Fibroscan exam	Good
Giraldi et al. (32)	Italy	Case-control study	815	51.37 ± 16.67	509/304	/	Presence of sonographic features of hepatic steatosis based on the presence of the bright liver pattern as recommended by the American Gastroenterology Association.	Good
Kim and Shin (25)	Korea	Cross-sectional study	52,280	40–79	15,588/36,692	4.2 years	NAFLD was diagnosed based on FLI Participants with FLI ≥60 were defined as having NAFLD.	Good
Noureddin et al. (29)	America	Case-control study	32,448	45–75	12,225/20,223	/	NAFLD cases among eligible participants were identified using Medicare claims	Good
Li et al. (33)	China	Cross-sectional study	26,891	≥18	12,727/14,164	/	Abdominal ultrasonography	Good
Tutunchi et al. (30)	Iran	Case-control study	210	30–60	90/120	/	Abdominal ultrasonography	Good
Du et al. (31)	China	Cross-sectional study	2,667	18–76	1,694/973	/	Abdominal ultrasonography	Good
Guo et al. (26)	UK	Case-control study	372,492	48.63–64.83	176,327/196,165	/	/	Good

References	Sources of intake assessment	Adjustment factors	Relationship between vegetables or fruits and NAFLD OR (LL, UL)	
			Vegetables	Fruits
Chan et al. (27)	FFQ	Age, sex, BMI, smoke, drink, central obesity, triglyceride >1.7 mmol/l, reduced HDL-cholesterol, hypertension, impaired fasting glucose or diabetes, the PNPLA3 genotypes (CC vs. CG vs. GG genotypes), and Energy intake	0.51 (0.3, 0.87)*	0.50 (0.3, 0.84)*
Liu et al. (28)	FFQ	Age, sex, BMI, economic income, smoking status, educational level, physical activity, family history of diabetes, stroke, and energy intake.	0.81 (0.66, 1.04)	0.84 (0.67, 1.07)
Tajima et al. (23)	BDHQ	Age, lifestyle factors, and BMI	0.83 (0.57, 1.21)	0.73 (0.5, 1.07)
Emamat et al. (24)	FFQ	Age, gender, BMI, energy intake, and physical activity	0.36 (0.22, 0.56)*	/
Giraldi et al. (32)	FFQ	Age, gender, total energy intake, diabetes status, smoking status, BMI, and physical activity.	1.81 (0.68, 4.78)	2.26 (0.97, 5.29)
Kim and Shin (25)	FFQ	Age, education level, smoking status, alcohol consumption, physical activity, energy intake, and red and processed meat intake, BMI	0.80 (0.69, 0.93)*	0.83 (0.72, 0.95)*
Noureddin et al. (29)	FFQ	BMI, alcohol intake, coffee intake, total soda intake, vigorous physical activity, and energy intake	0.99 (0.88, 1.1)	0.91 (0.81, 1.02)
Li et al. (33)	FFQ	Age, sex, smoking status, drinking status, education level, occupation, household income, physical activity, family history of disease (including cardiovascular disease, hypertension, hyperlipidemia, and diabetes), hypertension, hyperlipidemia, and diabetes, total energy intake, "fruits and sweet" dietary pattern score, "healthy dietary pattern score, and "animal foods" dietary pattern score, vegetable intake and fruit intake, BMI	0.81 (0.63, 1.05)	/
Tutunchi et al. (30)	Food diary	Sex, education, physical activity, BMI, and WC, the relationships and effect sizes for the residual effects of this variable	0.34 (0.16, 0.81)*	0.54 (0.19, 1.56)
Du et al. (31)	FFQ	Age, sex, educational attainment, BMI, WC, HC, BP, diabetes duration; family history, smoking, drinking, physical activity level, the consumption of bean products, salt, fish and sugary beverages, and biochemical index values (HbA1c, ALT, AST, and serum lipid levels).	0.67 (0.51, 0.88)*	1.15 (0.84, 1.59)
Guo et al. (26)	FFQ	Age, sex, race, education level, Townsend Deprivation Index (quartiles), drinking status, smoking status, exercise, BMI, and diabetes.	1.03 (0.93, 1.14)	0.89 (0.81, 0.98)*

*Indicates that the result is significant.

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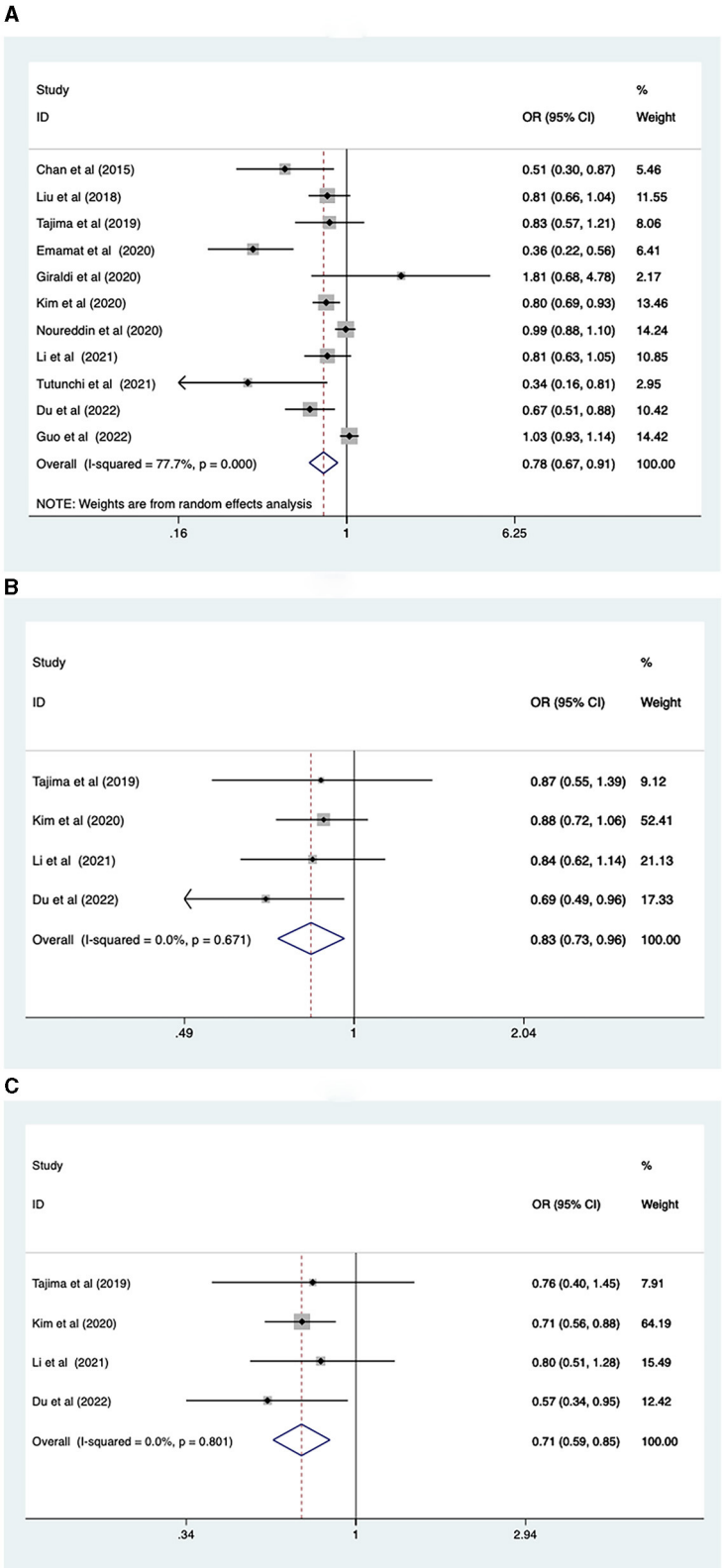

FIGURE 2
(A) Association between vegetable intake and risk of NAFLD; **(B)** NAFLD risk in men; **(C)** NAFLD risk in women.

the risk of NAFLD. After excluding each article one by one, the results remained stable (Figure 4). To further examine publication bias, Egger's ($p = 0.022$) and Begg's ($p = 0.161$) tests were conducted for the relationship between vegetable intake and the risk of NAFLD. The Egger's test showed evidence of publication bias (Figure 5A). Hence, the Trim and Fill method was employed

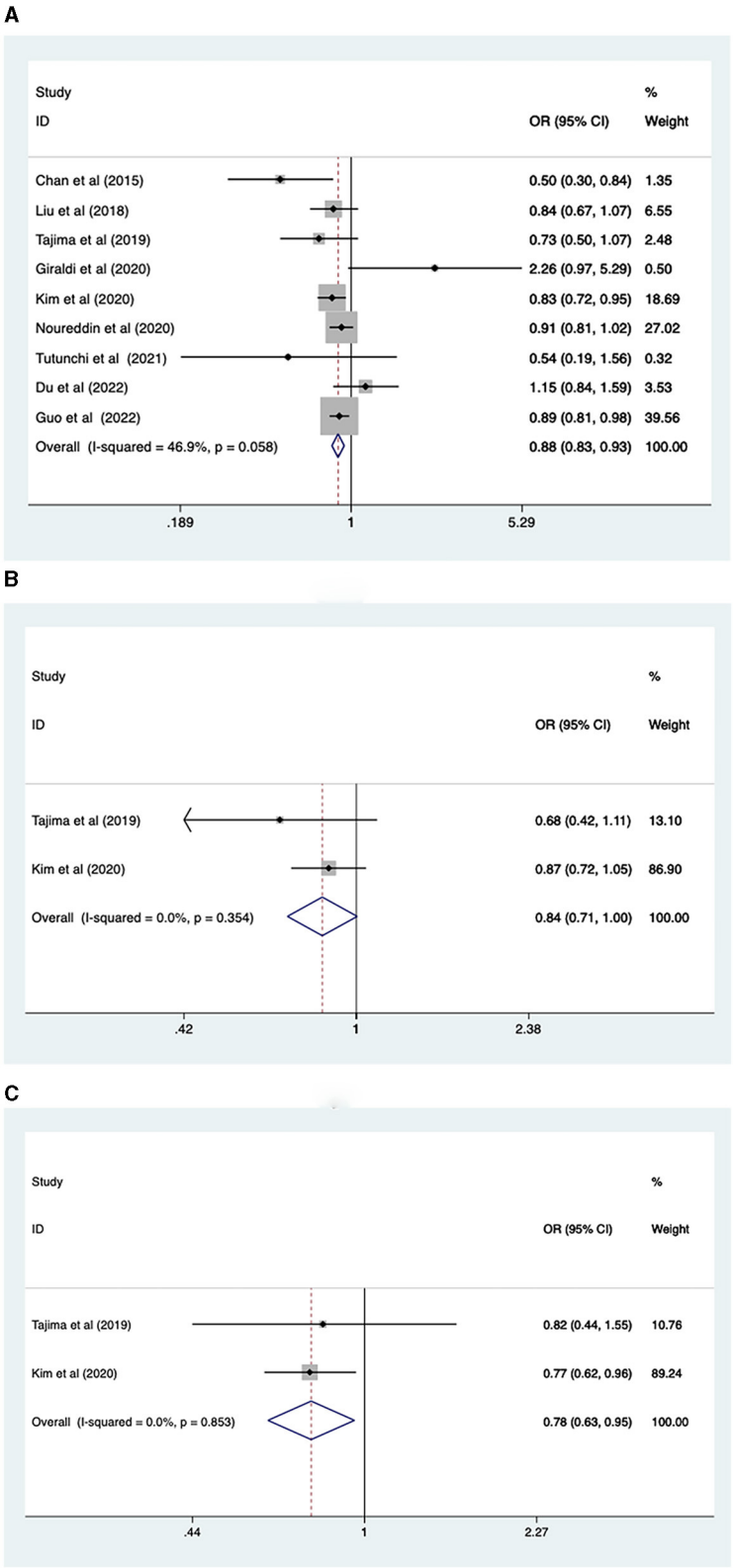

FIGURE 3
(A) Association between fruit intake and risk of NAFLD; **(B)** NAFLD risk in men; **(C)** NAFLD risk in women.

to adjust for the asymmetry in the funnel plot. However, the result indicated that no trimming was necessary and the data remained unchanged. When using Duval's Trim and Fill method, no new studies were added, suggesting that publication bias did not impact the study results (Figure 5B). Additionally, we analyzed publication bias using Egger's ($p = 0.822$) and Begg's

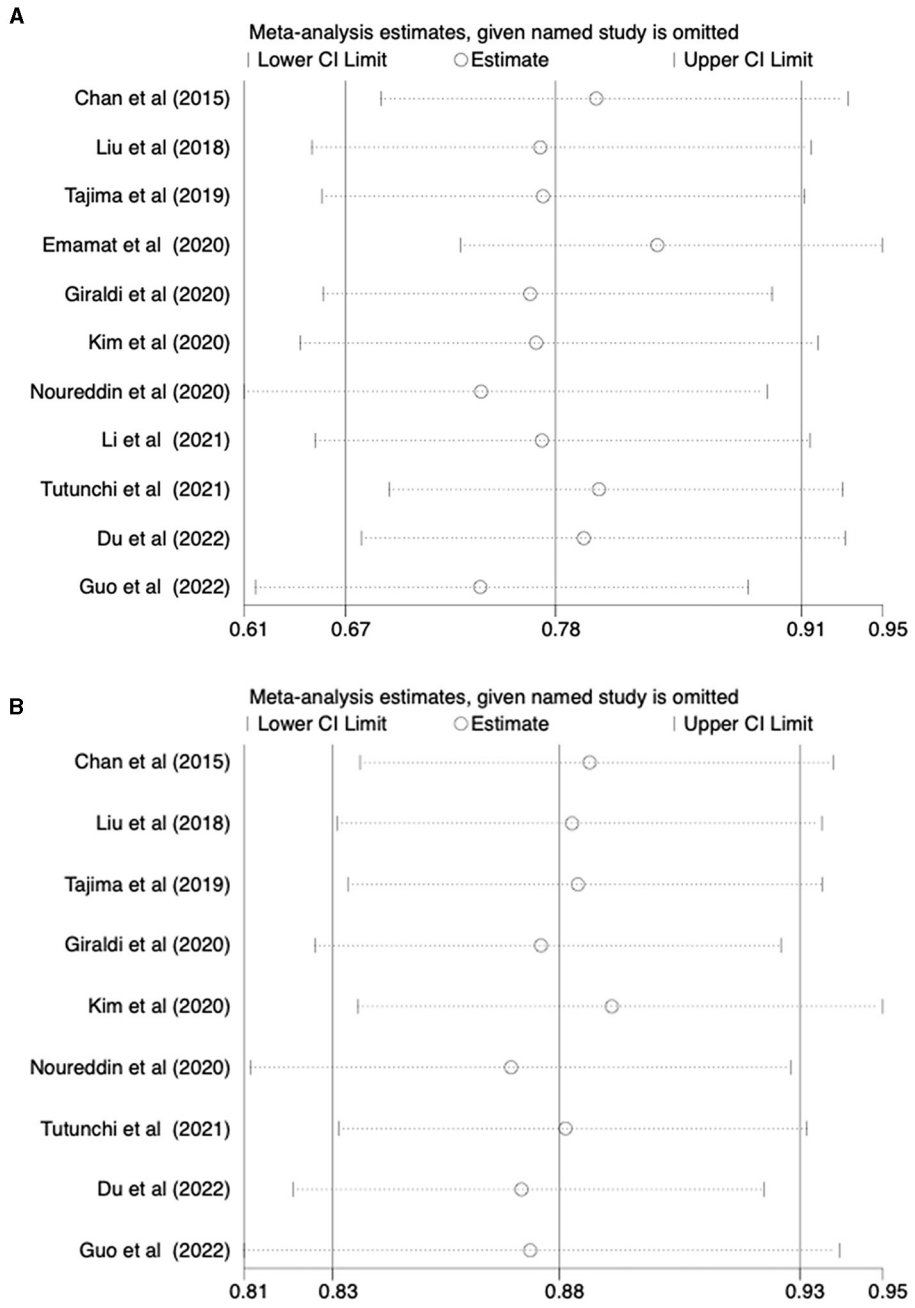

FIGURE 4
(A) Outcome sensitivity analysis of vegetable intake and risk of NAFLD; **(B)** Outcome sensitivity analysis of fruit intake and risk of NAFLD.

($p = 0.754$) tests for the relationship between fruit intake and the risk of NAFLD, finding no evidence of publication bias (Figure 5C).

4 Discussion

This represents the inaugural meta-analysis study investigating the connection between the intake of vegetables and fruits and the risk of NAFLD. A total of 11 studies involving 493,682 participants were included in the analysis, and the results suggest a negative correlation between the consumption of vegetables and fruits and the risk of NAFLD.

Dietary fiber, which is abundant in fruits and vegetables, is a type of short-chain fatty acids (SCFAs) produced by the fermentation of intestinal microorganisms in the gastrointestinal tract. These SCFAs, like propionic acid and butyric acid, have numerous benefits, including maintaining the integrity of the intestinal barrier, reducing the inflammatory reaction in the liver, regulating appetite, and maintaining glucose balance at the systemic level. This is all helpful in maintaining the energy balance of the liver, improving insulin sensitivity, and regulating liver

lipid metabolism (34, 35). Furthermore, dietary fiber also enhances satiety, thus promoting calorie restriction (36). Additionally, fruits and vegetables are rich in antioxidants, including vitamin C, vitamin E, beta-carotene, polyphenols, which neutralize free radicals and mitigate the detrimental effects of oxidative stress on the liver (37–41). It should also be noted that NAFLD is frequently accompanied by an inflammatory response. Therefore, inhibiting inflammation is crucial to alleviating NAFLD symptoms. Fruits and vegetables are rich in polyphenols and flavonoids, which possess anti-inflammatory properties and can reduce the severity of inflammation in the liver (41, 42).

It is observed that in some of the included studies, the relationship between intake levels of vegetables (23, 26, 28, 29, 32) and fruits (23, 28, 30–33) and the prevalence of NAFLD were not consistent with the conclusion in this meta-analysis. In particular, the proportion of related studies on fruit intake with inconsistent conclusions with this meta-analysis is relatively high. This discrepancy may be attributed to variations in disease diagnosis methods, study populations, adjustment factors, and dietary assessment methods.

The diagnosis of NAFLD differed across the included studies, and differences in the definition of fatty liver and the degree of medical diagnosis may have led to discrepancies in conclusions. Chan's study (27) measured intrahepatic triglyceride content (IHTG) using 1H-MRS within 8 weeks after the baseline visit of included participants, and the result showed that higher levels of vegetable and fruit intake were associated with a lower prevalence of NAFLD. Liu et al. (28) used B-ultrasound as the diagnostic basis, Giraldi et al. (32) used bright liver pattern and ultrasound features of liver steatosis as the diagnostic basis, and Noureddin et al. (29) identified eligible NAFLD patients through Medicare claims. Their results showed that vegetable and fruit intake levels were not associated with the prevalence of NAFLD. It is worth discussing that in the study with a large sample size conducted in South Korea by Kim and Shin (25), the incidence risk of NAFLD in the female population was related to vegetable and fruit intake, while in the male population, only fruit intake was found to be related. In the overall population, the incidence risk of NAFLD was associated with the intake of both vegetables and fruits. However, this conclusion may have significant bias because the diagnosis of NAFLD is based on the Fatty Liver Index (FLI), using a cutoff value of 60. However, in the study by Kim et al., 46% of the participants were of normal weight or underweight, and the accuracy of FLI in diagnosing NAFLD in lean NAFLD patients was low. This is because patients with low body mass index and NAFLD would not be able to reach the FLI limit for diagnosing NAFLD in the absence of increased GGT or triglycerides (included in the FLI calculation) (43). Furthermore, the accuracy of the critical value of 60 for diagnosing NAFLD is low in Asian populations, including in South Korea, because after the study by Kim et al., the ideal critical value for South Koreans was described to be equal to 29 (44–46).

Furthermore, results may vary between populations. Asians may have a stronger preference for leafy vegetables such as spinach and cabbage, while Europeans and Americans tend to consume vegetables like corn, squash, potatoes, onions, and broccoli, which contain higher levels of starch. This dietary difference could explain the diverse findings regarding the correlation between vegetable and fruit intake and NAFLD among participants in Asia, Europe, and America. Notably, there is limited literature from Europe and the Americas, necessitating cautious interpretation of these results and future confirmation through additional relevant studies. Additionally, we observed different results between males and females (23, 25, 31, 33), which may be attributed to notable differences in their dietary patterns (33). Studies have shown that females tend to increase their intake of fruits and

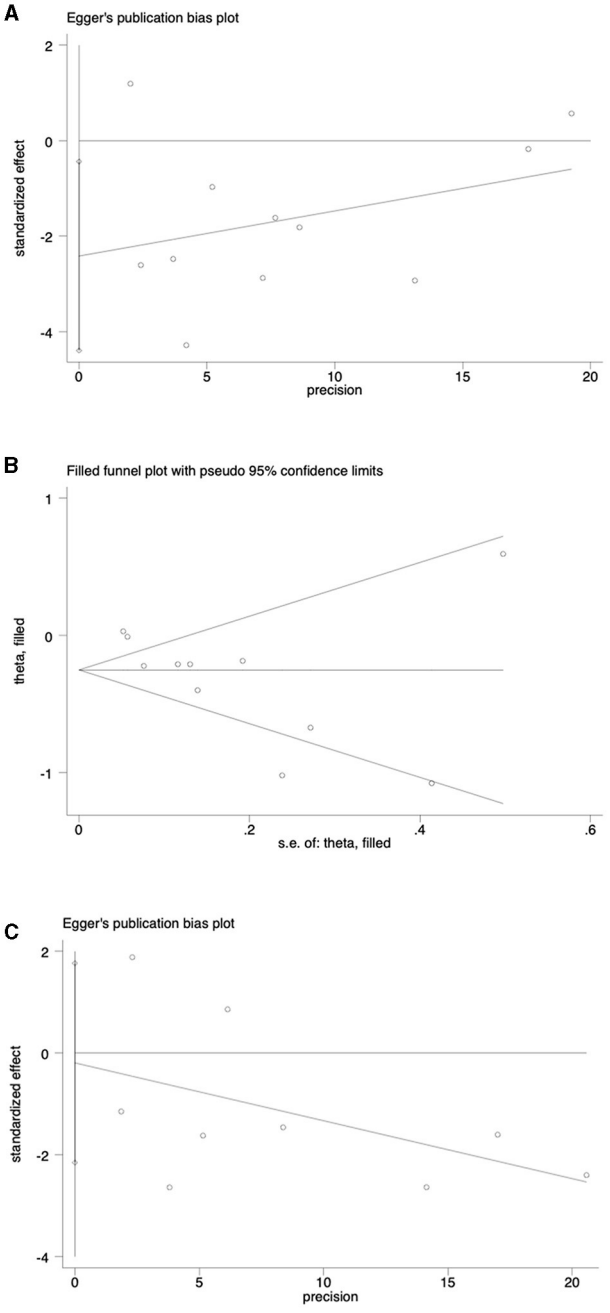

FIGURE 5
(A) Analysis of publication bias of outcomes associated with vegetable intake and risk of NAFLD; **(B)** Publication bias analysis was adjusted for the association between vegetable intake and NAFLD risk; **(C)** Analysis of publication bias for outcomes associated with fruit consumption and risk of NAFLD.

vegetables more than males (47, 48), and there are sex-specific disparities in fatty acid oxidation and regulation of liver *de novo* lipogenesis (DNL), with males inhibiting DNL less rapidly than females, leading to a shift in cellular metabolism from fatty acid oxidation to esterification (49). Since gender-specific studies are relatively scarce, further clinical data are required to validate these conclusions.

Results may vary when adjusting factors in a study. After adjusting for social and economic status and other factors in Li et al.'s study (33), green leafy vegetables (GLV) were negatively correlated with NAFLD. However, further adjustment for BMI eliminated this negative correlation. We found that adjusting for BMI did not significantly alter the results in multiple studies, including Noureddin et al.'s study (29). Although GLV intake is negatively correlated with NAFLD in normal/overweight individuals, obesity-related metabolic complications such as hyperlipidemia and insulin resistance may significantly increase liver lipids, resulting in decreased insulin sensitivity. These complications cannot be regulated by lipid metabolism and GLV intake (50). Additionally, some reports show that obese individuals significantly underestimate their dietary intake in self-recording or interview evaluation (51), which may explain why some studies did not observe the relationship between GLV intake and NAFLD after adjusting for BMI. Liu et al. (28) investigated the relationship between dietary patterns and NAFLD in Chinese adolescents, finding no association between fruit and vegetable intake and the occurrence of NAFLD. Teenagers usually have excellent physiological functions and efficiently absorb nutrients from food. Meanwhile, teenagers are more prone to unhealthy eating patterns that could affect their BMI and contribute to differences in conclusions. Therefore, additional studies are required to confirm the reliability of this conclusion.

