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Diabetes and non-alcoholic fatty liver disease: Points of physiological and mechanistic intersection and current co-therapeutic approaches

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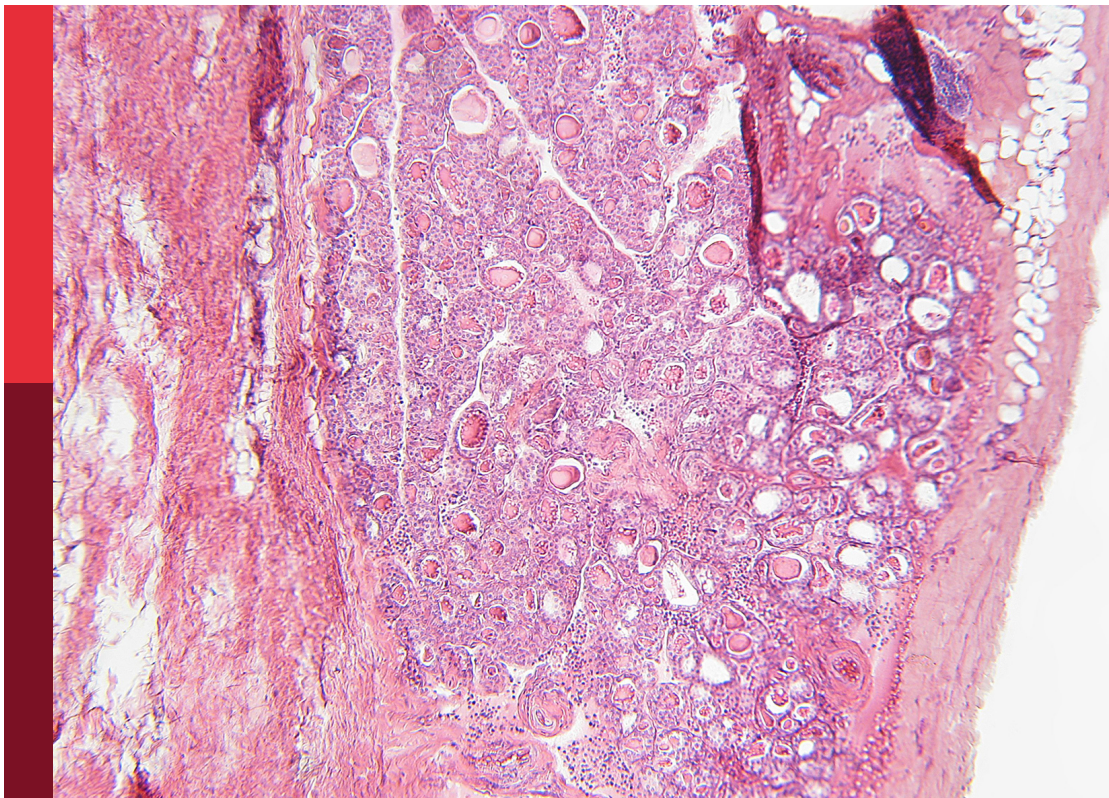

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Diabetes and non-alcoholic fatty liver disease: Points of physiological and mechanistic intersection and current co-therapeutic approaches

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Editorial: Diabetes and non-alcoholic fatty liver disease: points of physiological and mechanistic intersection and current co-therapeutic approaches

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KEYWORDS

NAFLD, NASH, diabetes, T2D, T1D, pharmacotherapeutics

Editorial on the Research Topic
Diabetes and non-alcoholic fatty liver disease: points of physiological and mechanistic intersection and current co-therapeutic approaches

Non-alcoholic fatty liver disease (NAFLD) has rapidly become the most prevalent liver disease across the globe, with estimates of ~25% of individuals globally having NAFLD (1). The term NAFLD covers a broad spectrum of severity, ranging from "benign" lipid accumulation, often referred to as "simple steatosis", to non-alcoholic steatohepatitis (NASH) which involves hepatocellular injury, inflammation, and fibrosis. If left untreated, NASH can further progress to cirrhosis, liver failure, hepatocellular carcinoma, and either necessary liver transplantation or death. It should be noted, that as of June 2023, the preferred nomenclature was updated to metabolic dysfunction-associated steatotic liver disease (MASLD) and metabolic dysfunction-associated steatohepatitis (MASH) (2). However, since this Research Topic was initiated well before this nomenclature update, for the purpose of this editorial we will use the NAFLD/NASH nomenclature. However, this updated nomenclature highlights the exact purpose of this special Research Topic.

One of the main factors contributing to the dramatic rise in incidence of NAFLD is its integral connection with obesity, insulin resistance, and diabetes, which are all undergoing their own pandemics. More recently, the mechanistic directionality of these associations has been debated. While hepatic lipid accumulation is believed to contribute directly to whole-body defects in insulin action (3), insulin resistance is also a driving factor for hepatic lipid accumulation due to both excessive adipose tissue lipolysis and increased hepatic *de novo* lipogenesis (4–6). With these controversies in mind, the aim of this special Research Topic was to share research findings in the areas of either type 1 diabetes (T1D) or type 2 diabetes (T2D) related to NAFLD. Additionally, since there are currently no

approved therapies for NAFLD, and due to the close relationship between insulin sensitivity and NAFLD, research on therapeutics to treat both diseases concomitantly were also encouraged.