In studies examining the intake levels of total vegetables and total fruits (25) and their association with the prevalence of NAFLD, results show a negative correlation. However, research by Liu et al. (28) and Giraldi et al. (32) indicates no relationship between vegetable and fruit intake levels and the prevalence of NAFLD. Guo et al.'s study (26) found significant results for fruit intake but not for vegetable intake. These studies included vegetables and fruits within dietary patterns, which are typically composed of independent or interactive foods and complex nutrient combinations that affect human metabolism. Therefore, it is challenging to exclude the synergistic effects of nutritional foods on NAFLD. Furthermore, not all types of vegetables and fruits are associated with a reduced risk of chronic diseases, as they contain different components and bioactive phytochemicals (52). While vegetables are generally considered low-carbohydrate foods and those with high dietary fiber levels may reduce the risk of NAFLD (33), excessive intake of starchy vegetables might increase blood glucose and insulin resistance, which is detrimental to NAFLD patients (53). Similarly, fruits contain natural fructose, and high fructose intake increases hepatic *de novo* lipogenesis (DNL), reduces fatty acid β-oxidation (FAO), and leads to fatty acid deposition (54). Excessive fructose intake can also promote the development of NAFLD. Additionally, the type of fructose—natural fructose from fruits vs. industrial fructose—might lead to different research conclusions. In Tajima et al.'s study (23), fruit intake was negatively correlated with the fatty liver index in the elderly, whose dietary fructose mainly came from fruits (55). Despite inquiries about fruit intake in studies, younger individuals might mistakenly count industrial fructose and fruit juice consumption as fruit intake. This could mean that, among the younger population, the harmful effects of industrial fructose and soft drinks on NAFLD might outweigh the protective effects of fruits on NAFLD (55). These factors may explain the inconsistencies between the conclusions regarding fruit intake in the included studies and the results of this meta-analysis.

In addition, there are some factors that cannot be avoided in the included studies. For example, in Giraldi et al. (32), there may be large differences in samples and high variability, resulting in wide confidence intervals, and outcomes may be affected. In Li et al.'s study (33), fruit intake was recorded for both NAFLD patients and the control group. However, the results were not significant, possibly due to differences in the study population, adjustment factors, dietary assessment methods, or recall bias in participants' reporting of fruit intake, leading to the omission of ORs. This does not meet the inclusion criteria for studies on fruit intake and NAFLD prevalence in our research and consequently contributes to a degree of selection bias in this study. Furthermore, the assessment questionnaire itself has self-reporting bias and subjectivity, and different assessment methods have different and limited contents, such as the lack of eating methods of vegetables and fruits, the choice of types of vegetables and fruits, the combination of food and the time of eating, which may also lead to different outcomes.

5 Strengths and limitations

Our study has various strengths. Firstly, it is the first meta-analysis to investigate the association between vegetable and fruit intake and incidence of NAFLD, utilizing large sample sizes. Secondly, in most of the studies included in the meta-analysis, the incidence of major NAFLD risk factors was controlled. Finally, we conducted subgroup analysis and stratification analysis of confounding factors to explore the sources of heterogeneity in the association between vegetable and fruit intake and NAFLD events.

Our study has a few limitations. Firstly, the included studies in our analysis encompassed case-control studies. As these studies employed food assessment questionnaires to estimate dietary intake, we cannot entirely rule out the possibility of measurement errors resulting from under- or over-reporting of food group intake due to participants' subjective judgments or memory biases. This could introduce recall and selection bias. Secondly, even though most studies adjusted for potential risk factors of NAFLD, residual confounding is always a concern in all observational studies. Finally, ORs were pooled from the highest and lowest intake levels, but intake levels were not always consistent across studies. Because of limited data, we were unable to include all studies in the dose-response analysis.

6 Conclusion

In conclusion, this systematic review and meta-analysis provide evidence that higher fruit and vegetable intake is linked to a lower

risk of NAFLD. However, given that the relationship between vegetable intake and NAFLD incidence varies across different populations (age, sex, and ethnicity), types of vegetables, and fruits, more high-quality prospective studies are desired to further elucidate this connection. Additionally, there are studies suggesting that excessive fruit intake may actually promote the development of NAFLD.

Data availability statement

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

Author contributions

RW: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. RY: Data curation, Formal analysis, Methodology, Software, Writing – review & editing. JJ: Investigation, Project administration, Software, Writing – review & editing. FL: Data curation, Investigation, Software, Writing – review & editing. HZ: Investigation, Software, Visualization, Writing – review & editing. ZC: Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. HW: Project administration, Supervision, Validation, Writing – review & editing. SY: Conceptualization, Methodology, Project administration, Supervision, Validation, Writing – review & editing. JL: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1398184/full#supplementary-material

APPENDIX 1
The complete retrieval formula.

SUPPLEMENTARY FIGURE 1
Subgroup of the association between vegetable intake and the risk of NAFLD. **(A)** Research type; **(B)** Nation state; **(C)** Assessment questionnaire.

SUPPLEMENTARY FIGURE 2
Stratified analysis of the association between vegetable intake and the risk of NAFLD (relevant). **(A)** age; **(B)** gender; **(C)** Smoking status; **(D)** Alcohol Consumption status; **(E)** physical exercise; **(F)** Energy intake; **(G)** BIM; **(H)** economic income; **(I)** educational level; **(J)** Family history of disease.

SUPPLEMENTARY FIGURE 3
Stratified analysis of the association between vegetable intake and the risk of NAFLD (irrelevant). **(A)** High blood pressure, high cholesterol; **(B)** diabetes; **(C)** Coffee intake; **(D)** Soft drink intake; **(E)** Vegetable intake; **(F)** Fruit intake; **(G)** waistline.

SUPPLEMENTARY FIGURE 4
Subgroup of the association between fruit consumption and the risk of NAFLD. **(A)** Study type heterogeneity; **(B)** National heterogeneity; **(C)** Heterogeneity of fruit intake was assessed.

SUPPLEMENTARY FIGURE 5
Stratified analyses of the association between fruit consumption and the risk of NAFLD (relevant). **(A)** Alcohol Consumption status; **(B)** BMI; **(C)** educational level; **(D)** energy intake; **(E)** physical exercise.

SUPPLEMENTARY FIGURE 6
Stratified analyses of the association between fruit consumption and the risk of NAFLD (irrelevant). **(A)** age; **(B)** gender; **(C)** smoking status; **(D)** Coffee intake; **(E)** Soft drink consumption; **(F)** diabetes; **(G)** Family history of disease; **(H)** waistline.

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Screening and assessment of malnutrition in patients with liver cirrhosis

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The development and advancement of malnutrition is associated not only with the progression of hepatic dysfunction, but also with cirrhosis-related complications. However, the prevalence of malnutrition reported in different studies varies widely due to differences in diagnostic methods and patient investigation settings. Therefore, we need to identify malnourished patients promptly and accurately. The purpose of this review was to compare the validity and reliability of nutritional screening tools and to select the most appropriate nutritional risk screening for patients with cirrhosis. We compared nutritional risk screening tools such as the Nutritional Risk Screening 2002 (NRS-2002), Malnutrition Universal Screening Tool (MUST), Royal Free Hospital-Nutritional Prioritizing Tool (RFH-NPT) and Liver Disease Undernutrition Screening Tool (LDUST). Royal Free Hospital-Nutritional Prioritizing Tool (RFH-NPT) is more feasible to screen cirrhotic patients for nutritional risk, and is highly reproducible, considering the impact of sodium and water retention; so it is practical to screen cirrhotic patients via RFH-NPT for nutritional risk, subsequently, to evaluate the nutritional status of patients with nutritional risk via the Global Leadership Initiative on Malnutrition (GLIM) diagnostic criteria. L3-SMI (third lumbar-skeletal muscle index) can accurately define sarcopenia in cirrhotic patients and also be used for clinical nutritional status assessment.

KEYWORDS

cirrhosis, risk assessment, assessment, malnutrition, prevalence

1 Introduction

Malnutrition is a frequent complication of liver cirrhosis and closely correlated with poor prognosis, especially in patients with decompensated cirrhosis (1); It is defined as changes in mental and physical functioning due to alterations in body composition and cellular quality, leading to poor clinical outcomes and reduced quality of life (2). The prevalence of malnutrition was 46 and 95% in Child-Turcotte-Pugh (CTP) A and C, respectively (3). The existing literature suggests that the prevalence of malnutrition in patients with decompensated cirrhosis may exceed 50% (4, 5).

The causes of malnutrition in cirrhotic patients can be categorized into two main aspects, namely decreased intake and increased consumption (Figure 1). Decreased intake includes: (1) Loss of appetite, early satiety and impaired consciousness leading to reduced intake serve as the most common causes (6, 7). Patients with cirrhosis are usually deficient in micronutrients. Several studies have shown that serum levels of zinc, selenium, and magnesium are significantly low in patients with cirrhosis and decrease dramatically in correspondence with the disease progression (8). This may partially account for the loss of appetite in these patients (9). In addition, diets which restrict sodium may result in unpalatable food and may be a contributing factor to inadequate nutrient intake. Moreover patients with cirrhosis usually have ascites and portal hypertension, leading to slowed bowel movements and limited gastric diastole, which may result in delayed feeling of hunger and reduction of food intake (10). On the other hand, some cirrhotic patients with impaired consciousness due to hepatic encephalopathy are primarily dependent on parenteral nutrition, giving rise to inadequate nutrient supply. (2) Continuous lactulose therapy and dysbiosis of intestinal flora may lead to malabsorption (11). Studies have shown that the development of malnutrition in patients with cirrhosis is associated with dysbiosis of the intestinal microbiota. There is an increase in pro-inflammatory flora such as Enterobacteriaceae, a phenomenon that usually leads to inflammation in cirrhotic patients, accompanied with increased protein metabolism and loss of muscle mass (12). Resting energy expenditure accounts for 60–70% of total energy expenditure in healthy individuals (13) and is often increased in patients with cirrhosis due to hypermetabolism, inflammatory response, and immunosuppression (14). Patients with cirrhosis tend to have increased protein metabolism and decreased synthesis in close relation to malnutrition (15). Hormonal mediation of malnutrition is complex, and it includes the major orexigenic (appetite) hormone, gastrin, as well as a variety of anorexigenic (satiety) hormones, including leptin, cholecystokinin, glucagon-like peptide-1, peptide YY, oxyntomodulin, and pancreatic polypeptide (16, 17). It seems unclear that how inflammation or hormones affect nutrition consumption. In addition, hyperammonemia appears to be one of the important causes of protein depletion in cirrhosis as well (18).

The development and advancement of malnutrition is associated not only with the progression of hepatic dysfunction, but also with cirrhosis-related complications, including infections, hepatic encephalopathy, and ascites (19, 20). Decompensated cirrhosis often presents with severe ascites and portal hypertension, which is particularly detrimental to oral nutrition. A negative balance of calories and protein can further deteriorate the already impaired synthetic function in cirrhotic patients (21). Furthermore, malnutrition independently serves as a prognostic indicator for mortality (20). It is imperative to properly identify malnourished subjects with the purpose of providing appropriate treatment to improve the prognosis. The estimated prevalence of malnutrition in patients with cirrhosis ranges from 5–92% due to considerable variation in the measuring tools (7). Table 1 summarizes studies concerning the prevalence of malnutrition in the most recent publications by using different nutritional assessment tool. To standardize and harmonize the diagnosis of malnutrition, the Global Leadership Initiative on Malnutrition (GLIM) reached a new global consensus on the criteria for the diagnosis of malnutrition in 2019, a two-step modality for nutritional assessment is recommended, that is, risk screening of subjects using validated tools prior to diagnostic assessment and intervention, and provide diagnostic criteria for

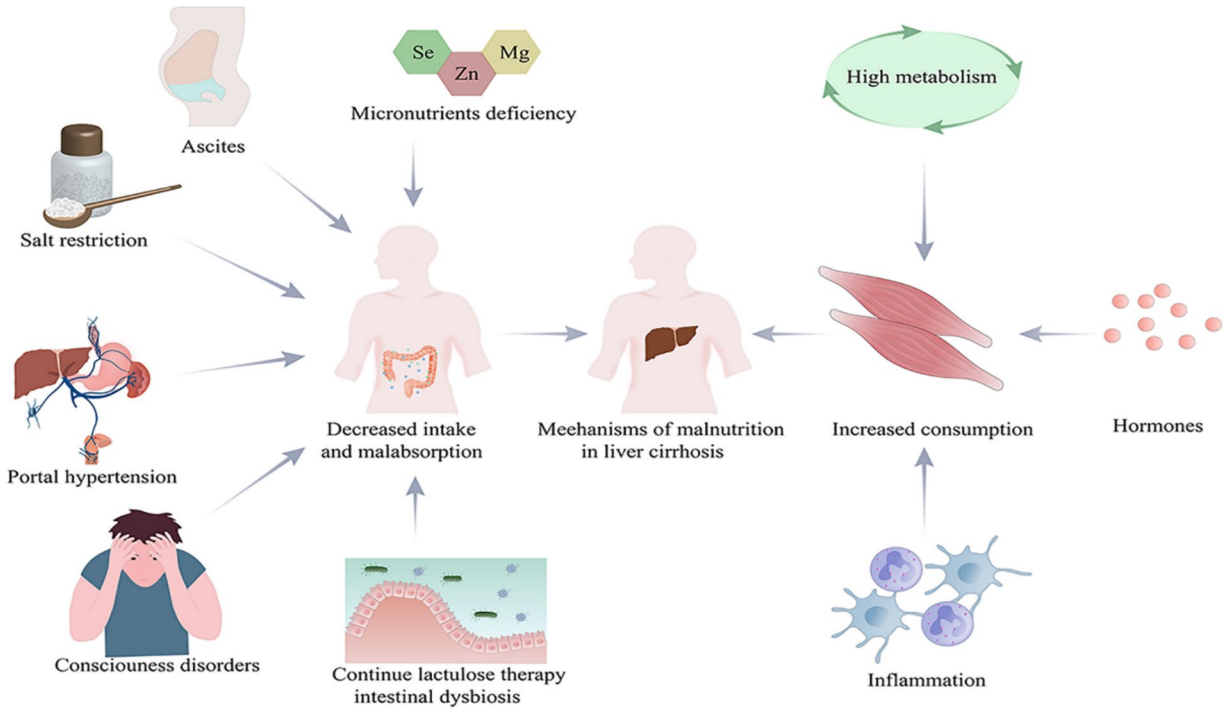

FIGURE 1
Causes of malnutrition in patients with cirrhosis. Created with Adobe Illustrator; Se, selenium; Zn, zinc; Mg, magnesium.

TABLE 1 Summary of studies showing the reported prevalence of malnutrition in patients with cirrhosis.

Authors, Year	Study population	Measure-ment tool	Prevalence of malnutrition (%)	Results summary
Oliveira et al., 2020 (22)	90 patients with cirrhosis	SGA PA	59.1 53.3	PA was a good method to assess prognosis.
Santos et al., 2022 (23)	152 patients with cirrhosis awaiting a liver transplant	SGA GLIM	63.2 0.7–30.9	The majority of GLIM combinations had poor agreement with SGA.
Chaney et al., 2020 (24)	134 patients with cirrhosis	SGA	47.8	Early treatment of malnourished patients with cirrhosis may reduce morbidity and LOS prior to transplantation.
Zambrano et al., 2020 (25)	118 patients with cirrhosis	PG-SGA	35.0	PG-SGA can be considered a good marker of sarcopenia that can be used in clinical practice.
Casas-Deza et al., 2023 (26)	57 clinically significant portal hypertension patients	LDUST	54.4	The LDUST has a solid ability to predict complications in cirrhosis outpatients with CSPH.
Topan et al., 2022 (1)	156 patients with cirrhosis	SGA RFH-NPT SMI MUAC	64.7 49.3 69.2 48.0	The combination between RFH-NPT and MUAC can be used as a valuable tool in daily practice.
Koulentaki et al., 2022 (27)	137 patients with cirrhosis	SGA MNA	60.0 43%	MNA was a strong predictor of mortality.
Javaid et al., 2022 (28)	83 patients with cirrhosis	SGA	88	Providing individualized nutritional intervention prevents further risk of malnutrition and related complications.
Wu et al., 2020 (29)	104 patients with cirrhosis	NRS-2002 RFH-NPT MUST LDUST SGA	51.0 63.2 38.1 70.3 63.0	The RFH-NPT was better able to predict the risk of malnutrition in patients with cirrhosis and had a superior prognostic value.
Boulhosa et al., 2020 (30)	166 patients with cirrhosis	NRS-2002 RFH-NPT GLIM	36.1 52.4 57.3	RFH-NPT has substantial agreement in identifying nutritional risk, good sensitivity and good value for diagnosing malnutrition in patients with advanced chronic liver disease.
Wang et al., 2022 (31)	135 patients with cirrhosis	RFH-NPT	65.2	Immune dysfunction measured by NLR was associated with malnutrition risk estimated by RFH-NPT in cirrhosis.
Yang et al., 2023 (32)	363 patients with decompensated cirrhosis	RFH-NPT GLIM	70.8 33.3	GLIM criteria may serve a specific proxy to diagnose malnutrition along with RFH-NPT screening.

SGA, Subjective Global Assessment; PA, phase angle; GLIM, Global Leadership Initiative on Malnutrition; PG-SGA, Patient-Generated Subjective Global Assessment; LOS, length of hospital stay; LDUST, Liver Disease Undernutrition Screening Tool; NRS-2002, Nutritional Risk Screening 2002; RFH-NPT, Royal Free Hospital-Nutritional Prioritizing Tool; SMI, Skeletal Muscle Index; MUAC, mid-upper arm circumference; CSPH, clinically significant portal hypertension; MNA, mini nutritional assessment.

malnutrition according to phenotypic and etiologic parameters (33). The definition of nutritional risk screening varies slightly from a variety of organizations, with the American Dietetic Association's Nutrition Care Process considering nutritional risk screening to be "those preventive services that use tests or standardized screening procedures to identify patients in need of specific interventions" (34). The American Society for Parenteral and Enteral Nutrition defines nutritional risk screening as "the process of identifying individuals who are malnourished or at risk of malnutrition in order to determine the need for a detailed nutritional assessment" (35). The European Society for Parenteral and Enteral Nutrition (ESPEN) states that nutritional risk screening is "a quick and simple process carried out by a medical practitioner," while ESPEN provides a more global definition (36). When selecting a nutritional risk screening tool, we judged the ability of the tool by sensitivity, specificity, negative predictive value, and positive predictive value; we also needed to consider the feasibility of the screening tool, as overly time-consuming or complex screening tools are likely to result in a lower rate of accurate completion (37).

According to the European Association for the Study of the Liver (EASL) Clinical Practice Guidelines, Child-Pugh score and Body Mass Index (BMI) should be calculated for all cirrhotic patients presenting to the clinic. Patients with cirrhosis often have sodium and water retention, which interferes with this calculation. The BMI was calculated using "dry weight" for patients with peripheral edema and ascites. For mild, moderate, or severe ascites, the current body weight was decreased by 5, 10% or 15%, respectively; for peripheral edema, another 5% reduction was applied (4). Cirrhotic patients with Child-Pugh C or BMI < 18.5 kg/m^2 are regarded as high nutritional risk and should undergo a complete nutritional assessment immediately, including an evaluation for sarcopenia as a complication of malnutrition. For obese patients (BMI > 30 kg/m^2), nutritional and lifestyle interventions targeting obesity are required. For cirrhotic patients with a BMI between 18.5–29.9 kg/m^2, the effect of fluid retention/ascites on BMI needs to be considered, therefore the use of nutritional risk screening that considers the effects of fluid retention is a prerequisite, and patients at intermediate/high nutritional risk are then subjected to a detailed nutritional assessment. Patients at low risk of malnutrition should be re-screened annually. Cirrhotic patients who are screened at high risk of malnutrition should be assessed and monitored every 1–6 months in the outpatient setting, and hospitalized patients should be assessed and documented on admission and at regular intervals throughout their hospitalization (4, 38). Figure 2 shows a synthesized protocol for screening and assessing malnutrition in liver cirrhosis, which was adapted from the EASL clinical practice guidelines.

However, many patients with cirrhosis nowadays tend to have a normal or even to be obese, so one of the major reasons why BMI does not necessarily reflect nutritional status is the loss of muscle mass. Sarcopenic obesity (Sa-O) refers to the coexistence of sarcopenia and obesity as measured by dual-energy X-ray absorptiometry, and describes the interactions between obesity and sarcopenia that are associated with decreased physical activity and reduced energy expenditure (39). One study found that patients with cirrhosis combined with Sa-O had a worse median survival than patients with normal body composition (40). Sarcopenia is a muscle disease that is defined as a reduction in the quantity, strength, and function of skeletal muscle (41). But the American Association for the Study of Liver Diseases (AASLD) practice guidelines present a consensus definition of sarcopenia in patients with cirrhosis as loss of muscle mass (42). As of 2021, the American Association for the Study of Liver Diseases considers sarcopenia and malnutrition to be interrelated, therefore, sarcopenia is considered to be a major component of malnutrition in cirrhotic patients (1, 42). The prevalence of sarcopenia in patients with cirrhosis is approximately 40–41% (43, 44).

A wide variety of nutritional risk screening and assessment tools are available in clinical practice; this review summarizes the nutritional screening and assessment tools commonly used in clinical practice, as well as the commonly used diagnostic methods for sarcopenia.

2 Screening of malnutrition

The widely used nutritional screening tools comprise Nutritional Risk Screening 2002 (NRS-2002), Malnutrition Universal Screening Tool (MUST), Royal Free Hospital-Nutritional Prioritizing Tool (RFH-NPT) and Liver Disease Undernutrition Screening Tool (LDUST).

NRS-2002 is a tool suggested by the ESPEN for screening the indications for nutritional support in hospitalized patients (45). The NRS-2002 intends to identify patients who may benefit from subsequent nutritional care support (46). The NRS-2002 scoring system estimates nutritional impairment, disease severity, and age (47). The three component total scores categorized patients into a no-risk group (< 3 points) and a malnutrition risk group (≥ 3 points). Notably, the NRS-2002 has been demonstrated in several studies to be a reliable predictor of clinical consequences, such as the occurrence of disease complications, prolonged length of hospitalization, and mortality (48). However, it is highlighted that the validity and effectiveness of the NRS-2002 to identify patients at malnutrition risk varies considerably across disease populations and age groups (49). Further research is therefore warranted to elucidate its utility in the context of cirrhosis. This is because cirrhotic patients usually have sodium and water retention, resulting in inaccurate scores.

The MUST was designed by the British Association for Parenteral and Enteral Nutrition to screen for the malnutrition risk of all adult patients (50, 51). It consists of three main components: unintended loss of weight, current BMI, and the existence of any acute illness that may affect nutritional intake for >5 days. Accordingly, the three component total scores categorized patients into a low-risk group (score = 0), medium-risk group (score = 1), or high-risk group (score ≥ 2). In 2015, ESPEN defined malnutrition as a state of altered body composition (reduced fat-free mass) attributable to reduced nutrient intake or absorption, resulting in reduced physical and mental functioning and affected clinical outcomes of disease (52). It has been shown that MUST scores correlate relatively well with the criteria for malnutrition as defined by the ESPEN; However, the sensitivity of MUST is much lower than that of NRS-2002, RFH-NPT, and LDUST (29).

The RFH-NPT was developed for patients with alcoholic cirrhosis; the assessment is divided into three steps. First, patients with acute alcoholic hepatitis or tube feeding are considered to be at high risk immediately. Second, it was evaluated for fluid overload and its impact on food intake and body weight; and third, the nutritional status of patients without fluid overload was assessed on the basis of body mass index, unplanned weight loss, and dietary intake per day. This metric has been verified in a multi-center study in the United Kingdom and

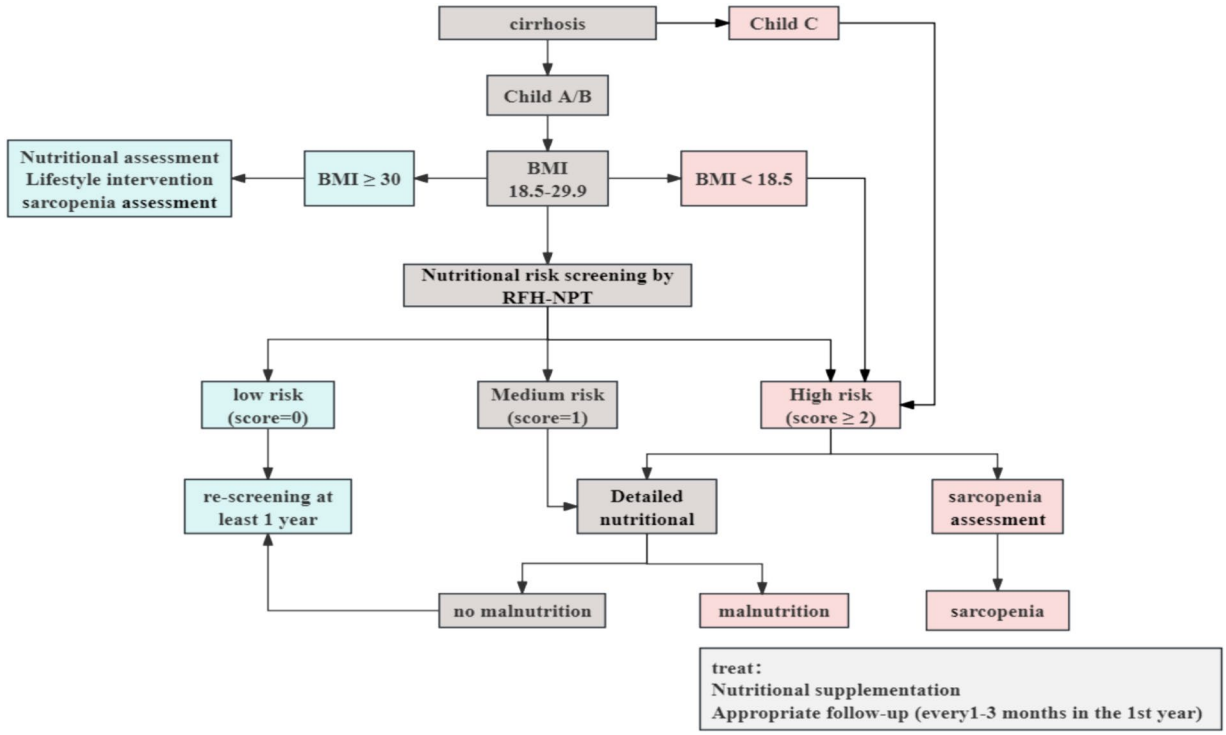

FIGURE 2
Nutritional screening and assessment in patients with cirrhosis. Adapted from the European Association for the Study of the Liver (EASL) clinical practices guidelines (4), Created with ProcessOn; BMI, body mass index; RFH-NPT, Royal Free Hospital-Nutritional Prioritizing Tool.

is considered an independent predictor of disease progression and survival (53, 54). The RFH-NPT takes into account the impact of sodium and water retention on nutritional screening in cirrhotic patients. However, RFH-NPT was originally developed for patients with alcoholic cirrhosis. In China, where viral cirrhosis is predominant, a prospective study assessing the nutritional status of cirrhotic patients attributable to hepatitis viral infections found that the RFH-NPT detected more patients with decompensated cirrhosis who may be at risk for malnutrition when compared with the NRS-2002 (29). One advantage of RFH-NPT is that it considers the impact of sodium and water retention on scoring, which is usually present in patients with cirrhosis at decompensated stage. Previous studies by our team have determined for the first time the relationship between serum micronutrient concentrations and the risk of malnutrition as assessed by the RFH-NPT in patients with cirrhosis (55). In addition, RFH-NPT is an independent predictor for disease progression. This emphasizes the importance of RFH-NPT to screen cirrhotic patients for malnutrition risk and its implication to predict patient prognosis (54). Taken together, RFH-NPT appears to be more valuable for nutritional risk screening in cirrhotic patients (56, 57).

LDUST has been identified for use in patients with cirrhosis. It was developed by the American Society for Parenteral and Enteral Nutrition and the Academy of Nutrition and Dietetics with limited available data in China. The LDUST comprises a total of six questions, suggestive of loss of weight, food intake, muscular loss, edema or fluids, and daily activities where nutritional grading is based on the final score; a score of 5 or more is graded as A and determined to be no risk; 2–5 is graded as B, < 2 is graded as C, and grades B and C are at risk for malnutrition (58). Since the assessment component of LDUST relies in part on the subjective judgment of the patient, this can lead to bias. LDUST has some limitations. Previous data indicate that LDUST has a relatively high positive and a relatively low negative predictive value for cirrhotic patients, thus some investigators consider it a negative screening tool that does not reliably identify patients with malnutrition (29). A study has shown that NRS2002 and RFH-NPT were superior to LDUST at detecting the malnutrition in cirrhosis patients diagnosed according to GLIM criteria (59).

Mini Nutritional Assessment-Short Form (MNA-SF) is mainly used in elderly patients and contains two components. Some studies have shown that MNA-SF has high sensitivity and specificity (60). Although it has been shown that MNA-SF can be used for nutritional risk screening in patients with cirrhosis (61), there are fewer relevant studies, and more studies are needed to validate MNA-SF for nutritional risk screening in cirrhotic patients. Simplified Nutritional Appetite Questionnaire (SNAQ) is mainly used to screen elderly patients for malnutrition due to decreased appetite. In cirrhotic patients, decreased appetite and intake due to ascites, portal hypertension, and salt restriction is one of the major causes of malnutrition in these patients. Therefore, it has been shown that SNAQ can be used to evaluate decreased appetite and predict weight loss in cirrhotic patients (62). Nutrition Risk in Critically ill (NUTRIC) is used to assess the nutritional risk of patients in the intensive care unit (ICU) and is used for early identification of patients most likely to benefit from intensive nutritional support. The score incorporates the variables age, comorbidities, days from admission to transfer to the ICU, Acute Physiology and Chronic Health Evaluation II (APACHE II), Sequential Organ Failure Assessment (SOFA), and

interleukin 6 (IL-6) (60). A study has demonstrated the high prognostic accuracy of NUTRIC in critically ill patients with cirrhosis (63). However, there are few reliable data on nutritional risk assessment in critically ill patients with cirrhosis.

3 Assessment of malnutrition

Commonly used nutritional assessment tools include Subjective Global Assessment (SGA), Patient-Generated Subjective Global Assessment (PG-SGA) and GLIM criteria.

The SGA questionnaire serves as the most widely used nutritional assessment tool in clinical (56), and SGA is one of the tools recommended by ESPEN and EASL for nutritional assessment of patients with liver disease (4, 64). SGA includes the following aspects: Weight loss, unintended reduction in dietary intake, gastrointestinal dysfunction, body functions, diseases and their relationship to nutritional needs, loss of muscle and fat mass, and fluid retention. Good nutritional status is graded A, moderate malnutrition is graded B, and severe malnutrition is graded C (65). One study showed that SGA-rated malnutrition was associated with increased number of unplanned hospital admissions (66). Other studies have also implicated a correlation between malnutrition and mortality in cirrhotic patients assessed by SGA (1, 67). However, more researches are needed to support the use of SGA in cirrhotic patients, because there are some limitations to the use of SGA, such as underestimation of the prevalence of sarcopenia (1, 67, 68).

The PG-SGA is a modified version of the nutritional assessment tool SGA. The PG-SGA consists of two parts, the first is a patient self-assessment including weight change, symptoms, functional capacity, and food intake; and the second is completed by both the professional and the patient including comorbidities, metabolic stress, and physical examination (69). The PG-SGA is a validated nutritional assessment tool recommended by ESPEN (70). Initially PG-SGA was used primarily in patients with tumors, PG-SGA has been validated in a wide range of patient populations and is often characterized as the "gold standard" for malnutrition diagnosis (69). However, cirrhotic patients usually experience sodium and water retention, which may affect correct judgment of weight change. The development of malnutrition is usually a long-term process, and the component within PG-SGA regarding unintentional weight loss covers a time frame of more than 1 month, which may interfere with the assessment. Additionally, weight change, nutritional impact symptoms, food intake, and physical functioning in SGA and PG-SGA may contribute to recall bias. The Royal Free Hospital Global Assessment (RFH-GA) was also derived from the SGA and is primarily used to determine the nutritional status of cirrhotic patients. However, this approach is time consuming and requires trained personnel to obtain consistent results, which limits its broad usage (15, 18).

In 2018, the GLIM reached a consensus on the diagnostic criteria for malnutrition and was proposed as the international consensus standard for diagnosing malnutrition (71). The GLIM consensus recommends that nutritional status be assessed on the basis of phenotypic criteria (low body mass index, unintentional weight loss, and loss of muscle mass) in combination with etiologic criteria (reduced intake or assimilation, and disease or inflammatory conditions); at least one of the phenotypic and one of the etiologic criteria must be present in order to make a diagnosis of malnutrition. A meta-analysis showed that the GLIM criteria have high diagnostic accuracy in differentiating malnutrition and have the potential to become the gold standard for diagnosing malnutrition in clinical practice (72). Malnutrition as defined by GLIM was associated with significantly higher in-hospital mortality and poor clinical outcomes (72, 73). It is worth noting that there is no uniformity in the GLIM diagnostic criteria for loss of muscle mass. Suggested methods of muscle mass assessment include bioelectrical impedance, ultrasound, dual-energy absorptiometry, CT, MRI, or other measurements such as calf muscle circumference or mid-arm muscle circumference (MAMC), as well as handgrip strength (HGS) as an ancillary measure (71). Some studies have also used fat-free mass index as an alternative measurement (74).

In addition to nutritional assessment using the Nutritional Assessment Tool, anthropometric, body composition analysis and laboratory indicators can also be used to assess the nutritional status of patients with cirrhosis. The main anthropometric indicators are Arm Circumference (AC), Triceps Skinfold (TSF) and Mid-Arm Muscle Circumference (MAMC). These indices are easy to perform and are effective methods of nutritional assessment in patients with liver disease at the bedside. AC, TSF and MAMC are more commonly used in nutritional assessment because they can be measured directly and are simple to perform, and are not affected by the patient's sodium and water retention. AC and TSF are sensitive indices of the patient's muscle and fat reserves. The cut-off value of AC for the diagnosis of malnutrition is 26 cm in men and women. The reference value of TSF is 8.3 mm for men and 15.3 mm for women, and the reference value of MAMC (MAMC = AC-3.14*TSF) is 24.8 cm for men and 21.0 cm for women. TSF and MAMC are used to determine malnutrition based on the percentage of the normal reference value, i.e., > 90% of the measured value/normal value is considered normal, and 80–90% is considered mild malnutrition; Between 60–80% is considered moderate malnutrition; < 60% is considered severe malnutrition. Commonly used laboratory indicators are mainly albumin and prealbumin, which reflect the function of hepatic synthesis; albumin is more affected by exogenous supplementation, so albumin is of low value in assessing the nutritional status of cirrhosis. Prealbumin changes are more sensitive than albumin, and prealbumin can still be synthesized during the decompensated phase of cirrhosis, whether prealbumin can be used as a measurement of malnutrition in cirrhosis remains to be studied (75).

The most researched and widely used body composition analysis is Bioelectrical Impedance Analysis (BIA), which is based on the principle of calculating the impedance, i.e., the electrical resistance of body water and the reactance of cell mass, by the conduction of electrical currents through the body, in order to estimate the measurements of body composition (76). The BIA includes nutritional indicators such as phaseangle (PA), skeletal muscle content, body fat mass, body fat percentage, extracellular water ratio, and other nutritional indices. The advantages of BIA are that the results are easy to obtain, are less affected by sodium and water retention, correlate well with liver function scores, and are more accurate in patients with cirrhosis who do not have sodium and water retention; however, BIA should not be performed in patients with a history of pacemaker or defibrillator implantation and amputation (77). PA is the magnitude of the change in AC phase in response to cell membranes in the human body and is based on the reactance and impedance values generated by the body. PA increases when the cell membrane structure

is intact and function increases, and decreases when the cell membrane structure is damaged or selective osmotic function decreases. PA reflects the amount of cells in the body and the integrity of the cell membrane structure and physiological function, and can be used as an indicator of nutritional judgment. In a research study, PA $\leq$ 4.9 was found to be a predictor of death in patients with cirrhosis, and PA is a useful and reliable tool for evaluating the prognosis of cirrhosis (78).

4 Sarcopenia

In patients with cirrhosis, malnutrition is characterized by depletion of skeletal muscle and adipose tissue mass, with the main nutritional consequences of the loss of skeletal muscle mass (79). Sarcopenia can be measured by handgrip strength (HGS) in addition to the Skeletal Muscle Index (SMI) (41). However, many factors affect HGS, patient's age, occupation may affect HGS. Therefore, the diagnosis of sarcopenia using HGS may be biased, so many studies typically use SMI to diagnose sarcopenia. SMI was expressed as the skeletal muscle area at the L3 or T12 level divided by the height squared (cm^2/m^2). There are differences in the cut-off values for differentiating sarcopenia in different countries and regions. Sarcopenia was defined as a SMI $\leq$52.4 cm^2/m^2 in male patients and SMI $\leq$38.5 cm^2/m^2 in female patients (80). However, this data is mainly derived from European and American populations. A study in China indicated 44.77 cm^2/m^2 for male patients and 32.50 cm^2/m^2 for female patients as the cut-off values for L3-SMI (81). Japanese scholars have defined sarcopenia in liver disease patients under 65 years of age as SMI < 42 cm^2/m^2 in men and SMI < 38 cm^2/m^2 in women (82). Although there are a variety of studies pertinent to sarcopenia in patients with liver disease, there is no standardized SMI criteria for diagnosing sarcopenia. Strong correlations have been demonstrated between individual cross-sectional magnetic resonance imaging (MRI) or computed tomography (CT) data and body composition (83). L3-SMI is the ratio of the cross-sectional area of the lumbar major muscle at the third lumbar vertebrae to the square of height on CT or MRI. L3-SMI has been identified for the quantitative assessment of loss of muscle mass and recognized as an objective, quantifiable parameter that can be used to assess nutritional status (81). It is a quantitative, objective, non-invasive, and simple method and is considered to be the gold standard for the assessment of sarcopenia in the context of cirrhosis (4, 84). Although it is costly to perform CT specifically to calculate SMI, patients are exposed to unnecessary radiation. Notably, patients with cirrhosis often undergo CT for other reasons (e.g., to screen for hepatocellular carcinoma), so this approach is clinically feasible; and SMI values calculated from thoracic spine 12 (T12) levels also showed a correlation with mortality (41, 79). Therefore, CT images at the T12 level can be used to calculate SMI for those patients who do not have abdominal CT. A review indicated that ultrasound testing for sarcopenia in patients with advanced liver disease is safe, feasible, and shows good correlation with gold standard measurements of sarcopenia and can be used as a valid tool in daily practice (85). The use of ultrasound for the evaluation of sarcopenia also has a number of limitations such as those related to the type of probe used (linear or convex), the anatomical site of measurement, the patient's posture during the examination, the position of the probe, the pressure exerted by the probe, and the type of parameters obtained (86). The strength, assistance walking, rise from a chair, climb stairs, and falls (SARC-F) questionnaire is a well-established tool for screening for sarcopenia and sarcopenia-related dysfunction. The SARC-F score also has good sensitivity as a bedside screening tool for sarcopenia in cirrhotic patients. Cirrhotic patients with high SARC-F scores and low MAMC require further evaluation for sarcopenia (87).

Sarcopenia is associated with poor prognosis and reduced survival rate before and after liver transplantation (88). Cirrhotic patients with sarcopenia were prone to experience worse prognosis and a significantly higher mortality rate when compared to those without sarcopenia (89, 90). Furthermore, the presence of sarcopenia is closely associated with the development of complications in patients with cirrhosis, such as ascites, esophageal varices, and hepatic encephalopathy (91). According to previous studies, sarcopenia increases the risk of ascites more triple fold, and cirrhotic patients with sarcopenia have a much higher risk of developing hepatocellular carcinoma (90, 92). Frailty, also very common in patients with cirrhosis, is a multidimensional concept that represents the ultimate manifestation of disorders of multiple physiologic systems, resulting in reduced physiologic reserves and increased vulnerability to health stressors (93). Frailty, malnutrition and sarcopenia overlap with each other in patients with cirrhosis, and there is a lack of evidence on whether the assessment of weakness contributes to the assessment of nutritional status in patients with cirrhosis.

5 Summary

Malnutrition is a crucial complication in patients with liver cirrhosis and is associated with the occurrence, development and deterioration of other complications. Nutritional interventions for these patients can curtail the complication and mortality to a certain extent and improve the quality of life among cirrhosis. Therefore, we need to identify malnourished patients promptly and accurately. RFH-NPT is a more feasible tool to screen cirrhotic patients for nutritional risk, and is highly reproducible, and considers the impact of sodium and water retention, thus making it practical to screen cirrhotic patients. Subsequently, GLIM diagnostic criteria may be used to evaluate the nutritional status of patients with nutritional risk via the GLIM diagnostic criteria.

L3-SMI can accurately define sarcopenia in cirrhotic patients and also be used for clinical nutritional status assessment. For malnourished patients identified by dietitians according to conditions of the patients to tailor specific nutrition program, regular follow-up, and timely adjustment of nutrition program.

6 Recommendations

Although studies have confirmed that RFH-NPT is suitable for nutritional risk screening in patients with viral cirrhosis, there is paucity of data available. Hopefully, more data will be available in the future to support this conclusion. Although the GLIM diagnostic criteria for malnutrition have been shown to be relatively accurate in identifying malnourished cirrhotic patients, this may lead to errors of judgment due to the ambiguity of the thresholds for the phenotypic criteria, especially for reduced muscle mass. This is expected to be followed by more studies in the future to propose a harmonized

diagnostic index for the GLIM diagnostic criteria. In addition, although L3-SMI has been proved to be used to evaluate sarcopenia, some studies have proposed that L3-SMI combined with HGS is more accurate in evaluating sarcopenia, and HGS is affected by many factors. More studies will hopefully be conducted in the future to propose more objective assessment criteria.

Author contributions

YH: Writing – original draft, Writing – review & editing. ZW: Writing – original draft, Writing – review & editing. SW: Writing – review & editing. LL: Writing – review & editing. JL: Writing – review & editing. YZ: Writing – review & editing. BC: Writing – review & editing. XS: Writing – review & editing. CS: Writing – review & editing. LW: Writing – review & editing.

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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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Dietary inflammatory index and the risks of non-alcoholic fatty liver disease: a systematic review and meta-analysis

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Background: Previous studies have suggested a correlation between dietary inflammatory potential and non-alcoholic fatty liver disease (NAFLD). Therefore, the study aimed to investigate the association between dietary inflammatory potential, measured by the dietary inflammation index (DII), and NAFLD.

Methods: From establishing the database to June 2023, a systematic search of PubMed, Web of Science, Embase and Cochrane Library were performed to identify relevant observational studies. These studies reported a correlation between DII and NAFLD. The meta-analysis used odds ratio (OR) with 95% confidence intervals (CI) to evaluate the relationship between DII and NAFLD.

Results: Eight studies were included in this meta-analysis after excluding irrelevant records. A summary of the results from the included studies showed that the risk of NAFLD was higher in those exposed to higher DII (OR = 1.26, 95%CI 1.12 to 1.40, $p < 0.001$), with a high degree of heterogeneity ($I^2 = 85.7\%$, $p < 0.001$). When DII was divided into 3 tertiles from low to high for comparison, the results showed that the risk of NAFLD was higher in Tertile 2 (T2) population compared to the Tertile 1 (T1) population (OR = 1.75, 95%CI 1.20 to 2.54, $p < 0.005$). The risk of NAFLD was significantly higher in Tertile 3 (T3) compared to the T1 population (OR = 3.07, 95%CI 1.63 to 5.77, $p = 0.001$).

Conclusion: The results suggest that high DII is associated with an increased risk of NAFLD, and conversely, low DII is associated with a decreased risk of NAFLD.

Systematic Review Registration: The study complies with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and is registered on PROSPERO (CRD42023455013).

KEYWORDS

dietary inflammatory index, non-alcoholic fatty liver disease, inflammation, diet, meta-analysis

Introduction

Over the past 40 years, non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease. The incidence rate of NAFLD ranges from 13.5% (Africa) to 31.8% (Middle East), with a world average incidence rate of 20%. Obesity, type 2 diabetes mellitus (T2DM) and hyperlipidemia are the major risk factors for NAFLD. Notably, the incidence of NAFLD in obese patients has reached a staggering 80%. In terms of the dangers of NAFLD, although less than 10% of patients will develop liver fibrosis or hepatocellular carcinoma within 10–20 years (1–3), the dangers cannot be ignored given the large number of patients. Moreover, based on the increasing obesity rate in the population and the extremely high incidence rate of NAFLD in the obese population, the economic burden of people on NAFLD increases year by year.

Inflammation, as a response of the body to tissue damage, involves massive recruitment of inflammatory factors and inflammation-associated cells. The pro-inflammatory mediators tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and interleukin-1β (IL-1β) contribute to the recruitment and activation of Kupffer cells, whilst the massive activation of Kupffer cells eventually leads to NAFLD (3). Besides, several studies have shown that specific dietary components are associated with inflammation. A recently published meta-analysis suggested that the Mediterranean diet (MD), as a dietary pattern with low inflammatory potential, significantly reduces the risk of NAFLD compared to other dietary patterns (4). Generally speaking, the MD is characterized by a diet rich in plant foods (vegetables, grains, legumes, fruits, and nuts), moderate amounts of fish and poultry, small amounts of red meat, and foods with a low degree of processing (5, 6). Additionally, high-sugar and high-fat dietary patterns have been shown to cause the body to produce high levels of inflammatory factors which can increase the risk of NAFLD or even exacerbate a patient's pre-existing liver disease (7, 8).

The dietary inflammation index (DII), a tool to assess the ability of diet to influence inflammatory processes, was first proposed by researchers at the University of South Carolina in 2009 and has been continuously updated and refined since then (9). The revised version of the DII currently in common use is based on literature on inflammation and diet published up to 2010. The DII categorizes an individual's inflammatory potential from maximally anti-inflammatory to maximally pro-inflammatory based on the potential of the diet. A higher DII indicates higher pro-inflammatory potential and a lower DII indicates higher anti-inflammatory potential. The DII consists of 45 food parameters, including 36 anti-inflammatory foods (fiber, alcohol, monounsaturated fatty acids, polyunsaturated fatty acids, omega 3, omega 6, niacin, thiamin, riboflavin, vitamin B6, zinc, magnesium, selenium, vitamin A, vitamin C, vitamin D, vitamin E, folic acid, beta carotene, anthocyanidins, flavan3ols, flavonols, flavanones, flavones, isoflavones, garlic, ginger, onions, thyme, oregano, saffron, turmeric, rosemary, eugenol, caffeine, and tea) and 9 pro-inflammatory foods (energy, carbohydrates, proteins, total fat, trans fat, cholesterol, vitamin B12, saturated fatty acids and iron) (9, 10). This meta-analysis is based on the results of published studies that aims at investigating the association between the DII and the risk of NAFLD.

Methods

The study complies with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (11) and is registered on PROSPERO (CRD42023455013).

Search strategy and study selection

From the inception of the database to June 2023, studies on the relationship between the DII exposed to NAFLD were searched in the electronic database including PubMed, Web of Science, Embase and Cochrane Library. The following was related terms for search use: (non-alcoholic fatty liver disease OR non alcoholic fatty liver disease OR NAFLD OR nonalcoholic fatty liver disease OR nonalcoholic fatty liver OR nonalcoholic fatty livers OR nonalcoholic steatohepatitis OR nonalcoholic steatohepatitides OR metabolic dysfunction-associated steatotic liver disease OR MASLD OR metabolic dysfunction-associated steatohepatitis OR MASH) AND (dietary inflammatory index OR inflammatory diet OR anti-inflammatory diet OR dietary score). The reference lists of relevant articles were searched by 2 researchers to avoid omissions.

Articles were independently assessed by 2 researchers as to whether they met the inclusion and exclusion criteria. Studies will be considered for inclusion if they meet each of the following inclusion criteria: (i) patients in the exposure group have a higher DII and patients in the non-exposed group experienced a lower DII; (ii) a correlation between DII and NAFLD is reported; (iii) the study provided an outcome of DII and NAFLD; and (iv) the study is an observational study (cohort study, case–control study and cross-sectional study) or a randomized controlled trial (RCT).

At the same time, articles meeting one of the following exclusion criteria would be excluded: (i) the full text cannot be obtained; (ii) articles were not written in English; (iii) the data was not available; and (iv) for the same cohort, the most comprehensive or latest article would be included.

Data extraction and quality assessment

The purpose of this study was to explore the risks of different DII-causing NAFLD in the population. NAFLD is an overarching term defined as a liver disease in which ≥5% of hepatocytes display macrovesicular steatosis in individuals who drink little or no alcohol (defined as <20 g/day for women and < 30 g/day for men) in the absence of readily identified alternative cause of steatosis (eg., medications, starvation, and monogenic disorders). The spectrum of disease includes nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH) (1, 12). According to the Delphi consensus statement, from 2023, the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), and the Latin American Association for the study of the liver (ALEH) have recommended the use of metabolic dysfunction-associated steatotic liver disease (MASLD) as an alternative to NAFLD (13, 14). However, in our research, we followed the previous convention of using the term NAFLD.

DII was defined as an indicator to assess the effects of diet on the inflammatory response of the human body. The DII proposed

by Shivappa et al. in 2014 included 45 different dietary factors (food parameters consisting of nutrients and whole foods) and their pro-inflammatory or anti-inflammatory effects (9). For the range of DII obtained from the included studies in different populations, we divided the DII into three different quartiles in ascending order. The populations were divided into three quartiles according to different DII, namely Tertile 1 (T1), Tertile 2 (T2), and Tertile 3 (T3).

The research data was extracted independently by 2 investigators. The following information was extracted using a pre-determined data collection form: (i) authors; (ii) year of publication; (iii) country; (iv) research cohort; (v) study duration; (vi); follow-up period (vii) participant profiles; (viii) measurement tool; (ix) adjusting factors; and (x) relevant data.

The quality of cohort and case–control studies was assessed based on the Newcastle-Ottawa Quality Assessment Scale, which consists of 3 quality parameters: selection (4 points), comparability (2 points) and results (3 points), with a score of 6 and above indicating high quality (15). Meanwhile, the quality of the cross-sectional study was assessed according to the Agency for Healthcare Research and Quality (AHRQ) cross-sectional research evaluation standard (16). There are 11 criteria, and a score of 8 points and above was considered high quality. Finally, the quality of included RCTs was assessed using the Cochrane collaboration recommendations (17).

Statistical analysis

The study used odds ratio (OR) with 95% confidence intervals (CI) to evaluate the relationship between DII and NAFLD. The chi-square test was used to determine heterogeneity, with I^2 values ≤30% indicating low heterogeneity; I^2 values between 30 and 60% indicating medium heterogeneity; the I^2 values ≥60% indicating high heterogeneity (18). Due to the potential heterogeneity among the inclusion studies, the study used a randomized model to improve the credibility of the results. Meanwhile, publication bias was assessed by using the Begg's test (19). In addition, the stability of the results was evaluated by sensitivity analyses. The two-tailed p test was used to define a p-value of less than 0.05 as statistically significant. All analyses were performed using STATA version 12.0.

Results

Study selection and data extraction

The entire process of finding and screening for the studies is presented in Figure 1. According to the title, abstract and full manuscript, a hierarchical approach was applied to assess the relevance of the research. First of all, 531 relevant records were identified through the pre-determined search strategy. A total of 398 studies were identified after eliminating duplicates. Then, 380 were excluded after a review of titles and abstracts. Next, of the 18 articles selected, 10 were not included for the following reasons: (i) not comply with the protocol; (ii) no interesting outcomes; (iii) data unavailable; and (iv) update post. Finally, 8 articles that conformed to the inclusion criteria were included in this meta-analysis.

Study characteristics

A total of 2 million individuals were included in the 8 studies selected for meta-analysis. Table 1 shows the basic information extracted from all 8 included studies (20–27). All studies were observational studies and published between 2018 and 2023. 2 of them were case–control studies (20, 25), the other 2 were cohort studies (21, 24) and 4 were cross-sectional studies (22, 23, 26, 27). The duration of the experiment ranged from 6 months to 144 months. Exposure tools comprised the food frequency questionnaire (FFQ) and Oxford WebQ. The DII consists of 45 dietary parameters. Among the eight included studies, two studies used 31 dietary parameters, two studies used 26 dietary parameters, one study used 18 dietary parameters, and the remaining study was not mentioned. In addition, there were 7 to 8 pro-inflammatory dietary parameters (such as Vitamin B12, iron and protein) and 11 to 23 anti-inflammatory dietary parameters (such as Vitamin A, Vitamin C and Vitamin D). Most studies were adjusted for covariates that may have a significant impact on NAFLD, including, but not limited to: age, sex, body mass index (BMI), education, smoking, alcohol consumption, physical activity, obesity, and ethnicity. The comprehensive set of covariates for which adjustments were made are described in Table 1.

Supplementary Table S1 shows the source of population, age at recruitment, median age and grouping subgroups of the different studies. Supplementary Table S2 (cohort study), Supplementary Table S3 (case–control study), and Supplementary Table S4 (cross-sectional study) show the evaluation quality of the 8 observational studies. Of these, 2 cohort studies scored 8 out of 10 for one case–control study scored 8 and the other scored 9 out of 10; and of the cross-sectional studies, 2 studies were 7 out of 10, one study scored 9 out of 10, and the other scored 10 out of 10. Collective, the quality of the included studies is high in general.

Quantitative analysis

In general, the results of pooling the 8 datasets indicated that the participants exposed to higher DII had a higher risk of developing NAFLD (OR = 1.26, 95%CI 1.12 to 1.40, $p < 0.001$) (Figure 2). At the same time, the heterogeneity among the included studies was assessed by the chi-square test, and the results showed a high degree of heterogeneity ($I^2 = 85.7\%$, $p < 0.001$) (Figure 2). Potential heterogeneity was assessed by subgroup analyses. The DII was divided into 3 different quartiles in ascending order, namely T1, T2 and T3. Compared with T1, the risk of NAFLD in the T2 population was higher (OR = 1.75, 95%CI 1.20 to 2.54, $p < 0.005$). The risk of NAFLD was significantly higher in T3 participants compared to T1 (OR = 3.07, 95%CI 1.63 to 5.77, $p = 0.001$) (Figure 3).

In addition, when populations were classified by region (Table 2), there was no statistically significant difference in the risk of NAFLD among those affected by different DII in Europe, Iran and the US (Europe: OR = 2.37, 95%CI 0.47 to 11.94; Iran: OR = 2.25, 95%CI 0.92 to 5.49; US: OR = 1.19, 95%CI 0.94 to 1.49). By gender (Table 2), there was no obvious difference in the risk of NAFLD with different DII in the male population (OR = 2.64, 95%CI 0.66 to 10.56). However, for the female population, those exposed to higher DII had a higher risk

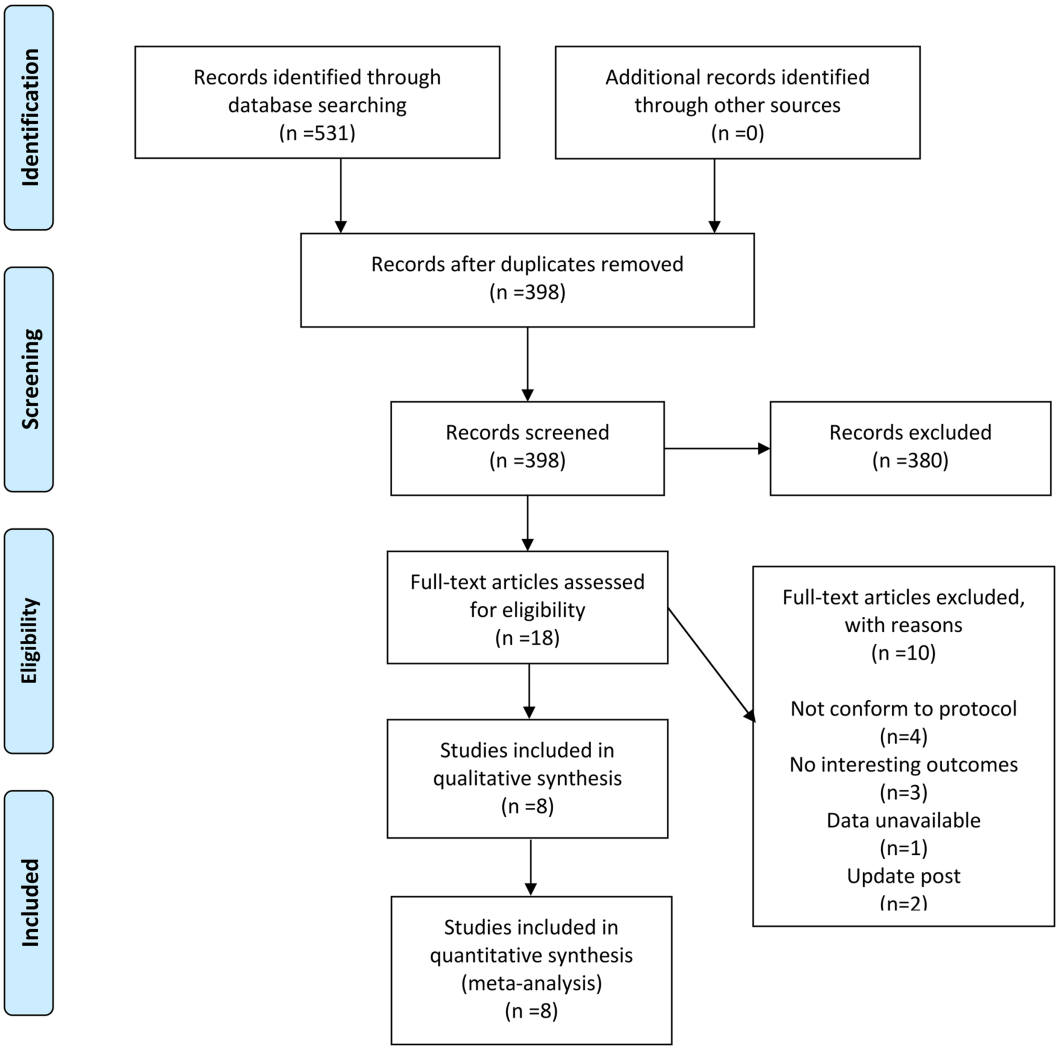

FIGURE 1
Flow diagram describing inclusion and exclusion criteria.

of NAFLD (OR = 1.26, 95%CI 1.08 to 1.47). Secondly, among those with hypertension (Table 2), there was no statistical difference between participants with different DII (OR = 0.85, 95%CI 0.31 to 2.36). Meanwhile, in the smoking population (Table 2), those with high DII exposure had a higher risk of NAFLD (OR = 1.20, 95%CI 1.05 to 1.37). Finally, in the data we have obtained, when DII was the same, the male had a higher risk of NAFLD than females (OR = 1.11, 95%CI 1.00 to 1.24, $p = 0.053$).

Sensitivity analysis and publication bias

To evaluate the effect of publication bias, the relationship between higher and lower DII in NAFLD was evaluated using Begg's test (Supplementary Figure S1). Overall, there was no significant publication bias among studies ($p > 0.1$). Finally, sensitivity analyses were used to evaluate the stability of the results, where each study was excluded individually and was pooled for stability.

Discussion

To our knowledge, there has been no previous meta-analysis of the relationship between DII and NAFLD been published so far. Our meta-analysis found a possible correlation between the risk of DII and NAFLD based on relevant cohort studies. The results suggested that people exposed to higher levels of DII may have a higher risk of NAFLD (OR = 1.26, 95%CI 1.12 to 1.40, $p < 0.001$). Correspondingly, the lower the exposure to DII, the lower the risk of NAFLD in the population.

Previous studies have shown that diet could affect NAFLD indirectly or directly by causing changes in inflammation in the body in various ways.

Pro-inflammatory diets, such as high-fat diets, can lead to the accumulation of ectopic lipids that cause the recruitment of macrophages (M1) and pro-inflammatory cytokines. It also activates inflammatory pathways in the brain by activating M1 macrophages and upregulating circulating pro-inflammatory cytokines (28). In conclusion, high-fat diets can cause oxidative stress in the body by

TABLE 1 Characteristics of included observational studies in the meta-analysis.

Author, year	Country	No. of participants	Duration of experiment (year)	Exposure assessment	Adjust parameters	Study design
Moradi, F. 2022	Iran	240	Sept. 2019–Feb. 2020	FFQ	Age, sex, education, physical activity, BMI and SES, energy intake, taking medication, and supplements	Case–control study
Petermann-Rocha, F. 2023	UK	171,544	2006–2010	Oxford WebQ	Age, sex, poverty, ethnicity, obesity, blood glucose, hypertension, HDL, triglyceride, inflammatory diseases, smoking and physical activity	Cohort study
Vahid, F. 2018	Iran	999	Jan. 2015–Dec. 2015	FFQ	Age, BMI, LDL, TG, AST/ALT, education, smoking, alcohol consumption, and blood glucose	Case–control study
Zhang, Z. 2023	US	10,052	2005–2016	MEC subsample weight (WTMEC2YR for 2005–2016)	Ethnicity, sex, poverty, marital, education, smoking, BMI, obesity, AST, ALT, and GGT	Cross-sectional study
Soltanieh, S. 2023	Iran	200	/	FFQ	Age, sex, blood glucose, smoking, physical activity, energy intake, BMI, WHtR, triglyceride, cholesterol, and HOMA	Cross-sectional study
Ramírez-Vélez, R. 2022	US	4,189	2017–2018	A review of the literature published up to 2010 linking diet and inflammatory markers	Sex, age, ethnicity, citizenship status, energy intake, alcohol consumption, smoking, physical activity, hypertension, HDL, obesity, and blood glucose	Cross-sectional study
Tyrovolas, S. 2019	Greece	2,992	2001–2012	FFQ	Age, sex, education, hypertension, blood glucose, cholesterol, obesity, and smoking	Cohort study
Valibeygi, A. 2023	Iran	9,792	Nov. 2014–Jun. 2019	FFQ	/	Cross-sectional study

No., number; FFQ, food frequency questionnaire; MEC, Mobile Exam Center; NHANES, National Health and Nutrition Examination Survey; BMI, body mass index; SES, socioeconomic status; HDL, high density lipoprotein cholesterol; LDL, low-density lipoprotein; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transferase; WHtR, waist to height ratio; HOMA, homeostasis model assessment.

activating a large number of signaling pathways. The level of inflammatory response in the body can have a direct impact on liver health. Pro-inflammatory diets may genetically interfere with hepatic β-oxidation, resulting in a massive upregulation of pro-inflammatory molecules (e.g., IL-1β, IL-17, and IL-18) and oxygen-responsive substances, which in turn increases the production of endogenous lipids and increases the risk of NAFLD, as well as exacerbating the damage related to chronic disease (1, 29, 30).

Anti-inflammatory diets, such as those rich in vegetables and fruits, are thought to be effective in preventing and ameliorating NAFLD (31). It contains flavonoids, α-tocopherol (vitamin E) and vitamin C which act as antioxidants to reduce oxidative stress in the body (32). The results of a randomized double-blind

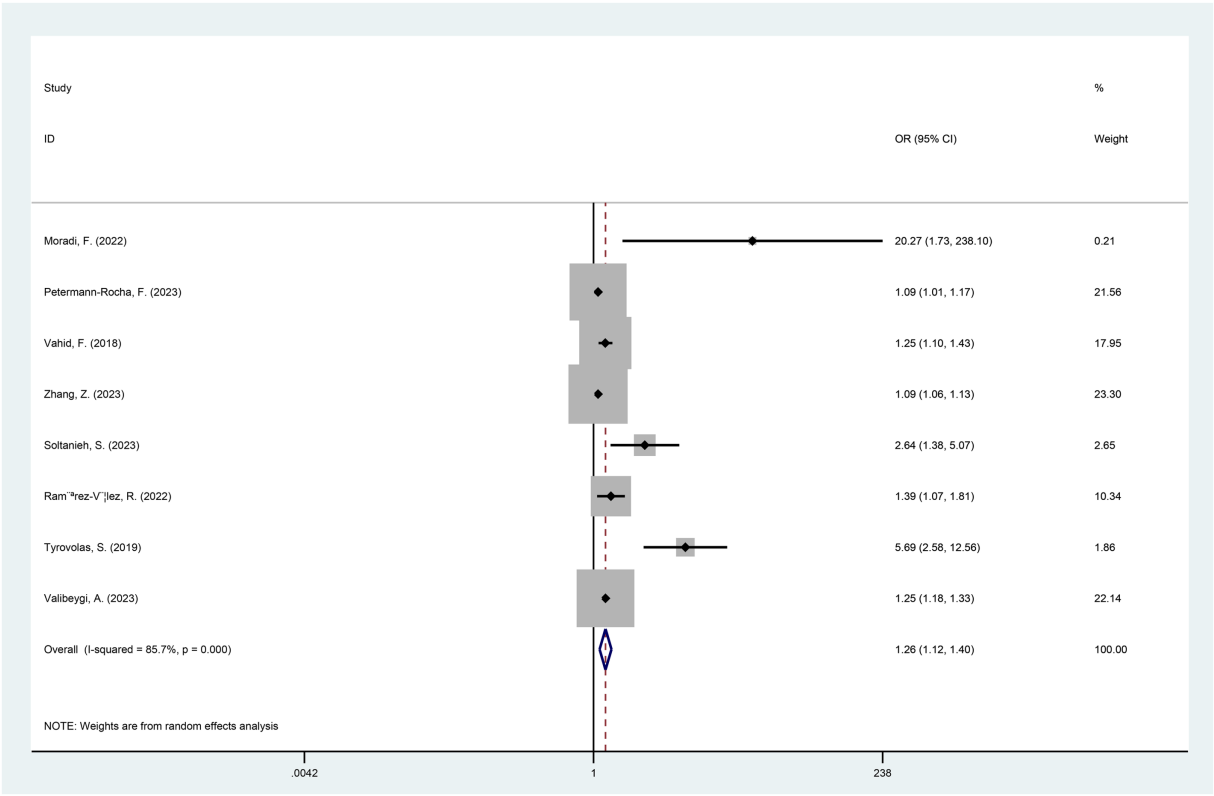

FIGURE 2
Forest plot of NAFLD risk induced by exposure to higher versus lower DII ($p < 0.001$).

placebo-controlled trial showed that α-tocopherol (vitamin E) and vitamin C are excellent and potent antioxidants with anti-inflammatory properties (33). In addition, flavonoids have been reported extensively to be potent antioxidants in the prevention of NAFLD (34, 35). Their protective nature is attributed to their ability to accelerate the oxidation of fatty acids in the liver, enhance the antioxidant capacity of the cells and inhibit the production of nuclear factor-κ B, which in turn attenuates the release of inflammatory cytokines (36). As a result, it can reduce the risk of NAFLD through the mechanisms described above.

In addition, diet plays a crucial role in the regulation of inflammation levels in the body by influencing the maintenance of a balance of gut flora in the gut (37). Plant-based dietary patterns that have a lower potential to stimulate inflammation have been associated with the production of short-chain fatty acids (SCFA), a byproduct of gut flora, which have anti-inflammatory capacity. SCFA can affect energy metabolism and immunity by mediated via binding to G-protein coupled receptors expressed in the immune system and endocrine cells of the gut. In this way, SCFA can reduce the level of inflammation in the body (1, 38). Conversely pathogen-associated molecular proteins (PAMPs), products of intestinal microbes, also can increase inflammation levels in the liver. Recent studies in mice have shown that PAMPs, as a danger-associated molecular pattern (DAMPs), activate the hepatocyte inflammasome (a multiprotein cytoplasmic complex) in hepatocytes. Activation of inflammasomes in hepatocytes may be important for hepatocytes to cause initial stress, which subsequently leads to hepatocyte death and fibrosis (39–41).

Diets can also modulate inflammation levels through metabolic syndrome (MetS), including obesity and diabetes (42), which can lead to further development of NAFLD. A cross-sectional study of middle-aged and older adults found that higher DII was associated with a higher risk of MetS (43). Therefore, we guess that a high DII dietary pattern may indirectly increase the risk of NAFLD by increasing the risk of MetS.

In the case of obesity, for example, the state of obesity is often considered to be a causative factor of inflammation in the body (44), but there is growing evidence that inflammation can also contribute to the development of obesity (45). A longitudinal study of the MD showed that the higher the DII score in the exposed population, the progressively higher the incidence rate of overweight and obesity (46). Thus, pro-inflammatory diets trigger obesity by raising inflammation levels in the body. Obesity is an important risk factor for NAFLD. Hepatocytes in obese patients are exposed to high lipid and carbohydrate levels, leading to hepatic dysfunction and liver injury through dual processes of lipotoxicity and glucotoxicity (7, 30, 47).

Other MetS (including hypertension, hyperuric acid, and hyperlipidemia) have a bi-directional relationship with NAFLD, and these disorders can not only increase the risk of NAFLD but also increase some features and complications of these disorders (29, 48).

The genetic and environmental factors are known to influence the onset and development of NAFLD in individuals (49). A meta-analysis showed that the risk of NAFLD was lower in the black population and higher in the Hispanic population (50). In this regard, we analyzed subgroups of the population from different regions. However, our results were not statistically significant (Europe: OR = 2.37, 95%CI 0.47 to 11.94; Iran: OR = 2.25, 95%CI 0.92 to 5.49; US: OR = 1.19, 95%CI 0.94 to 1.49). This may be due to insufficient data on the

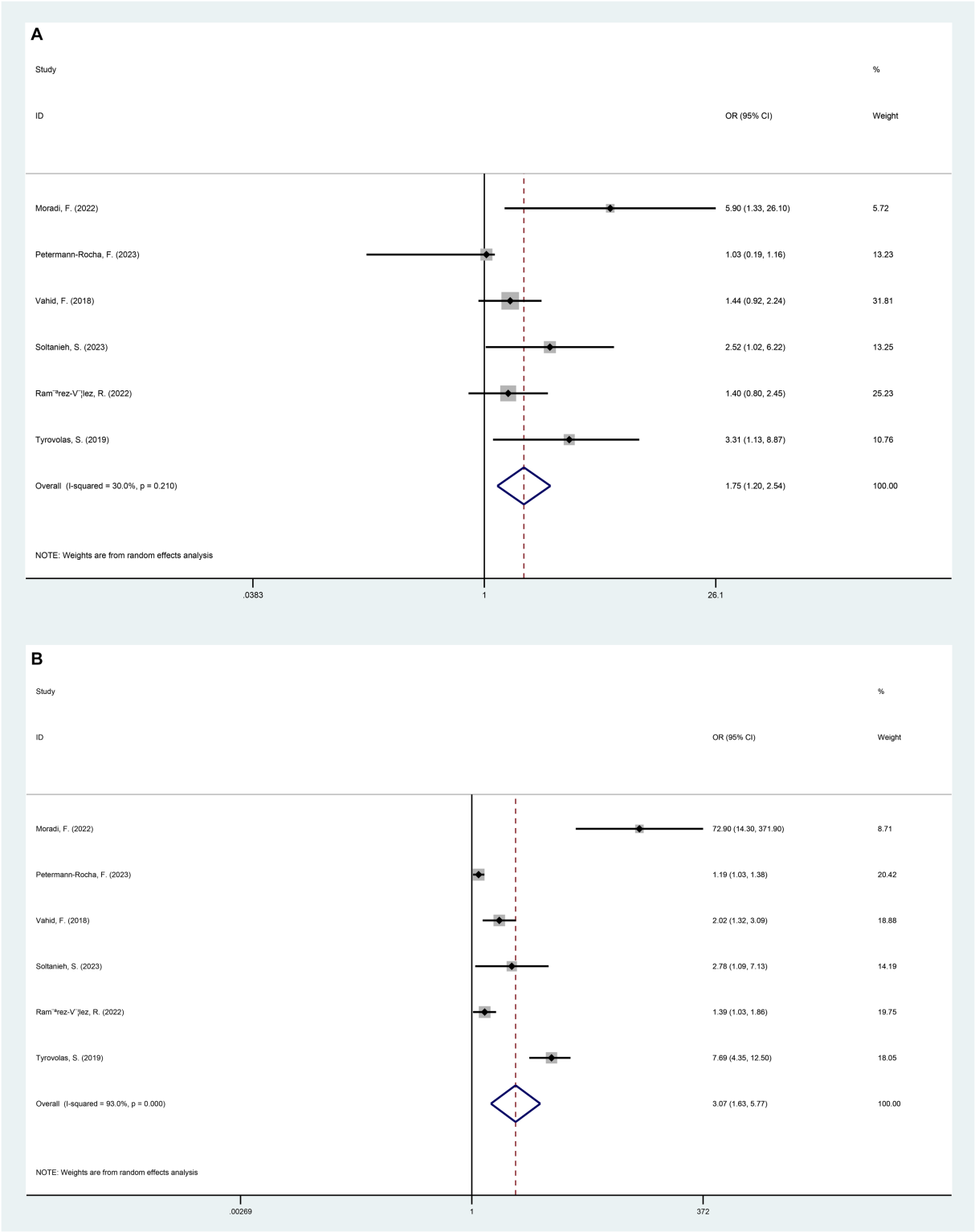

FIGURE 3
Forest plot of NAFLD risk induced by exposure to T1 versus T2/T3 by categorizing DII from low to high into different tertiles of T1, T2, and T3. **(A)** T1 versus T2 ($p = 0.004$); **(B)** T1 versus T3 ($p = 0.001$).

subgroups in the included studies and the possibility that the same area contains multiple races.

Data from previous studies have shown that men have a significantly higher risk of developing NAFLD than women (51), and our results of subgroup analysis are consistent with these results (OR = 1.11, 95%CI 1.00 to 1.24). It suggests that gender is also an influential factor in NAFLD development. Estrogen may be a major contributor to the above phenomenon. A prospective study in Italy

TABLE 2 Subgroup analysis of the association between dietary inflammatory index and nonalcoholic fatty liver disease.

Subgroup	No. of studies	OR	95%CI	*p*	I^2 (%)
Regional					
Europe	2	2.37	[0.47, 11.94]	0.295	94.0
Iran	4	2.25	[0.92, 5.49]	0.075	79.3
US	2	1.19	[0.95, 1.49]	0.140	69.1
Sex					
Male	2	2.64	[0.66, 10.56]	0.169	83.3
Female	2	1.26	[1.08, 1.47]	0.003	0.0
Other influencing factors					
Hypertension	2	0.85	[0.31, 2.36]	0.758	93.1
Smoking	2	1.20	[1.05, 1.37]	<0.001	34.3
Male versus Female	2	1.11	[1.00, 1.24]	0.053	0.0

No., number; OR, odds ratio; 95%CI, 95% confidence intervals.

included 5,408 women with hysterectomies who were randomly assigned to tamoxifen (an estrogen inhibitor) or placebo. The results showed that tamoxifen was associated with an increased risk of NAFLD, especially in obese women (52). Additionally, we found that a pro-inflammatory diet increased the risk of NAFLD in the female population (OR = 1.26, 95%CI 1.08 to 1.47). This may be related to the fact that pro-inflammatory diets are associated with the modulation of inflammation levels in the body. A cross-sectional study of adult women in the United States found that a pro-inflammatory diet led to a decrease in sex hormone binding globulin (SHBG) (53). It has also been suggested that pro-inflammatory diets may be associated with decreased testosterone and estradiol levels in adolescent males (54). However, our findings were not statistically significant in the male population (OR = 2.64, 95%CI 0.66 to 10.56), which may be related to the paucity of relevant studies. More well-designed clinical trials are still needed to validate this aspect.

Our subgroup results suggest that pro-inflammatory diets increase the risk of NAFLD in chronic smokers (OR = 1.20, 95%CI 1.05 to 1.37). During smoking, nicotine accumulates in large quantities in gastric juices and saliva, producing pathogenic effects (55). On the one hand, cytotoxic chemicals produced by smoking may accelerate the growth and proliferation of fibroblasts, leading to the formation of scar tissue (56). On the other hand, smoking may also induce a massive recruitment of pro-inflammatory factors (IL-1, IL-6, and TNF-α) involved in hepatocyte injury (57). In addition, nicotine strongly stimulates hepatic injury and hepatic fibrosis by activating the nicotinic acetylcholine receptor (nAChR) in the liver (58). Smoking can increase the risk of NAFLD through multiple pathways along with pro-inflammatory diets.

Because of the high degree of heterogeneity among the included studies, for this, we performed subgroup analyses. Based on the results of the subgroups, we speculate that the different percentages of smoking, hypertension, and female characteristics in the included study populations may contribute to the potential heterogeneity. In addition, the different ethnicity and dietary habits of the sources of the included study population are also an important cause of heterogeneity. Finally, the differences in study design could also be an objective reason for the high degree of heterogeneity.

This study systematically explored the relationship between DII and NAFLD with some subgroup analysis. Meanwhile, our meta-analysis proves that the inflammatory potential of the diet might positively correlate with the risk of NAFLD. Additionally, the results of this meta-analysis can be used in clinical practice and public health interventions that aim to reduce the risk of NAFLD. Finally, our review may provide some reference for future clinical and basic trials on the relevant topics. There are also some limitations in this study. Due to the small sample size, more subgroup analysis could not be performed or the results were inaccurate. At the same time, the small number of studies included and the presence of a high degree of heterogeneity in this meta-analysis may affect the credibility of the results. Well-designed, larger clinical trials are required to prove this point.

Conclusion

In conclusion, the results of our meta-analysis suggest an association between DII and the risk of NAFLD. The more pro-inflammatory the diet, the higher the risk of NAFLD; conversely, the more anti-inflammatory the diet, the lower the risk of NAFLD. Therefore, promoting foods low in DII could help reduce the risk of NAFLD in the population. In addition, more and further well-designed, large, multicenter clinical studies are needed to confirm the correlation between DII and NAFLD.

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

XZ: Conceptualization, Data curation, Formal analysis, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. JR: Data curation, Formal analysis, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. YH: Data curation, Formal analysis, Resources, Software, Writing – original draft, Writing – review & editing. AX: Resources, Software, Supervision, Writing – original draft, Writing – review & editing. YF: Data curation, Supervision, Writing – original draft, Writing – review & editing. QZ: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. LG: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. XL: Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing.

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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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1388557/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Begg's funnel plot with pseudo 95% confidence limits (p=0.108).

SUPPLEMENTARY FIGURE S2
Sensitivity analysis.

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Glossary

DII	dietary inflammation index
NAFLD	non-alcoholic fatty liver disease
TNF-α	tumor necrosis factor-α
IL-6	interleukin-6
IL-1β	interleukin-1β
MD	Mediterranean diet
PRISMA	the Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RCT	randomized controlled trials
AHRQ	the Agency for Healthcare Research and Quality
OR	odds ratio
CI	confidence interval
FFQ	food frequency questionnaire
BMI	body mass index
NAFL	nonalcoholic fatty liver
NASH	nonalcoholic steatohepatitis
AASLD	the American Association for the Study of Liver Diseases
EASL	the European Association for the Study of the Liver
ALEH	the Latin American Association for the study of the liver
MASLD	metabolic dysfunction-associated steatotic liver disease
T1	Tertile 1
T2	Tertile 2
T3	Tertile 3
SCFA	short-chain fatty acids
PAMPs	pathogen-associated molecular proteins
DAMPs	danger-associated molecular pattern
MetS	metabolic syndrome
SHBG	sex hormone binding globulin
nAChR	nicotinic acetylcholine receptor

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Bidirectional relationship between *Helicobacter pylori* infection and nonalcoholic fatty liver disease: insights from a comprehensive meta-analysis

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Background: *Helicobacter pylori* (*H. pylori*) infection and nonalcoholic fatty liver disease (NAFLD) represent significant concerns in global health. However, the precise relationship between *H. pylori* and NAFLD remains a subject of ongoing debate. This study endeavors to elucidate the association between *H. pylori* infection and the susceptibility to NAFLD. Furthermore, we aim to investigate the interplay among *H. pylori* infection, NAFLD, and metabolic syndrome (MetS).

Methods: We conducted an extensive search of the PubMed, EMBASE, and Web of Science databases spanning from inception to January 2024. Our examination focused on rigorous studies investigating the correlation between *H. pylori* infection and NAFLD. Utilizing a random-effects model, we computed the pooled odds ratio (OR) and corresponding 95% confidence interval (CI). Additionally, we assessed statistical heterogeneity, performed sensitivity analyses, and scrutinized the potential for publication bias.

Results: Thirty-four studies involving 175,575 individuals were included in our meta-analysis. Among these, 14 studies (involving 94,950 patients) demonstrated a higher incidence of NAFLD in *H. pylori* infection-positive individuals compared to *H. pylori* infection-negative individuals [RR = 1.17, 95% CI (1.10, 1.24), $Z = 4.897$, $P < 0.001$]. Seventeen studies (involving 74,928 patients) indicated a higher positive rate of *H. pylori* infection in patients with NAFLD compared to those without NAFLD [RR = 1.13, 95% CI (1.02, 1.24), $Z = 2.395$, $P = 0.017$]. Sensitivity analyses confirmed the robustness of these findings, and funnel plot analysis revealed no significant publication bias. Furthermore, we observed associations between *H. pylori* infection or NAFLD and various metabolic factors, including body mass index (BMI), blood pressure, lipids, liver function, and kidney function.

Conclusion: Our meta-analysis presents evidence supporting a reciprocal relationship between *H. pylori* infection and the susceptibility to NAFLD.

Nevertheless, additional investigations are warranted to bolster this correlation and unravel the underlying mechanisms involved.

KEYWORDS

Helicobacter pylori, nonalcoholic fatty liver disease, meta-analysis, metabolic syndrome, incidence, risk factors

1 Introduction

Helicobacter pylori (*H. pylori*) is a bacterium with a Gram-negative structure known for its colonization of the gastric epithelium. This bacterium has been linked to the development of peptic ulcers, gastric cancer, and gastric mucosa-associated lymphoid-tissue (MALT) lymphoma (1, 2). *H. pylori* infection is known to be one of the most common gastrointestinal infections in humans (1, 2). A survey conducted on family units in China revealed a prevalence rate of approximately 40.66% for *H. pylori*, with rates of 43.45% in adults and 20.55% in children and adolescents (3). Despite a global decline in the prevalence of *H. pylori* infection from 58.2% in the period of 1980–1990 to 43.1% in the period of 2011–2022 (4), it continues to pose a significant clinical and public health burden. Furthermore, beyond its correlation with gastric disorders, *H. pylori* infection has been associated with a range of extragastric conditions, including stroke, Alzheimer's disease, and nonalcoholic fatty liver disease (NAFLD) (5).

NAFLD is a significant public health concern affecting approximately 25% of the global population (6). According to a study published in 2022, the global prevalence of NAFLD was found to be 32.4% (7). Additionally, there is a projection indicating that the prevalence of NAFLD is expected to rise to 56% over the next decade (8). NAFLD includes both simple steatosis (SS) and non-alcoholic steatohepatitis (NASH), with the latter having the potential to advance to cirrhosis and hepatocellular carcinoma (HCC). In addition, NAFLD is closely associated with various extrahepatic conditions, including cardiovascular disease, obesity, diabetes, and hyperuricemia (9). Consequently, tackling NAFLD is paramount. Despite continuous research endeavors, the exact causes and mechanisms underlying NAFLD remain incompletely understood.

Insulin resistance and metabolic syndromes (MetS) such as hypertension, obesity, dyslipidemia, and type 2 diabetes mellitus are well-established risk factors for NAFLD (10). The correlation between *H. pylori* infection and the susceptibility to NAFLD has been explored in numerous studies; however, the results have been inconclusive. The findings of Polyzos et al. (11) suggest that *H. pylori* infection is an independent risk factor for NAFLD progression. In contrast, a cross-sectional study found that *H. pylori* infection was not listed as a risk factor for NAFLD (12). Furthermore, another observational study found no association between *H. pylori* infection and NAFLD diagnosis in a central European cohort (13). To our knowledge, the number of studies evaluating the impact of *H. pylori* eradication on NAFLD is limited, and the results of these studies are inconsistent. Some studies have found that *H. pylori* eradication may play a role in reducing the risk of NAFLD (14). Another study evaluated 13 patients with biopsy-proven NAFLD and showed that eradication of *H. pylori* had no significant long-term effect on hepatic steatosis (15).

Considering the escalating worldwide prevalence of NAFLD and its significant clinical and economic ramifications, it becomes crucial to elucidate the possible detrimental impacts of *H. pylori* infection on the risk of NAFLD. Therefore, we undertook a recent meta-analysis to investigate the association between *H. pylori* infection and NAFLD.

2 Materials and methods

2.1 Registration

This study was registered on the PROSPERO with a registration number CRD42023488399.

2.2 Literature search

The correlation between *H. pylori* infection and NAFLD was investigated by accessing the following databases: CNKI, VIP, Wanfang, PubMed, and Web of Science. The search period encompassed the establishment of these databases up until January 2024. Subject terms used in the search included "*Helicobacter pylori*," "*Helicobacter pylori* infection," "*Helicobacter*," "*H. pylori*," "HP," "Nonalcoholic fatty liver disease," "Nonalcoholic steatohepatitis," "NAFLD," "NASH," "NAFL," and others. The search was confined to full-text articles, and language restrictions were not imposed.

2.3 Eligibility criteria

(i) The study population should include patients with a diagnosis of NAFLD and detectable *H. pylori* infection; (ii) the study methodology should clearly report the diagnosis of *H. pylori* infection and NAFLD; and (iii) the study outcomes should include the counts of patients positive and negative for *H. pylori* infection, both with and without NAFLD.

2.4 Exclusion criteria

(i) Studies that did not exclude individuals with heavy alcohol consumption (usually defined as < 20 g/day for women and < 30 g/day for men) or other competing chronic liver

diseases (e.g., viral hepatitis, iron overload, and use of potentially hepatotoxic drugs); (ii) Laboratory and animal studies; studies in pediatric populations (< 18 years); (iii) reviews, case studies, survey analyses, conference abstracts, and irrelevant literature; and (iv) duplicates of published literature.

2.5 Data extraction

Two independent evaluators reviewed the titles, abstracts, and full text of the literature obtained from each database. They assessed the eligibility of each article based on the criteria stated above. In cases of disagreement, the original articles were reviewed again, and consensus was reached through discussion. Pertinent information was extracted from the screened literature, including details such as authors, year, country, study type, sample size, gender, age, *H. pylori* testing method, and NAFLD diagnostic method.

2.6 Diagnosis

H. pylori infection can be detected through either invasive methods, such as endoscopic biopsy, or noninvasive tests including serology, the 13C or 14C urea breath test, and fecal antigen test. NAFLD diagnosis can involve histology, ultrasonography, or surrogate markers like the hepatic steatosis index (HSI), NAFLD-liver fat score (NAFLD-LFS), and/or fatty liver index (FLI).

2.7 Study quality assessment

Two evaluators used the JBI scale to assess the quality of cross-sectional study literature in ten areas. These areas included the purpose of the study, selection of the population, sample characteristics, inclusion and exclusion criteria for the sample, credibility and validity of data collection, authenticity of the data, ethical considerations, correctness of the statistical methodology, accuracy of the findings, and elaboration of the study's value. A score of 14 or higher was considered indicative of high-quality literature. The quality of cohort study literature was evaluated using the NOS score, which assessed eight aspects. These aspects included the selection of the exposed and non-exposed populations, the method of measuring exposure factors, whether the outcome of interest occurred before the intervention, comparability of the exposed and non-exposed groups, accuracy and unbiasedness of outcome assessment, whether the follow-up duration was sufficient, and the adequacy of the follow-up process. For case-control studies, the NOS score evaluated their quality based on several factors. These factors included the appropriateness of case identification, representativeness of cases, selection of controls, identification of controls, comparability of cases and controls, identification of exposure factors, method of identification of exposure factors, and non-response rate. Scores ranging from 1 to 3, 4 to 6, and 7 to 9 were used to evaluate the low, medium-high, and high quality of the literature, respectively.

2.8 Statistical analyses

The extracted data were subjected to meta-analysis utilizing STATA 16.0 software. The specific process was as follows: (i) Effect size selection: dichotomous variables were evaluated using relative risk (RR), while continuous variables were assessed through weighted mean difference (WMD). Their corresponding 95% confidence intervals (CI) were then computed. (ii) Heterogeneity test: taking P-value and I^2 as criteria, when $P > 0.1$ and $I^2 \leq 50\%$, heterogeneity was small, and a fixed effect model (Fixed Effect, FE) was used for analysis. When $P \leq 0.1$ and $I^2 > 50\%$, heterogeneity was large, and a random effect model (Random Effect, RE) was used for analysis. (iii) Evaluation of publication bias: Funnel plots and egger tests were drawn for the literature on the main indicators to evaluate whether there was a possibility of publication bias. (iv) Sensitivity analysis: In the case of notable heterogeneity among the primary indicators' studies, sensitivity analysis ought to be conducted, and efforts made to identify the root cause of such heterogeneity.

3 Results

3.1 Study characteristics

The initial review identified a total of 544 documents. After removing duplicates and other unqualified documents, 243 studies were retained. Eventually, 34 studies were included in the analysis (Figure 1) (16–50). These 34 studies were published between 2011 and 2024. The majority of the studies were carried out in China, with the United States, Japan, Iran, Korea, and Brazil following suit. Study designs primarily consisted of cross-sectional, cohort, and case-control studies. In total, there were 175,575 patients included in the analysis. The distribution of male and female participants was approximately equal, and their ages ranged from 20 to 70 years. *H. pylori* infection was predominantly identified through the C13/C14 breath test, serum *H. pylori*-specific antibody assay, or urease test. The diagnosis of NAFLD was mainly established through abdominal ultrasound, abdominal CT, abdominal MRI, or liver biopsy. More detailed information can be found in Table 1.

3.2 Study quality

Sixteen out of the 19 cross-sectional studies attained JBI scores of 14 or higher, while the remaining three achieved scores of 10, 13, and 13, respectively, indicating the overall high quality of the cross-sectional studies included. Among the seven cohort studies, two received NOS scores of 3, deemed as low quality; one study scored 4, two scored 5, and one scored 6, categorized as medium quality, while one study with an NOS score of 7 was considered high quality. Additionally, in the eight case-control studies, one study with an NOS score of 5 and two studies with a score of 6 were classified as medium quality. Two studies with an NOS score of 7 and three with a score of 8 were deemed high quality among the remaining case-control studies (Supplementary Tables 1–3).

Sixteen out of the 19 cross-sectional studies were rated as high quality with JBI scores of 14 and above. The remaining three studies

TABLE 1 Characteristics of studies regarding the association of *H. pylori* infection with NAFLD.

References	Country	Type of study	Sample size	Gender		Age		Diagnosis of Hp infection	Diagnosis of NAFLD
				Male	female	NAFLD/control	hp+/hp−		
Chen et al. (16)	China	Cross-sectional study	962	529	433	–	–	^{14}C-UBT	Abdominal ultrasound
Lisa et al. (17)	China	Case-control	504	306	198	41.91 ± 8.29/ 42.61 ± 8.17	–	^{14}C-UBT	Abdominal ultrasound
Pazizge (18)	China	Case-control	2,323	1,010	1,313	46.25 ± 10.52/ 43.01 ± 14.30	–	^{13}C/ ^{14}C-UBT	Abdominal ultrasound
Shen (19)	China	Cross-sectional study	2,650	1,169	1,481	–	–	Serum Hp specific antibodies	Abdominal ultrasound
Wang (20)	China	Cross-sectional study	3,447	1,945	1,502	–	45 (36,53)/ 46 (36,54)	^{13}C-UBT	Abdominal ultrasound
Zhen (21)	China	Cohort	2,063	1,091	972	51.38 ± 13.87/ 48.04 ± 15.08	48.36 ± 14.80/ 48.71 ± 15.03	^{13}C-UBT	Abdominal ultrasound
Zhang (22)	China	Cross-sectional study	3,947	2,022	1,925	50.63 ± 11.43/ 50.61 ± 12.38	–	^{14}C-UBT	Abdominal ultrasound
Wang (23)	China	Cohort	1,902	1,219	683	–	–	^{13}C-UBT	Abdominal ultrasound
Xu and Li (24)	China	Case-control	1,000	565	435	53.43 ± 5.12/ 53.38 ± 5.06	–	^{13}C-UBT	Abdominal ultrasound
Peng et al. (25)	China	Case-control	250	161	89	53.05 ± 9.62/ 53.45 ± 10.06	–	^{13}C-UBT	Diagnostic criteria for non-alcoholic fatty liver disease
Wu et al. (26)	China	Case-control	114	–	–	–	45.6 ± 10.15/ 49.3 ± 10.45	^{13}C-UBT	Abdominal ultrasound
Zhang and Ding (27)	China	Cross-sectional study	3,635	–	–	–	–	^{13}C-UBT	Abdominal ultrasound
Xie and Yie (28)	China	Cohort	198	127	71	–	58.4 ± 1.4/ 57.1 ± 1.9	^{14}C-UBT	Diagnostic criteria for non-alcoholic fatty liver disease
Yang et al. (29)	China	Cohort	160	78	82	–	52.378 ± 9.486/ 53.965 ± 6.685	^{13}C-UBT	Abdominal ultrasound
Chen et al. (30)	China	Cohort	109	61	48	–	42.35 ± 12.34/ 42.65 ± 12.46	^{13}C-UBT	Abdominal ultrasound
Wang et al. (31)	China	Cohort	848	441	407	–	59.49 ± 11.49/ 58.23 ± 12.15	^{14}C-UBT	Abdominal ultrasound/ CT/ MRI
Guo (32)	China	Cross-sectional study	960	570	390	54.10 ± 12.72/ 52.02 ± 12.39	54.10 ± 12.72/ 52.02 ± 12.39	^{14}C-UBT	Abdominal ultrasound
Zhang et al. (33)	China	Cross-sectional study	5,889	3,432	2,457	39.00 ± 15.00/ 32.00 ± 11.00	34.00 ± 15.0/ 33.00 ± 13.0	^{13}C-UBT	Abdominal ultrasound

(Continued)

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TABLE 1 (Continued)

References	Country	Type of study	Sample size	Gender		Age		Diagnosis of Hp infection	Diagnosis of NAFLD
				Male	female	NAFLD/control	hp+/hp−		
Liu (34)	China	Cross-sectional study	2,026	1,284	742	45.75 ± 11.25/ 42.50 ± 12.31	–	^{14}C-UBT	Abdominal ultrasound
Liu et al. (35)	China	Cross-sectional study	14,373	9,164	5,209	50.32 ± 13.79/ 49.88 ± 14.00	–	^{13}C-UBT	Abdominal ultrasound
Dong (36)	China	Case-control	296	163	133	46.18 ± 14.85/ 43.18 ± 14.46	–	Rapid Urease Test	Diagnostic criteria for non-alcoholic fatty liver disease
Shi et al. (37)	China	Cross-sectional study	5,000	2,990	2,010	50 ± 10/ 51 ± 9	–	^{13}C-UBT	Abdominal ultrasound
Xu (38)	China	Case-control	112	60	52	44.2 ± 5.8/ 43.4 ± 6.2	–	Serum Hp specific antibodies	CT
Mohammadifard et al. (39)	China	Case-control	130	62	78	37.6 ± 5.6/ 36.6 ± 6.1	–	ELISA	Abdominal ultrasound
Jiang et al. (40)	China	Cross-sectional study	4,081	1,887	2,194	47.18 ± 13.33/ 42.37 ± 13.36	–	^{13}C-UBT	Abdominal ultrasound
Wang et al. (41)	China	Cross-sectional study	71,633	30,086	41,547	–	46.5 ± 12.5/ 44.8 ± 13.3	^{13}C-UBT	Abdominal ultrasound
Yan et al. (42)	China	Cross-sectional study	1,185	778	407	43.50 ± 9.94/ 40.90 ± 11.28	–	^{13}C-UBT	Abdominal ultrasound
Baeg et al. (43)	South Korea	Cross-sectional study	3,663	–	–	–	–	^{13}C-UBT	Hepatic steatosis index (HSI) and NAFLD liver fat score (NAFLD-lfs)
Valadares et al. (44)	Brazil	Cross-sectional study	88	13	72	–	39.1 ± 11/ 39.4 ± 8.1	Rapid Urease Test	liver biopsy
Kang et al. (45)	USA	Cross-sectional study	5,404			46.1 ± 0.15/ 41.5 ± 0.18	48.4 ± 0.26/ 40.2 ± 0.12	Serum Hp specific antibodies	Abdominal ultrasound
Han et al. (46)	South Korea	Cohort	1,784	1,483	301	–	55.9 ± 9.3/ 54.9 ± 10.1	Serum Hp specific antibodies	CAP
Yu et al. (47)	China	Cross-sectional study	20,389	11,969	8,420	50.20 ± 12.13/ 46.45 ± 13.60	–	^{13}C-UBT	Abdominal ultrasound
Okushin et al. (48)	Japanese	Cross-sectional study	13,737	7,419	6,318	–	–	Serum Hp specific antibodies	Abdominal ultrasound
Wang et al. (49)	China	Cross-sectional study	1,898	1,217	681	–	37.04 ± 0.289/ 37.28 ± 0.210	^{13}C-UBT	Abdominal ultrasound

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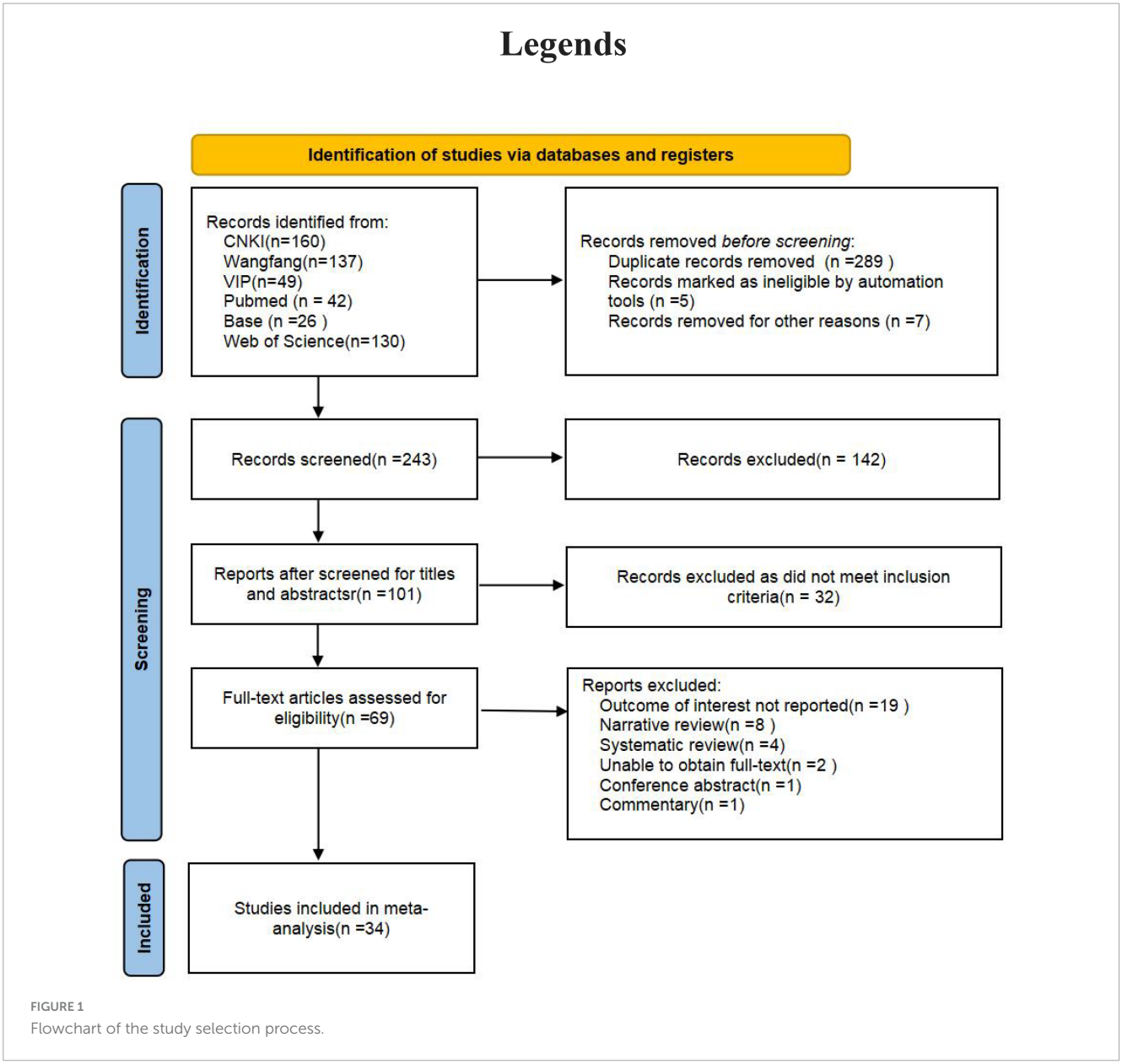

FIGURE 1
Flowchart of the study selection process.

obtained JBI scores of 10, 13, and 13, respectively, suggesting a generally high quality of the cross-sectional studies included. Regarding the cohort studies, two out of the seven were assessed as low quality, each receiving NOS scores of 3. One study had a NOS score of 4, two studies had a NOS score of 5, and one study had a NOS score of 6, all of which were evaluated as medium quality. Furthermore, one study with an NOS score of 7 was assessed as high quality. Among the case-control studies, one study with an NOS score of 5 and two studies with an NOS score of 6 were considered of medium quality. Additionally, two studies with a NOS score of 7 and three studies with a NOS score of 8 were evaluated as high quality (Supplementary Tables 1–3).

3.2.1 *H. pylori* infection and occurrence of NAFLD

Seventeen studies, encompassing 74,928 patients, reported the incidence of *H. pylori* infection in individuals with NAFLD. The prevalence of *H. pylori* infection was found to be higher in NAFLD patients compared to those without NAFLD [RR = 1.13, 95% CI (1.02, 1.24), $Z = 2.395$, $P = 0.017$] (Figure 2).

3.2.2 NAFLD and occurrence of *H. pylori* infection

Fourteen studies reported the incidence of NAFLD in *H. pylori* infection involving 94,950 patients. The incidence of NAFLD was higher in *H. pylori* infection than in *H. pylori* negativity [RR = 1.17, 95% CI (1.10, 1.24), $Z = 4.897$, $P < 0.001$] (Figure 3).

3.2.3 Bias assessment

To assess publication bias, a funnel plot and Egger's test were employed to scrutinize the incorporation of literature concerning the association between *H. pylori* infection and the onset of NAFLD, as well as the presence of *H. pylori* infection in individuals with NAFLD. The funnel plot exhibited an asymmetrical distribution of data points on both sides of the symmetry axis, suggesting

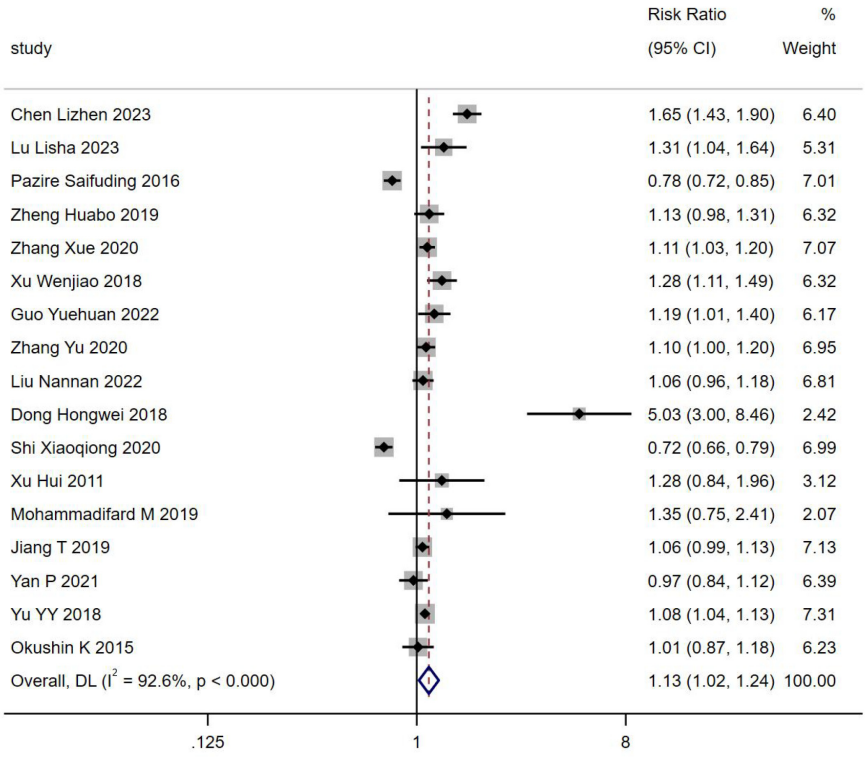

FIGURE 2
Forest plots for *H. pylori* infection and occurrence of NAFLD.

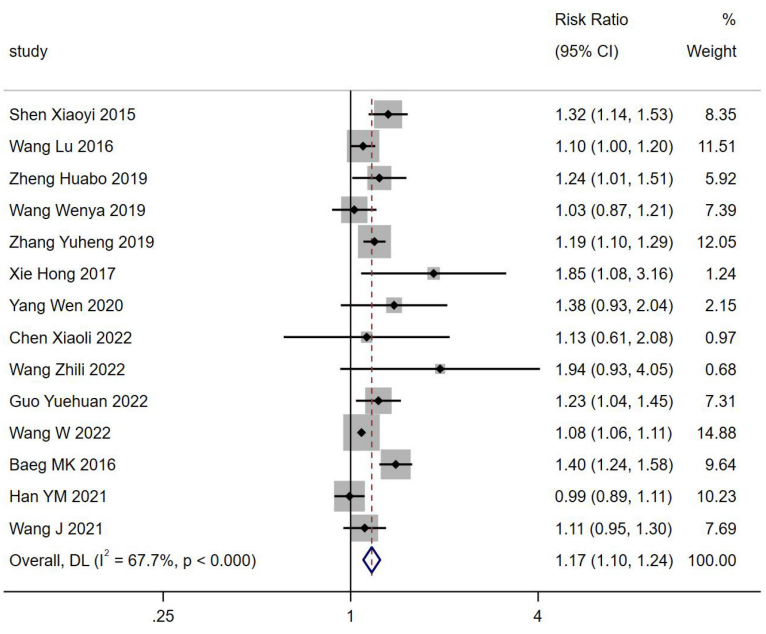

FIGURE 3
Forest plots for NAFLD and occurrence of *H. pylori* infection.

the potential presence of publication bias among the literature included in this analysis (Figures 4A–D). Furthermore, the egger test demonstrated an asymmetrical distribution of scatter points above and below the symmetry axis with the middle line, adding further evidence to the presence of publication bias.

3.2.4 Sensitivity analysis

All point estimates for the occurrence of NAFLD in *H. pylori* infections and the occurrence of *H. pylori* positivity in NAFLD fell within the 95% CI of the combined effect sizes, which indicates that the results of the present study are stable (Figures 5A, B).

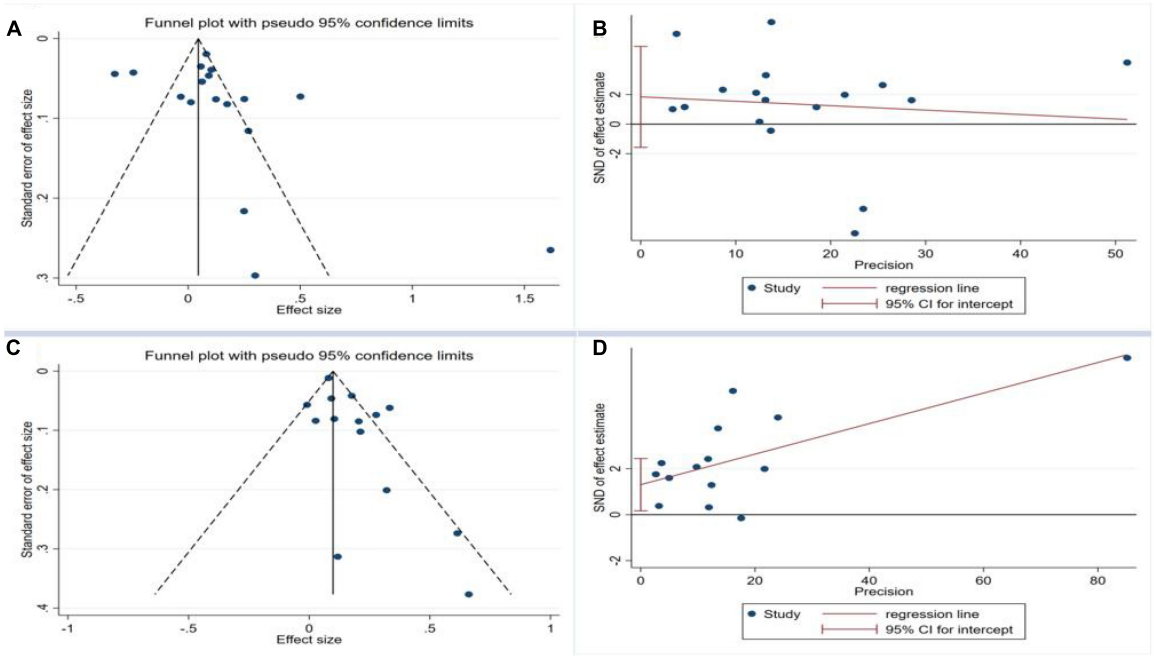

FIGURE 4
Funnel plot and Egger test for the publication bias test of the included studies. **(A)** Funnel plot for *H. pylori* infection and occurrence of NAFLD; **(B)** Egger test for *H. pylori* infection and occurrence of NAFLD; **(C)** Funnel plot for NAFLD and occurrence of *H. pylori* Infection; **(D)** Egger test for NAFLD and occurrence of *H. pylori* infection.

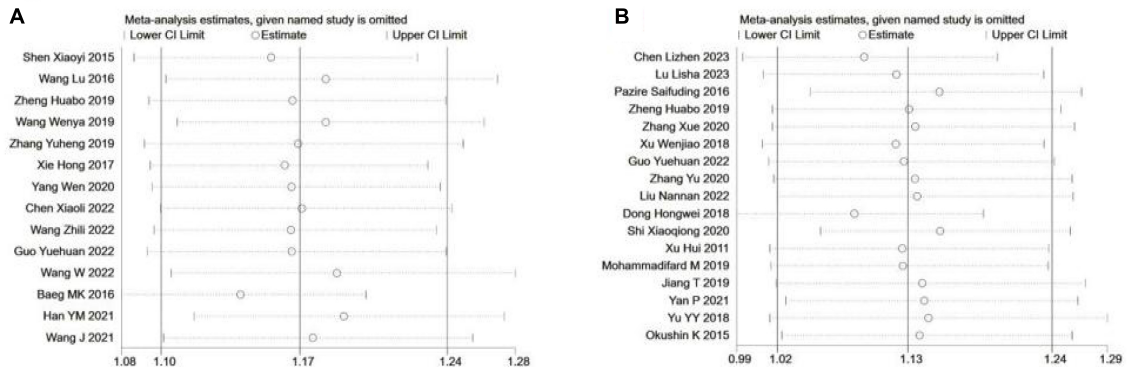

FIGURE 5
Sensitivity analysis of included studies. **(A)** *H. pylori* Infection and OCCURRENCE of NAFLD; **(B)** NAFLD and occurrence of *H. pylori* infection.

3.2.5 *H. pylori* Infection and BMI

The BMI was found to be elevated in patients positive for *H. pylori* infection compared to those negative for the infection [WMD = 0.92, 95% CI (0.55, 1.29), Z = 4.859, $P < 0.001$] (Supplementary Figure 1).

3.2.6 NAFLD and BMI

BMI was higher in NAFLD patients than in non-NAFLD patients [WMD = 3.05, 95% CI (2.13, 3.97), Z = 6.508, $P < 0.001$] (Supplementary Figure 2).

3.2.7 *H. pylori* Infection and FPG

There was no statistically significant variance in FPG levels between patients with positive *H. pylori* infection and those without the infection [WMD = 0.54, 95% CI (−1.13, 2.22), Z = 0.635, P = 0.525] (Supplementary Figure 3).

3.2.8 NAFLD and FPG

It showed no statistically significant difference in FPG in NAFLD patients compared to non-NAFLD patients [WMD = 1.86, 95% CI (−1.83, 5.56), Z = 0.987, P = 0.324] (Supplementary Figure 4).

3.2.9 *H. pylori* Infection and liver function (ALT, AST, GGT)

ALT and GGT were higher in *H. pylori* infection-positive patients than in *H. pylori*-negative patients [WMD = 0.60, 95% CI (0.59, 0.61), Z = 155.549, $P < 0.001$; WMD = 2.37, 95% CI (1.29,

3.45), $Z = 4.307$, $P < 0.001$]. AST did not differ between the two groups (Supplementary Figure 5).

3.2.10 NAFLD and liver function (ALB, ALT, AST, GGT, TBIL)

ALT, AST and GGT was higher in NAFLD patients than in non-NAFLD patients [WMD = 10.21, 95% CI (6.59, 13.83), $Z = 5.533$, $P < 0.001$; WMD = 3.95, 95% CI (3.11, 4.78), $Z = 9.311$, $P < 0.001$; WMD = 12.88, 95% CI (6.5, 19.26), $Z = 3.958$, $P < 0.001$]. TBIL and ALB did not differ between the two groups [WMD = 0.69, 95% CI (−0.24, 1.62), $Z = 1.460$, $P = 0.144$; WMD = 0.01, 95% CI (−0.04, 0.06), $Z = 0.480$, $P = 0.631$] (Supplementary Figure 6).

3.2.11 *H. pylori* Infection and kidney function (UA, Cr, BUA)

UA, Cr and BUA was higher in *H. pylori* infection-positive patients than in *H. pylori*-negative patients [WMD = 6.19, 95% CI (0.50, 11.87), $Z = 2.133$, $P = 0.033$; WMD = 0.59, 95% CI (0.30, 0.88), $Z = 3.966$, $P < 0.001$; WMD = 0.07, 95% CI (0.05, 0.09), $Z = 6.649$, $P < 0.001$] (Supplementary Figure 7).

3.2.12 NAFLD and kidney function (UA, BUA)

UA of NAFLD patients was higher than that of non-NAFLD patients [WMD = 63.77, 95% CI (47.58, 79.97), $Z = 7.717$, $P < 0.001$]. There was no statistical significance in BUA between the two groups [WMD = 0.14, 95% CI (−0.11, 0.39), $Z = 1.117$, $P = 0.264$] (Supplementary Figure 8).

3.2.13 *H. pylori* Infection and blood lipid (TG, TC, HDL, LDL)

TC, HDL, LDL of *H. pylori* infection-positive patients was higher than that of *H. pylori*-negative patients [WMD = 0.84, 95% CI (0.10, 0.59), $Z = 2.213$, $P = 0.027$; WMD = −0.27 95% CI (−0.49, −0.05), $Z = -2.427$, $P = 0.015$; WMD = 0.11 95% CI (0.06 0.17), $Z = 3.882$, $P < 0.001$] (Supplementary Figure 9). There was no significant difference in the TG between the two groups [WMD = 0.81, 95% CI (−1.59, 3.21), $Z = 0.661$, $P = 0.508$].

3.2.14 NAFLD and blood lipid (TG, TC, HDL, LDL)

TG and LDL were higher in NAFLD patients than in non-NAFLD patients [WMD = 0.94, 95% CI (0.80, 1.08), $Z = 13.296$, $P < 0.001$; WMD = 0.35 95% CI (0.17 0.53), $Z = 3.793$, $P < 0.001$]. There was no significant difference in TC and HDL between the two groups [WMD = 2.22, 95% CI (−0.54, 4.99), $Z = 1.574$, $P = 0.115$; WMD = −1.05 95% CI (−2.25 0.16), $Z = -1.707$, $P = 0.088$] (Supplementary Figure 10).

3.2.15 *H. pylori* and blood pressure

DBP was higher in *H. pylori* infection-positive patients than in *H. pylori*-negative patients [WMD = 1.01, 95% CI (0.11, 1.91), $Z = 2.211$, $P = 0.027$]. SBP did not differ between the two groups[WMD = 1.51, 95% CI (−1.18, 4.19), $Z = 1.101$, $P = 0.271$] (Supplementary Figure 11).

3.2.16 NAFLD and blood pressure

SBP and DBP were higher in NAFLD patients than in non-NAFLD patients [WMD = 8.03, 95% CI (6.50, 9.55), $Z = 10.298$, $P < 0.001$; WMD = 5.71, 95% CI (4.02, 7.39), $Z = 6.645$, $P < 0.001$] (Supplementary Figure 12).

4 Discussion

Our comprehensive meta-analysis has identified *H. pylori* as a significant risk factor for individuals prone to NAFLD. Additionally, the prevalence of *H. pylori* infection among NAFLD patients was determined to be 1.13 times higher compared to those without NAFLD. Through a bidirectional meta-analysis of the latest published studies, employing a meticulous search strategy and stringent selection criteria, we have amassed substantial evidence supporting the correlation between *H. pylori* infection and NAFLD. Notably, our meta-analysis boasts a larger sample size compared to previous investigations, enhancing the robustness and currency of our findings.

MetS, comprising overweight/obesity, type 2 diabetes mellitus (T2DM), and metabolic dysregulation, plays a pivotal role in the onset of NAFLD (50, 51). Moreover, a strong correlation exists between MetS and *H. pylori* infection (52). Through our meta-analysis, we have established a relationship between *H. pylori* infection and NAFLD. This enhances our comprehension of the underlying mechanisms linking *H. pylori* infection, MetS, and NAFLD, an aspect that has not been extensively explored in prior meta-analyses.

There is a lack of direct experimental mechanistic evidence to support the effect of *H. pylori* infection on NAFLD. Disruption of the gastrointestinal epithelium and transport of *H. pylori*-associated metabolites through the portal flow to the liver activate the toll-like receptor inflammatory process that may develop NAFLD (53). In particular, low-grade chronic inflammation in the gastric mucosa may exacerbate and promote the local and systemic release of several pro-inflammatory cytokines, thereby exacerbating systemic insulin resistance (IR), increasing disorders of lipid metabolism (adipocytokines and lipid metabolism), increasing intestinal permeability, and altering the composition of the gut microbiome. Inflammatory cytokines are key in the pathogenesis of both *H. pylori* infection and NAFLD (54, 55). Persistent *H. pylori* infection may lead to chronic low-level inflammation and increased expression of NOD-like receptor protein 3 (NLRP3) inflammatory vesicles, as well as inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and TNF-α (54, 55). However, the exact relationship between *H. pylori* infection and serum adipocytokines remains uncertain, and further extensive prospective studies are needed to establish a conclusive link.IR is a key factor in the development of NAFLD, contributing significantly to hepatic triglyceride accumulation, inflammatory cascade response and progression of liver fibrosis (56). Meta-analyses suggest a possible correlation between *H. pylori* infection and IR (57). Hepatocellular steatosis, characterized by disturbances in hepatocellular lipid metabolism, is the main pathological manifestation of NAFLD (9). A comprehensive analysis using a large cohort propensity score-matched study suggested that eradication of *H. pylori* may mitigate the deterioration of lipid metabolism. However, lipid levels did not fully recover to those observed in uninfected individuals (58). There is strong evidence that *H. pylori* infection affects the integrity of the intestinal barrier. In an experiment involving mice fed a high-fat diet and infected with *H. pylori*, a significant reduction in the expression of tight junction proteins in the intestinal barrier was observed. This reduction was attributed to an increase in CagA-containing exosomes, leading to increased intestinal permeability

(59). *H. pylori* infection may alter the composition of the intestinal microbiota by altering the anaplasmosis, Lactobacillus, Aspergillus, Rickettsia and Actinomycetes groups, as observed in obese patients (60).

Our meta-analysis has several limitations. Firstly, the majority of the studies included only presented cross-sectional data, which could introduce recall and selection biases. As a result, the findings can only suggest a potential association between *H. pylori* infection and NAFLD. Secondly, only a subset of the studies accounted for confounding factors in multivariate regression analyses. This lack of adjustment may introduce confounding variables and affect the accuracy of the results. Thirdly, the presence of significant heterogeneity across the studies could potentially undermine the reliability of the pooled odds ratio estimates. Fourthly, there are disparities in the diagnostic methods for *H. pylori* infection and NAFLD among the included studies. Fifthly, although we included all available studies in our meta-analysis, the number of studies and participants may still be insufficient. Therefore, it is essential to interpret the results of this meta-analysis critically and cautiously, and further multicenter prospective studies are necessary to validate the main findings.

Despite these limitations, our meta-analysis also has important strengths. We implemented a rigorous search strategy and strict inclusion criteria, including all available evidence published to date. To the best of our knowledge, our meta-analysis is the largest and most recent updated meta-analysis to date designed to investigate the association between *H. pylori* infection and NAFLD risk. Second, we used standardized risk estimates from all included studies to achieve a consistent combination of estimates between studies. In addition, our study was registered in advance on the PROSPERO platform, and most of the included studies were of high quality, indicating that our results are reliable.

Besides, we also proved the relationship between NAFLD and metabolic disorders such as BMI, ALT, AST, GGT, UA, TG, LDL, DBP and SBP by meta-analysis. MetS has been shown to be the strongest risk factor for NAFLD and NASH (61). In 2020, the term NAFLD was replaced with metabolism-associated fatty liver disease (MAFLD), a change that garnered widespread recognition within the academic community globally (62–65).

In summary, there is clear evidence of a substantial and bidirectional relationship between *H. pylori* infection and the susceptibility to NAFLD. This underscores the importance for clinicians to pay close attention to this correlation. However, additional research is needed to bolster and clarify this association, as well as to elucidate the underlying mechanisms.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

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

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Registration and protocol

This study was registered on the PROSPERO with a registration number CRD42023488399.

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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Diet and physical exercise as key players to tackle MASLD through improvement of insulin resistance and metabolic flexibility

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Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) has emerged as a prevalent health concern, encompassing a wide spectrum of liver-related disorders. Insulin resistance, a key pathophysiological feature of MASLD, can be effectively ameliorated through dietary interventions. The Mediterranean diet, rich in whole grains, fruits, vegetables, legumes, and healthy fats, has shown promising results in improving insulin sensitivity. Several components of the Mediterranean diet, such as monounsaturated fats and polyphenols, exert anti-inflammatory and antioxidant effects, thereby reducing hepatic steatosis and inflammation. Furthermore, this dietary pattern has been associated with a higher likelihood of achieving MASLD remission. In addition to dietary modifications, physical exercise, particularly resistance exercise, plays a crucial role in enhancing metabolic flexibility. Resistance exercise training promotes the utilization of fatty acids as an energy source. It enhances muscle glucose uptake and glycogen storage, thus reducing the burden on the liver to uptake excess blood glucose. Furthermore, resistance exercise stimulates muscle protein synthesis, contributing to an improved muscle-to-fat ratio and overall metabolic health. When implemented synergistically, the Mediterranean diet and resistance exercise can elicit complementary effects in combating MASLD. Combined interventions have demonstrated additive benefits, including greater improvements in insulin resistance, increased metabolic flexibility, and enhanced potential for MASLD remission. This underscores the importance of adopting a multifaceted approach encompassing dietary modifications and regular physical exercise to effectively manage MASLD. This narrative review explores the biological mechanisms of diet and physical exercise in addressing MASLD by targeting insulin resistance and decreased metabolic flexibility.

KEYWORDS

MASLD, physical exercise, insulin resistance, metabolic flexibility, Mediterranean diet, resistance exercise

1 The high prevalence of MASLD urges effective treatment options based on optimization of lifestyle changes

Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) is a prevalent condition that affects approximately 25% of the global population (1). It is commonly considered as the liver-related manifestation of the metabolic syndrome and is closely linked to obesity and type 2 diabetes (T2D) (2). MASLD is a disease spectrum spanning from simple excess triacylglycerol (TAG) accumulation within the hepatocytes (steatosis) to inflammation (metabolic dysfunction-associated steatohepatitis, MASH), cirrhosis and hepatocellular carcinoma (HCC) (3). MASLD carries a burden of morbidity and mortality, primarily due to liver-related complications, but more significantly due to unfavorable cardiovascular events. Glucose lowering drugs based on the incretin system are often used off-label to promote the optimization of glucose control when type 2 diabetes is an issue, or for the management of weight loss (4, 5). The primary approach to managing the disease involves substantial weight loss and intensive reduction of cardiovascular risk factors.

Nonetheless, lifestyle changes aimed at weight loss remain the mainstay of MASLD treatment in general population.

As far as weight loss for the treatment of MASLD is concerned, the recommendation is a targeted weight reduction of 7–10%, a measure found to be effective in mitigating lipid accumulation, enhancing metabolic flexibility, and ameliorating insulin resistance (6).

Weight loss exceeding 10% has demonstrated superior efficacy in resolving MASLD and MASH, exhibiting regression of fibrosis and inflammation (6, 7). Notably, the synergistic benefits of weight loss are contingent on the adoption of a combined dietary and exercise approach (8).

Resmetirom, an oral, liver-directed, thyroid hormone receptor beta (THR-β)–selective agonist, were superior to placebo with respect to MASH resolution and improvement in liver fibrosis in a stage 3 trial (9). Resmetirom may represent the first approved liver specific drug aimed at MASH resolution (10), on top of lifestyle changes aimed at weight loss.

With this article we would like to review the mechanisms by which dieting and physical exercise may benefit metabolic health and fatty liver through improved insulin resistance and metabolic flexibility. While insulin resistance has been considered one of the most relevant pathogenetic feature of MASLD and the driver for the progression of liver inflammation (11), reduced metabolic flexibility remains a less explored mechanism (12).

1.1 Energy expenditure at rest and metabolic adaption as determinants of weight loss programs success

Basal metabolic rate (BMR) refers to the amount of energy expended by an organism during a state of physical and psychological rest, measured in the morning while fasting (after a 10-h period since the last meal), without preceding physical exertion after eating, and in a thermoneutral environment (13).

Understanding how human bodies oxidize calories at rest can help us understand why some people are more susceptible to MASLD. Conditions like obesity and diabetes, which are linked to BMR reduction, are also known to contribute to MASLD. However, the exact impact of BMR on MASLD needs further investigation (14, 15).

In individuals who lead a sedentary lifestyle, BMR accounts for a significant proportion, ranging from 60 to 70% of the total energy expenditure, whereas in those who regularly engage in physical activities, this percentage decreases to approximately 50% (16).

The factors that influence basal metabolism have demonstrated their crucial role, as changes in these parameters have been causatively associated with an increased susceptibility to specific clinical conditions, such as obesity, thereby raising the risk of developing MASLD (17).

According to existing literature, modifications in lean mass and adipose tissue mass are recognized as the primary influencers of BMR (13).

Contemporary research is increasingly focused on developing strategies for weight loss in individuals with overweight or obesity, while simultaneously preserving lean mass, which plays a significant role in basal energy expenditure. Conversely, divergent studies have revealed that after periods of calorie restriction, BMR may exhibit an adaptive response known as "metabolic adaptation," which negatively affects the rate of BMR and predisposes individuals to a higher risk of weight regain (17–19).

The precise mechanisms underlying metabolic adaptation remain incompletely understood, although it is hypothesized to potentially involve reduced sympathetic drive or decreased thyroid activity (20). Caloric intake, dietary patterns and specific dietary components influence BMR. For instance, sufficient protein intake is considered crucial for maintaining lean mass in low-calorie dietary regimens (21, 22).

Additional factors that have emerged in the literature as influential in modulating BMR include exercise, height, general weight, age, and circulating thyroid hormones (23).

Physical activity has a profound impact on the rate of BMR by affecting changes in lean mass among individuals (see section 4). The influence of thyroid hormones on BMR has long been acknowledged, with hyperthyroidism or hypothyroidism conditions exerting noticeable effects on BMR. A recent study demonstrated that elevated plasma concentrations of T4 were associated with increased BMR. The role of triiodothyronine (T3) in this context remains a subject of debate (23).

1.2 Definition of metabolic flexibility

In order to understand the regulatory mechanisms of metabolism at rest, it is helpful to introduce the concept of metabolic flexibility. Metabolic flexibility refers to the body intrinsic ability to utilize readily available substrates as an energy source. During periods of rest, the body can shift between using glucose and lipids as primary fuel sources. After a meal, insulin promotes the storage of glucose as glycogen in the liver and facilitates glucose uptake in skeletal muscle. Conversely, during fasting conditions, glucagon stimulates hepatic gluconeogenesis and glycogenolysis, while also promoting lipolysis in adipose tissue to release free fatty acids for use as an energy substrate. This coordinated regulation by insulin and glucagon allows the body to maintain metabolic homeostasis by adapting its fuel utilization to the prevailing nutritional state.

Disruptions to this metabolic flexibility, such as in metabolic disorders like MASLD, can impair the body ability to efficiently switch between glucose and lipid oxidation. This can lead to the accumulation of lipids in non-adipose tissues like the liver, exacerbating disease progression.

1.3 Regulatory mechanisms of energetic metabolism at rest revolve around insulin and glucagon insulin

1.3.1 Insulin

Metabolic regulation during periods of rest involves complex molecular signaling pathways.

We begin our examination with the role of insulin. After a meal, pancreatic β cells release insulin into the bloodstream, resulting in an elevated insulin-glucagon ratio. Insulin exerts its effects on various tissues, promoting glycolysis and glycogen synthesis in the liver while inhibiting gluconeogenesis and glycogenolysis. In skeletal muscle, insulin facilitates glucose uptake by translocating the insulin-dependent glucose transporter GLUT 4 to the plasma membrane, and it also promotes glycogen synthesis and glycolysis. Moreover, in adipose tissue, insulin, upon binding to its receptor, suppresses lipolysis while simultaneously stimulating the production of lipids (24).

Additionally, insulin exerts its effects by promoting protein synthesis, regulating mitochondrial biogenesis, and inhibiting autophagy (25). This hormone plays a crucial role in the regulation of glucose homeostasis and the storage of energy in the form of lipids and glycogen. Acting as a peptide hormone, insulin interacts with the tyrosine kinase receptor (INS-R), initiating a signaling cascade that encompasses the aforementioned effects.

1.3.2 Glucagon

Glucagon is released by pancreatic α-cells and acts as antagonist to insulin. Glucagon secretion is stimulated during fasting conditions and primarily exerts its effects at the hepatic level by promoting gluconeogenesis, glycogenolysis, lipolysis, and ketogenesis. Simultaneously, glucagon inhibits the pathways activated by insulin, such as glycolysis and lipogenesis. The decrease in insulin levels during fasting conditions suppresses malonyl-coenzyme A synthesis in liver cells, leading to the activation of fatty acid oxidation as the predominant energy source (26).

The regulation of energy homeostasis in the body relies on the interplay between insulin and glucagon. The contribution of gut-secreted incretin hormones, such as GLP-1, in stimulating insulin secretion has been extensively demonstrated in mechanicistic and clinical studies (27–29). Furthermore, there is evidence suggesting a reciprocal relationship, where glucose-induced insulin secretion inhibits glucagon secretion from α cells in a paracrine manner (26).

1.3.3 Mitochondria

Insulin inhibits enzymes responsible for fatty acid oxidation, thereby directing mitochondria to preferentially utilize glucose as a fuel source.

When glucagon levels rise during fasting or starvation, the hormone signals mitochondria to shift away from glucose oxidation and toward increased fatty acid beta-oxidation.

Disruptions in mitochondrial function can lead to diminished energy production and an increased tendency for the accumulation of lipids within the liver (30). Dysfunctional mitochondria exhibit reduced efficiency in oxidizing fatty acids, resulting in an overflow of lipids and the formation of toxic lipid intermediates such as diacylglycerols and ceramides. These lipid species interfere with insulin signaling pathways, compromising the ability of hepatocytes to respond appropriately to insulin and regulate glucose metabolism (31).

1.4 Insulin resistance and gut-liver axis

Insulin resistance is a pathophysiological condition characterized by the decreased ability of target tissues, such as the liver, skeletal muscle, and adipose tissue, to respond appropriately to insulin (10). This impairment in insulin signaling and action results in impaired glucose and lipid metabolism (11). Insulin resistance can manifest at both the systemic and hepatic levels. Systemic insulin resistance refers to a whole-body phenomenon, where peripheral tissues exhibit reduced sensitivity to insulin, leading to hyperglycemia and compensatory hyperinsulinemia (11). In contrast, hepatic insulin resistance is the specifically impaired insulin action within the liver, which fails to properly suppress gluconeogenesis and increase glucose uptake in response to insulin. Hepatic insulin resistance contributes to the dysregulation of glucose and lipid homeostasis, further exacerbating metabolic disturbances and the development of MASLD.

Concurrently, targeting the gut-liver axes can improve systemic insulin sensitivity. The gut microbiome plays a pivotal role in this gut-liver axis, influencing metabolic flexibility through several mechanisms (12). Gut bacteria produce a variety of metabolites, including short-chain fatty acids (SCFAs), that can modulate hepatic and peripheral insulin sensitivity. SCFAs, for instance, can activate AMP-activated protein kinase (AMPK) in the liver, enhancing mitochondrial fatty acid oxidation and glucose metabolism (13) (see section 1.5).

Furthermore, the gut microbiome shapes the production of bile acids, which act as signaling molecules to regulate energy homeostasis. Specific bile acid species can activate the Farnesoid X Receptor (FXR) and G-protein coupled bile acid receptor (TGR5) in the liver, leading to improved gluconeogenesis, lipid metabolism, and insulin sensitivity (14, 15). Dysbiosis of the gut microbiome, as seen in MASLD, can disrupt this delicate balance and contribute to impaired metabolic flexibility (16).

Targeting the gut-liver axis through dietary interventions, prebiotics, probiotics, or pharmacological modulation of the microbiome and bile acid signaling may therefore represent a promising strategy to improve metabolic flexibility and mitigate insulin resistance in MASLD patients.

1.5 PGC-1α is a sensor of energy expenditure

Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1alpha (PGC-1α) is a critical regulator of cellular processes and energy metabolism. Its expression is highest in tissues that have a high capacity for oxidative metabolism, such as skeletal muscle, liver,

brown adipocytes, and myocardium (32). The role of PGC-1α varies depending on the specific tissue. In the liver, extensive research has been conducted on the effects of PGC-1α on mitochondrial and energy metabolic activities of hepatocytes. During fasting, there is an increase in the ratio of AMP to ATP, which activates AMPK and subsequently leads to the activation of PGC-1α. The primary effect of PGC-1α activation is the induction of mitochondrial biogenesis through its stimulation of nuclear transcription factors NRF1 and NRF2 (33). These transcription factors control the expression of important genes involved in energy metabolism, leading to increased expression of mitochondrial proteins and enhanced enzymatic capacity in oxidative metabolic pathways such as beta-oxidation, the tricarboxylic acid cycle, and oxidative phosphorylation (34). Furthermore, PGC-1α activation also induces the expression of antioxidant molecules that protect against reactive oxygen species (ROS) (34).

In individuals with obesity and insulin resistance, the liver is chronically exposed to high levels of free fatty acids from the portal circulation. Additionally, hyperinsulinemia, which is associated with insulin resistance, promotes the synthesis of fatty acids in the liver. These conditions contribute to the excessive accumulation of fatty acids, surpassing the mitochondrial oxidative capacity of hepatocytes and resulting in MASLD. In the context of MASLD, appropriate levels of PGC-1α play a positive role. This is because an increase in both mitochondrial mass and function has been observed, leading to enhanced beta-oxidation. Studies have shown a correlation between reduced levels of PGC-1α and an increased risk of developing hepatic steatosis, which can be attributed to impaired interaction with NRF promoters (34). This impaired interaction leads to decreased levels of mitochondrial proteins and antioxidants, resulting in further increased oxidative stress (35). Both *in vivo* and *in vitro* studies have demonstrated that overexpression of PGC-1α leads to increased hepatic beta-oxidation, causing a significant reduction in hepatocyte triglyceride accumulation. Exercise has also been identified as a useful tool for increasing PGC-1α levels in the liver, as well as in skeletal muscle tissue (36).

In skeletal muscle, contraction plays a crucial role in shaping its characteristics, including mitochondrial content and function, gene transcription, and intracellular signaling related to contractile proteins (12). However, in individuals with obesity and insulin resistance or T2D, there is a limited ability to switch from glucose oxidation to fatty acid oxidation, impairing metabolic flexibility (12). This is influenced by factors such as increased free fatty acid (FFA) intake, which leads to elevated mitochondrial acetyl CoA and cytosolic citrate levels. The accumulation of citrate inhibits phosphofructokinase, resulting in increased glucose-6-phosphate and inhibition of glycolysis by suppressing glucokinase. Furthermore, metabolic by-products of FFA degradation, such as ceramides and diacylglycerol, interfere with insulin receptor signaling, hindering GLUT 4 translocation to the cell membrane and exacerbating insulin resistance (37).

Elevated plasma FFA levels and increased muscle lipid content contribute to insulin resistance by disrupting mitochondrial regulation and promoting ectopic fat deposition (12). In the skeletal muscle of people with obesity, there is a predominance of type IIB fibers characterized by lower mitochondrial density and oxidative capacity, along with reduced expression of PGC-1α, a key regulator of mitochondrial biogenesis. PGC-1α is strongly induced by muscle contraction and, through positive feedback, upregulates the expression of MEF2, promoting myocyte enhancement. People with insulin resistance and obesity generally exhibit decreased expression of PGC-1α and NRF-1 in myocytes, resulting in reduced oxidative phosphorylation capacity and impaired fatty acid oxidation (38). Specifically, individuals with type 2 diabetes and glucose intolerance exhibit inhibited PGC-1α transcription, leading to reduced mitochondrial protein expression (39).

Intervention studies have shown that physical activity can positively impact mitochondrial metabolism and improve metabolic flexibility. In one study, aerobic exercise in elderly prediabetic subjects led to enhanced metabolic flexibility, as evaluated by the respiratory quotient during a hyperinsulinemic euglycaemic clamp (40). Considering the various roles of PGC-1α in energy metabolism, its induction by physical activity, and its reduced expression in obesity, diabetes, and insulin-resistance, it is evident that PGC-1α plays a critical role in the metabolic flexibility of skeletal muscle tissue.

White adipose tissue (WAT) serves not only as an energy reservoir in the form of triglycerides but also as an active regulator of metabolic health and substrate flow. Excessive WAT accumulation and dysfunction are closely associated with metabolic impairments, contributing to adipose-related diseases (12). Similar to muscle tissue, adipose tissue relies on insulin for its proper functioning. Insulin binding to its receptor initiates a phosphorylation cascade that ultimately leads to the translocation of GLUT4-containing vesicles to the cell membrane, facilitating glucose uptake. Insulin also inhibits lipolysis, reducing the release of non-esterified fatty acids (NEFA) into the circulation (41). However, insulin-resistant individuals experience dysregulation in these tightly controlled mechanisms, resulting in impaired modulation of lipolysis and persistent release of NEFA into the bloodstream (12).

There are two main types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). Brown adipocytes, characterized by their smaller size, rich cytoplasm, and high mitochondrial density, exhibit a remarkable oxidative capacity. The high expression of uncoupling protein 1 (UCP1) in brown adipocytes enables thermogenesis by dissipating the proton gradient in the mitochondrial intermembrane space, generating heat instead of ATP (42).

Studies have shown that white adipocytes can undergo conversion to brown-like adipocytes, and the key transcriptional factors involved in this process are PPAR-γ and PGC-1α (43). Treatment with rosiglitazone, a PPAR-γ agonist, has been found to significantly increase the expression of UCP1 and PGC-1α (44). PGC-1α, a coactivator, plays a crucial role in thermogenesis during cold exposure by increasing the AMP/ATP ratio, which activates AMPK. AMPK then phosphorylates PGC-1α, orchestrating thermogenesis and oxidative metabolic processes. Analysis of subcutaneous WAT in individuals with obesity reveals downregulation of PGC-1α (45). Moreover, experiments in mice with a specific deletion of PGC-1α in adipose tissue demonstrate reduced gene expression related to oxidative phosphorylation, beta-oxidation, glucose tolerance, and insulin resistance (46).

It is worth noting that aerobic and resistance exercise promotes increased lipolysis in white adipose tissue, leading to reduced adiposity and altered expression of key proteins involved in energy metabolism, such as the GLUT4 transporter and PGC-1 coactivator (46).

1.6 PPARα as crucial regulator of energy metabolism gene expression

PCG-1α can activate Peroxisome proliferator–activated receptor alpha (PPARα) through a complex regulatory mechanism. PCG-1α interacts with PPARα and enhances its transcriptional activity by acting as a coactivator. PCG-1α binds to PPARα and promotes the recruitment of other coactivators, such as CBP/p300, leading to the activation of target genes.

The activation of PPARα by PCG-1α has important hepatic effects. PPARα plays a crucial role in regulating lipid metabolism in the liver. When activated by PCG-1alpha, PPARα promotes fatty acid oxidation, leading to increased mitochondrial beta-oxidation and subsequent energy production. This activation also stimulates the expression of genes involved in fatty acid transport and metabolism, as well as ketogenesis.

PPARα is a transcription factor belonging to the nuclear receptor family. It is known for its affinity for various endogenous ligands, primarily lipid molecules such as fatty acids, prostaglandins, leukotrienes, and fatty acid-derived metabolites, as well as antidiabetic drugs (47). PPARα is predominantly found in tissues with high metabolism and energy demand, such as the liver, heart, skeletal muscle, and kidney. However, its presence in other tissues, including adipose tissue, highlights its significant role in lipid metabolism regulation (48).

PPARα plays a central role in lipid metabolism by regulating a key molecular pathway involved in beta-oxidation. It stimulates the expression of enzymes such as CPT1 and acyl-CoA oxidase (ACOX), which are crucial for the transport and subsequent oxidation of fatty acids within the mitochondrial matrix (49). Activation of PPARα also leads to the transcription of genes responsible for ketogenesis and bile acid metabolism during fasting, promoting the synthesis of ketone bodies (50). Additionally, PPARα positively affects lipoprotein metabolism by regulating the expression of apolipoproteins A1 and A2 (APOA1, APOA2), leading to increased plasma levels of these proteins and contributing to the modulation of HDL (51).

PPARα is involved in the regulation of the inflammatory response by negatively regulating leukotriene synthesis and trans-repressing the activities of inflammatory regulators such as NF-kB, AP-1, and STAT. This overall reduces the expression of inflammatory molecular pathways (52). Interestingly, an inverse correlation is observed between PPARα and the development of NASH. This suggests that increased inflammation may contribute to the suppression of PPARα expression (53).

Dysregulation of PPARα could have important consequences in hepatic lipid accumulation and increase the risk of cardiovascular disease. Therefore, PPARα could be a therapeutic target for resolving altered lipid metabolism.

1.7 PPARα functions as a lipid sensor in white adipose tissue

PPARα serves as a critical lipid sensor that coordinates the transcriptional response to fatty acids and plays a vital role in controlling oxidative capacity and thermogenesis, especially in brown adipose tissue. As such, PPARα is also expressed in adipose tissue and regulates several metabolic processes, including lipolysis, beta-oxidation, adipocyte differentiation, thermogenesis, and the inflammatory response. Activation of PPARα induces the expression of molecules involved in lipid metabolism.

PPARα functions as a lipid sensor, as it increases the transcription of PPARα-regulated genes in response to elevated fatty acid levels and chronic stimulation of beta-adrenergic receptors. This leads to an increase in oxidative capacity and thermogenesis (54). PPARα is mainly expressed in brown adipose tissue and has low expression in white adipocytes. However, studies have shown a negative correlation between PPARα mRNA levels in human adipose tissue and body mass index (55). Interestingly, some studies have found that PPARα agonists increase beta-oxidation and glycerol kinase expression in human white adipocytes (56, 57). PPARα has also been shown to induce subcutaneous white adipose tissue browning and stimulate thermogenesis, improve insulin resistance, and reduce inflammation in animal models when treated with fenofibrate (58). However, it is important to note that results obtained in animal models may not directly translate to humans due to species differences (55).

2 Physical exercise drives several mechanisms aimed at improved usage of energy substrates

2.1 Physical exercise as part of the interventions aimed at MASLD regression

Aerobic exercise, such as walking and cycling, is a cost-effective and widely accessible non-pharmacological intervention for the general public. It is characterized by increased energy expenditure during exercise sessions and has been shown to have positive effects on various factors associated with MASLD, including hemoglobin A1c, resting blood pressure, and serum cholesterol levels (59). Nonetheless, it is important to note that aerobic exercise can lead to fatigue and discomfort, potentially compromising long-term adherence to exercise regimens.

Several informative reviews have been published in the medical literature, discussing the role of exercise prescription for MASLD and highlighting the potential benefits of both aerobic and resistance exercises (60–64). However, there remains a lack of clarity regarding the optimal exercise protocol, including the recommended frequency, intensity, and duration of aerobic and resistance exercises for improving MASLD. Furthermore, considering the high prevalence of cardiovascular diseases in individuals with MASLD (65, 66), a comparison of exercise types based on energy consumption has yet to be conducted. Additionally, it is important to target the pathogenesis of MASLD, specifically insulin resistance and decreased metabolic flexibility, through physical exercise as potential mechanisms for mitigating liver damage. In the following paragraphs, we will analyze the available evidence to better understand the benefits of aerobic and resistance exercise in relation to the metabolic changes they elicit.

2.2 Frequency, intensity, and duration of exercise for improving hepatic steatosis in MASLD

In a comprehensive review of clinical trials, Hashida et al. included twenty-four exercise protocols from 18 articles,

demonstrating a decrease in hepatic steatosis in 91.7% of the protocols (64). The median age of patients was 48 years, with a median BMI of 30.9 kg/m^2. All protocols had a frequency of exercise of 3 times per week. Moderate-intensity aerobic exercise for a duration of 40 min over a 12-week period was the typical protocol. Aerobic exercise led to improvements in BMI and serum alanine aminotransferase levels.

Most protocols focused on conventional aerobic exercises, such as walking or cycling at a constant intensity. However, a study on high-intensity interval training (HIIT) demonstrated reductions in whole-body fat mass, serum alanine aminotransferase levels, and hepatic lipids. HIIT involved high-intensity exercise intervals followed by recovery periods (67).

Also, very short or low-intensity exercises may be insufficient for improving hepatic steatosis, emphasizing the importance of exercise energy consumption (68, 69).

Aerobic exercise is known to promote lipolysis in adipose tissues, leading to the production of acetyl-CoA through increased beta-oxidation (70). Acetyl-CoA is then metabolized in the tricarboxylic acid cycle, resulting in ATP production in the mitochondria's electron transport system. Aerobic exercise also upregulates certain proteins, such as uncoupling protein-1 and peroxisome proliferator-activated receptor gamma (71), which further enhance lipolysis in adipose tissues (72). Studies have shown that aerobic exercise can decrease serum levels of resistin and increase levels of high molecular weight adiponectin, indicating potential benefits for individuals with conditions like hypertension and MASLD (73–75).

2.3 Resistance exercise for improving hepatic steatosis in MASLD

In the same work from Hashida et al. (64) resistance exercise resulted overall beneficial in MASLD. Based on seven protocols from seven studies, a reduction in hepatic steatosis was observed in 85.7% of the protocols, including five randomized controlled trials (76–82). The median age of patients was 49.2 years, with a median BMI of 30.6 kg/m^2. The exercise frequency was consistently three times per week, with a median metabolic equivalent (METs) of 3.5, exercise duration of 45 min, and a 12-week period.

Resistance exercise resulted in modest changes in BMI and serum alanine aminotransferase (ALT) levels. Three protocols demonstrated improvements in hepatic steatosis without significant weight loss (76, 80, 83). MR spectroscopy showed a decrease in intrahepatic lipid levels in three studies (13%; 3%; and 25% reduction from baseline (76–78)).

While most studies used weight machines, one study with 53 individuals who trained for 12 weeks evaluated the effects of simple bodyweight resistance exercises such as push-ups and squats, demonstrating improvements in muscle mass, ALT levels, and hepatic steatosis (83).

One study did not show improvements in hepatic steatosis through resistance exercise (82). The longer duration (32 weeks) and significant increase in body weight in that study may have influenced the results.

The optimal duration for resistance exercise to improve hepatic steatosis appears to be 12 weeks. General recommendations include three sets of 8–12 repetitions, three times per week, targeting major muscle groups. Using a variety of weight training exercises is recommended.

Resistance exercise may have distinct therapeutic characteristics compared to aerobic exercise in MASLD patients, as it showed modest changes in BMI and demonstrated steatosis improvement independent of body weight reduction.

Further research is needed to explore the long-term effects of resistance exercise and to better understand its mechanisms in improving hepatic steatosis.

Resistance exercise offers benefits for hepatic steatosis with less energy consumption. The exact mechanisms behind these benefits are not fully understood, but it may involve muscle fiber type-specific adaptations. Muscle fibers can be categorized into type I (slow oxidative) and type II (fast glycolytic) based on their energy metabolism. Resistance exercise has been found to specifically promote hypertrophy in type II muscle fibers, while not significantly affecting type I fibers. Additionally, resistance exercise has been shown to increase the expression of GLUT-4 in type II fibers and enhance intracellular insulin sensitivity (84–87). These changes may contribute to improvements in hepatic steatosis and insulin resistance.

Moreover, resistance exercise may improve MASLD through muscle-liver crosstalk mediated by a myokine called irisin (88, 89). Irisin is released by skeletal muscles and has been shown to increase thermogenesis and energy expenditure by promoting the browning of subcutaneous adipocytes. It also exhibits regulatory effects on lipid metabolism in hepatocytes (88).

Studies have demonstrated that recombinant irisin inhibits the expression of key regulators of lipogenesis, such as sterol regulatory element-binding protein-1c, and lipogenic enzymes in hepatocytes (90). Overexpression of irisin has been found to improve hepatic steatosis in obese mice. Interestingly, individuals with MASLD have been reported to have lower serum irisin levels compared to healthy individuals (91).

Recent research by Kim et al. investigated the effects of aerobic and resistance exercises on circulating irisin levels. They found that resistance exercise significantly increased circulating irisin levels, while aerobic exercise did not produce the same effect. This suggests that resistance exercise specifically influences lipid metabolism in the liver through the action of irisin (92).

Therefore, one additional explanation for the improvement of MASLD through resistance exercise, despite lower energy consumption compared to aerobic exercise, could be attributed to the muscle-liver crosstalk facilitated by irisin. These mechanisms are key players in the switch from impaired metabolic flexibility to enhanced metabolic flexibility and might be responsible for pleiotropic, and not only liver-focused, benefits of resistance exercise. In details, reverting the futile cycle of *de novo lipogenesis* and beta-oxidation that is typical of decreased metabolic flexibility, may improve the oxidation of glucose and foster the flux of energetic intermediates of lipid metabolism toward mitochondrial oxidation therefore accelerating the de-fatting of the liver (see Figure 1).

2.4 Skeletal muscle contractions enhance glucose uptake and by-passes insulin resistance

During resistance exercise, muscle contractions activate several molecular signaling pathways that influence glucose uptake. One key pathway involves the activation of AMPK. AMPK activation stimulates glucose uptake by promoting the translocation of glucose transporter

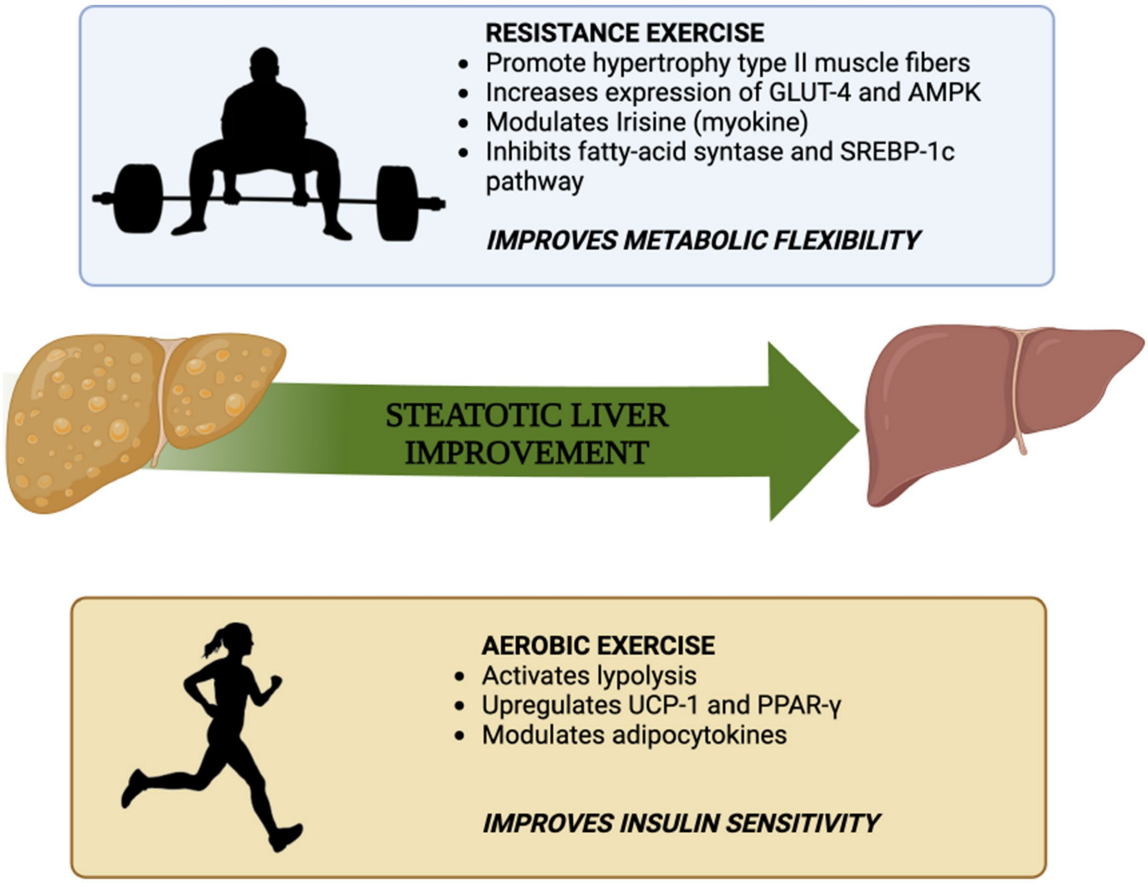

FIGURE 1
Summary of the mechanisms by which aerobic exercise and resistance exercise improve insulin sensitivity and metabolic flexibility, respectively [adapted from Hashida et al. (64)].

GLUT4 to the cell surface, thereby enhancing the uptake of glucose into muscle cells (93).

The mechanisms underlying this increase are complex, involving the docking and fusion of vesicles containing GLUT4 with the cellular surface membrane. It has been demonstrated that muscular contraction stimulates the vesicle-associated SNARE proteins, facilitating the merging of GLUT4-containing vesicles with the cellular surface membrane (94).

Additionally, resistance exercise activates the insulin signaling pathway, which plays a crucial role in glucose uptake. Insulin, released by the pancreas in response to elevated blood glucose levels, binds to insulin receptors on muscle cells, leading to downstream signaling events that result in the translocation of GLUT4 to the cell surface. This allows for increased glucose uptake by skeletal muscle cells (94).

Overall, increased blood glucose clearance and increased liver fat mobility derived from resistance exercise may exert a positive effect in liver health preventing and reverting MASLD progression (Figure 2).

3 Dietary components of Mediterranean diet can act as promoter of insulin sensitivity

Certain dietary components, such as specific nutrients and dietary patterns, have been associated with improvements in insulin sensitivity. Examining the evidence surrounding these components enhances our understanding of how dietary interventions may influence insulin resistance in the context of MASLD.

Contemporary literature is focusing on evaluating dietary patterns aiming to comprehend the collective impact of the entire diet on specific health conditions. This is in contrast to the focus of the past studies which were more aimed to individualize the role of specific nutrients on health.

A systematic review conducted by Tanase et al. (95) explores the intricate relationship among T2D, insulin resistance (IR), and MASLD. The review compared nine different dietary patterns, including vegetarian, Mediterranean diet, high-protein diet, moderate-carbohydrate diet, low-carbohydrate diet, low-glycaemic index/GL diet, paleolithic diet, low-fat diet, and a control diet. More specifically, the review revealed that, while all dietary patterns induced a significant reduction in Hb1Ac levels after 12 weeks, the traditional Mediterranean diet emerged as the most efficacious in improving postprandial hyperglycaemia and insulin sensitivity at an equivalent body weight. This underscores the role of dietary quality in enhancing these metabolic parameters.

The impact of the Mediterranean diet on insulin resistance stems from the synergistic interplay of its constituent elements, characterized by elevated consumption of olive oil, nuts, fruits, legumes, vegetables, and fish, combined with reduced intake of red meat, processed meats, and sweets. Notably, mono-and polyunsaturated fatty acids (MUFAs and

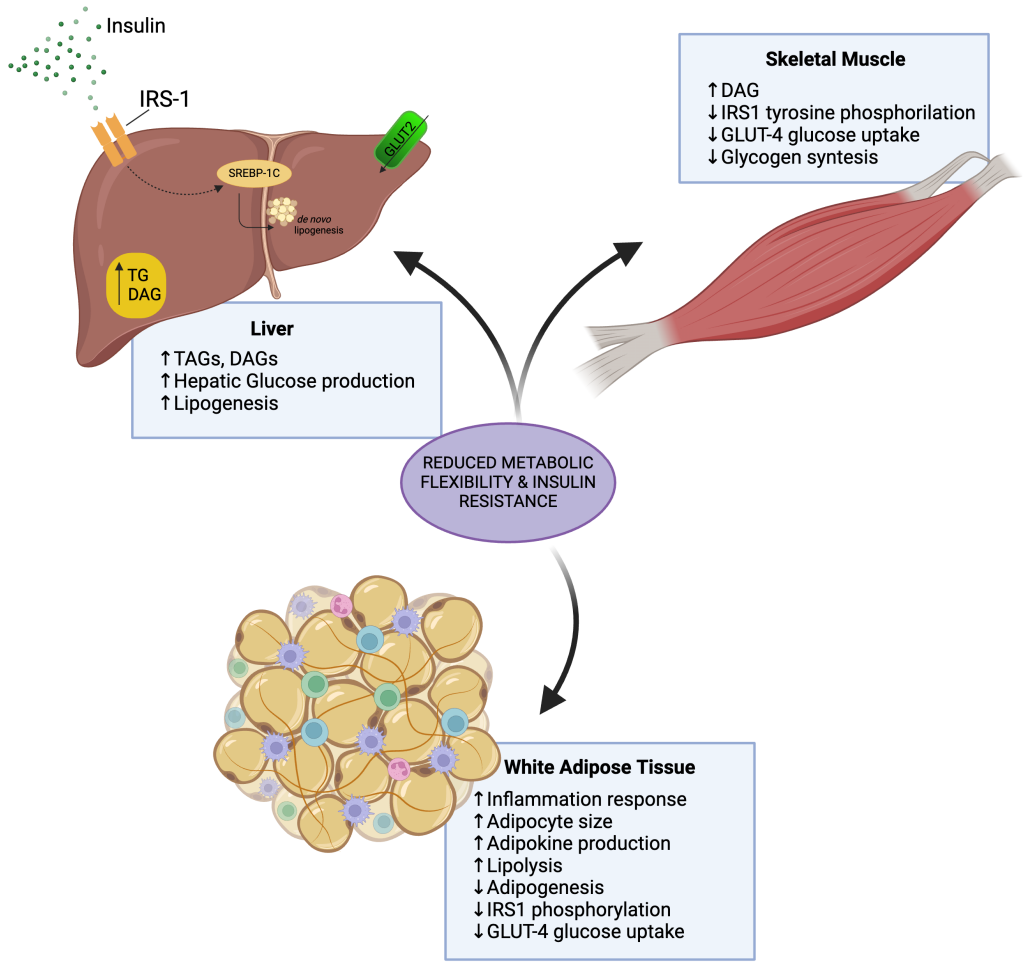

FIGURE 2
Effects of insulin resistance in the liver, white adipose tissue, and skeletal muscle.

PUFAs), primarily sourced from nuts and olive oil within this dietary pattern, gained a lot of interest through the years. A particular significance were acquired by omega-3 PUFAs, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are renowned for their antioxidant and anti-inflammatory properties (95–97).

Moreover, these fatty acids have demonstrated efficacy in enhancing insulin sensitivity, ameliorating steatohepatitis, and reducing intrahepatic triglyceride content. The mechanistic underpinnings involve the downregulation of SREBP-1c and activation of PPARα (98).

PUFAs also play a significant influence on glucose metabolism by binding and stimulating G-protein-coupled receptors (GPCRs), such as GPR120. This stimulation leads to an increased secretion GLP-1 by enteroendocrine L cells. GLP-1 stimulates insulin release from pancreatic β-cells, enhances glucose uptake in skeletal muscles, and may modulate satiety perception by attenuating appetite through central nervous system actions. A noteworthy observation from the review is that the favorable impact of unsaturated fatty acids on insulin sensitivity over saturated fatty acids is evident when total fat intake is below 37% of energy, while higher fat intake increases the risk of insulin resistance irrespective of fat quality (99).

Focusing on the carbohydrate sources prevalent in the Mediterranean diet, these primarily consist of legumes and whole grains. These components exhibit efficacy in postprandial hyperglycaemia, which increase more gradually over time, and enhance insulin sensitivity. The beneficial effects can be attributed to the high fibre content, especially soluble fibre. Soluble fibre attenuates the pronounced glycaemic peaks characteristic of a Western diet, thereby mitigating oxidative stress, preserving pancreatic beta cell integrity, delaying gastric emptying, and correlating with reduced insulin requirements (100).

Furthermore, the Mediterranean diet is distinguished by its elevated consumption of flavonoids derived from vegetables, nuts, and whole grains. A review reported that individuals with heightened flavonoid intake exhibited an 11% reduced risk of developing T2D during follow-up, a condition frequently associated with MASLD (99).

It is essential to note that the Mediterranean diet, as a dietary pattern, not only incorporates elements conducive to improving insulin sensitivity but also entails a reduction in the consumption of sugar-rich foods, particularly added sugars such as fructose. The latter has been linked to the development of MASLD and insulin resistance, attributed to increased hepatic lipogenesis, alterations in intestinal microflora, heightened intestinal permeability, endotoxemia, elevated hepatic Tumor Necrosis Factor-α production, and lipid peroxidation (79).

With regards to other dietary patterns investigated in the existing literature, the majority of studies fail to independently explore the impact of dietary quality aside from weight loss—a critical factor in enhancing insulin sensitivity. Consequently, there is a lack of consistent

evidence demonstrating the direct role of these patterns in insulin sensitivity under conditions of normocaloric diets.

Notably, irrespective of dietary quality, weight loss has consistently demonstrated improvements in postprandial hyperglycaemia and insulin resistance. This is evidenced by enhanced serum liver enzymes, reduced hepatic lipid accumulation, and alleviation of liver inflammation following weight loss interventions (100). This is also supported by the joint EASL-EASD-EASO European Clinical Practice Guidelines for the Management of MASLD (101).

Moreover, a crossover randomized controlled trial sought to assess the impact of distinct dietary patterns on insulin sensitivity within the context of hepatic steatosis. The study involved twelve non-diabetic subjects (6 females/6 males) with biopsy-proven MASLD, subject to a randomized, cross-over 6-week dietary intervention. The dietary patterns examined were the Mediterranean diet and a low-fat, high-carbohydrate diet (LF/HCD) as a control. Notably, the findings demonstrated that, even in the absence of weight loss, adherence to the Mediterranean diet led to a reduction in hepatic steatosis and an improvement in insulin sensitivity (102).

4 Resistance exercise can foster the switch from impaired metabolic flexibility to enhanced flexibility through activation of AMPK while aerobic exercise promotes insulin sensitivity

Exercise induces acute changes in the molecular and biochemical processes involved in mobilizing energy from carbohydrates and fatty acids, leading to an enhanced energy supply. AMPK, a key regulator of energy sensing, is stimulated by regular physical activity (103). Drug-induced activation of AMPK produces gene expression patterns similar to those observed during exercise (104).

In response to resistance exercise, healthy individuals can efficiently switch between glucose and fatty acid oxidation for energy production, depending on exercise intensity and duration. As exercise intensity increases, there is a greater reliance on glucose oxidation through oxidative phosphorylation, while anaerobic glycolysis becomes the predominant pathway during higher intensity exercise (105). These metabolic shifts occur independently of insulin, as circulating insulin levels remain suppressed during exercise, and the contribution of fatty acid oxidation to energy production decreases proportionally (106, 107).

During acute exercise, the release of free fatty acids (FFAs) from adipose tissue is primarily driven by increased levels of catecholamines, with abdominal subcutaneous white adipose tissue (WAT) being the main source (108). In contrast, visceral adipose tissue plays a minor role due to its smaller size, particularly in healthy metabolic states (108).

On the other hand, the increased energy demand during aerobic exercise can upregulate the activity of PPAR-α within muscle cells. As discussed in the section 1.7, high PPARα activity in skeletal muscle up-regulates the delivery of fatty acids from WAT to skeletal muscle.

Studies blocking β-adrenergic receptors in abdominal subcutaneous WAT have demonstrated the role of WAT in metabolic flexibility during exercise by reducing lipolysis (109). Furthermore, the deletion of adipose triglyceride lipase (ATGL), a key enzyme involved in lipolysis, has been shown to impair exercise performance in a mouse model due to a decreased supply of FFAs to skeletal muscle (110).

Obesity may lead to a decrease in β2-adrenergic receptor density in adipocytes, resulting in resistance to catecholamine action and a blunted release of FFAs from WAT during fasting and exercise. The capacity of WAT to mobilize FFAs during acute exercise and in response to chronic training plays a crucial role in facilitating overall fat oxidation, particularly within skeletal muscle (111). Resistance physical activity is therefore essential for increasing energy supply to tissues and improving metabolic flexibility. At the same time, aerobic exercise can improve insulin resistance mainly through the re-arrangement of FFAs from WAT to tissues on a state of intense mitochondrial lipids oxidation. Ultimately, both kind of training, through different mechanisms contribute to improvement of steatotic liver.

Metabolic flexibility encompasses a diverse array of pathways and mechanisms related to fuel selection, energy expenditure, and overall metabolic function. These aspects present potential targets for therapeutic interventions, particularly in the context of MASLD. Conflicting data have emerged regarding the impact of increasing mitochondrial FFA flux and oxidation on insulin resistance (112, 113). Notably, these strategies do not replicate the same energy expenditure or demand observed during exercise. Consequently, while interventions aimed at modifying substrate metabolism or metabolic flexibility may hold implications for obesity and metabolic diseases associated with nutrient overload, they cannot be considered genuine exercise mimetics unless accompanied by an elevated energy demand (12).

5 Conclusion

Impaired metabolic flexibility impacts the body ability to properly adjust to different physiological conditions, including exercise. Insulin resistance involves various tissues and organs, with the liver being a key organ where the shift from metabolic flexibility to inflexibility occurs. During exercise and under other changing conditions, decreased metabolic flexibility can compromise the body ability to handle different substrates.

Restoring metabolic flexibility and reducing insulin resistance are important targets for improving health outcomes. Exercise, combined with dietary and pharmacological interventions, may be an effective way to help restore metabolic flexibility and mitigate the liver damage driven by insulin resistance.

Author contributions

SPM: Conceptualization, Supervision, Writing – review & editing, Writing – original draft. AG: Writing – original draft. SC: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. FZ: Writing – original draft. GP: Writing – original draft. DF: Writing – original draft. GA: Writing – original draft. SB: Supervision, Writing – original draft, Writing – review & editing.

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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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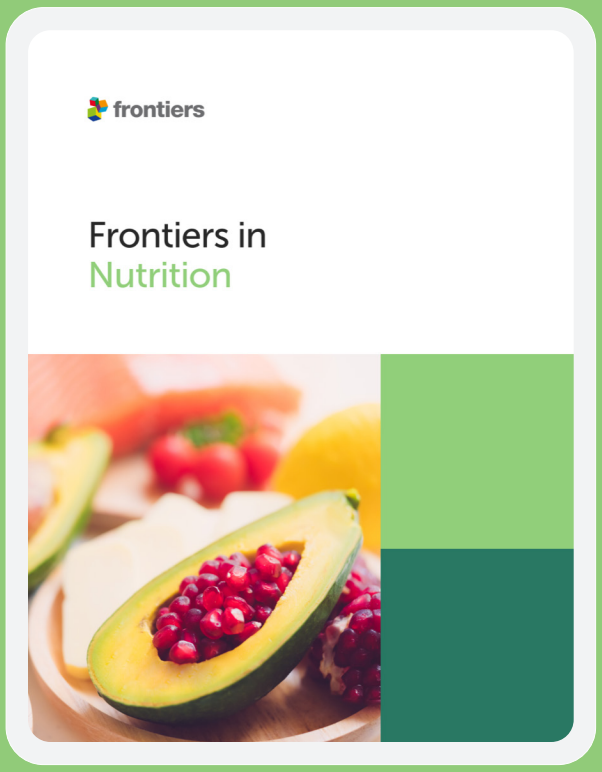

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