A review article by Memaj and Jornayvaz summarized the current knowledge of the prevalence and pathophysiology of NAFLD in T1D which is much less understood compared to insulin resistance/T2D. This review concluded that NAFLD is more prevalent in T1D subjects compared to the general population, however, notes the difficulty in comparing studies with different criteria for determining NAFLD. This article also notes interesting pathophysiological mechanisms which could drive NAFLD in T1D subjects such as altered insulin delivery and hepatic clearance, as well as noting the association between poor glycemic control and the risk of NAFLD.

Related to the potential for NAFLD driving T2D, an article by Chen C. et al. reported that NAFLD progression associated with the development of incident diabetes. Similarly, Chen Y. et al. reported that in a large Taiwanese population, the presence of high serum markers of liver injury was significantly associated with development of incident diabetes. Additionally, an article by Li, et al. reported that even lean individuals with NAFLD were more susceptible to development of T2D. In another study of lean NAFLD, Zhu et al. describe that the association of high circulating lipids or lipid ratios and NAFLD risk is true in both obese and lean individuals. Other studies in this special Research Topic investigated more specific aspects of diabetes and the role NAFLD may play in the association. Basnet et al. describe the presence of high serum uric acid, or hyperuricemia in T2D, and suggest that the prevalence of NAFLD increases the risk of development of diabetes with hyperuricemia. Lastly, studying a cohort of T2D patients, Deravi et al. found that the presence of NAFLD associated with the diabetic microvascular complications such as diabetic neuropathy, nephropathy, and retinopathy. In summary, these studies suggest that the presence of NAFLD is associated with the later development of T2D or worsening of T2D co-morbidities such as hyperuricemia and microvascular disease.

Provided the profound connection between diabetes and NAFLD, a number of articles in this special Research Topic described the therapeutic options for concomitantly treating both diabetes and NAFLD. In a specific population of individuals with both metabolic syndrome-related NAFLD with sarcopenia, Yi et al. noted that physical activity, more-so than dietary factors, was key to preventing sarcopenia. Several studies investigated pharmacotherapeutic options for treating NAFLD and diabetes. Two studies investigated the potential of incretin-related therapies to improve NAFLD. Tan et al. performed a prospective analysis in T2D subjects treated with the glucagon-like peptide-1 receptor agonist (GLP1-RA), liraglutide, and report that liraglutide use decreased hepatic fibrosis in these T2D subjects. Wang X. et al. performed a prospective study on the use of the dipeptidyl peptidase-4 inhibitor, sitagliptin, and reported that while sitagliptin improved glucose metabolic parameters, there was no significant improvement in hepatic fat content. Yan et al. also discuss the efficacy of GLP1-RAs and compare to the effects of sodium-glucose cotransporter-2 inhibitors (SGLT2i) which alternatively reduce glycemia by preventing renal glucose reabsorption. In this systematic review and meta-analysis, the authors describe that in NAFLD patients, only GLP1-RAs improve markers of insulin resistance, while SGLT2i did not significantly reduce fasting glycemia or insulin resistance. Wang Z. et al. performed a meta-analysis of studies regarding the treatment of NAFLD with the thiazolidinedione insulin sensitizer pioglitazone in patients with and without T2D. This analysis concluded that pioglitazone improved insulin resistance and plasma lipids, and also improved NAFLD in both subjects with and without T2D. Conversely, Huang et al. studied T2D subjects treated with or without metformin, and report that long-term metformin use may actually increase susceptibility to developing NAFLD. Lastly, a review article by Niranjan, et al. summarized the therapeutic options for improving hepatic insulin sensitivity to treat NAFLD, including the potential importance of anti-inflammatory agents. Altogether, these studies suggest that agents that improve insulin action, are also associated with improved NAFLD.

The sole "basic" research study published within this Research Topic was performed by Wu et al. In this study, livers from high-fat diet-fed mice with or without overexpression of the G0/G1 switch gene (G0S2) were subjected to proteomics analysis. G0S2 overexpression led to the differential expression of 125 proteins in these livers, with pathway analysis indicating that G0S2 disrupts the "response to insulin", which is supported by decreased glucose tolerance and insulin tolerance in these mice. Overall, the authors suggest that G0S2 should be considered a potential target for the treatment of diabetes and NAFLD.

This interesting Research Topic certainly highlights the strong connection between diabetes and NAFLD. With the ongoing pandemics of obesity, diabetes, and NAFLD, research on the vital connections between these diseases will only continue to rise. Additionally, in-depth studies and reviews on therapeutic options to concomitantly treat both diabetes and NAFLD will be of utmost importance due to the current lack of approved treatments for NAFLD. Articles from this Research Topic suggest that therapeutic agents that improve insulin sensitivity associate with NAFLD improvements, whereas agents that may only improve glycemia do not improve NAFLD.

Author contributions

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

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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GLP-1 RAs and SGLT-2 Inhibitors for Insulin Resistance in Nonalcoholic Fatty Liver Disease: Systematic Review and Network Meta-Analysis

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Objective: Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) and sodium-glucose cotransporter-2 (SGLT-2) inhibitors reduce glycaemia and weight and improve insulin resistance (IR) *via* different mechanisms. We aim to evaluate and compare the ability of GLP-1 RAs and SGLT-2 inhibitors to ameliorate the IR of nonalcoholic fatty liver disease (NAFLD) patients.

Data Synthesis: Three electronic databases (Medline, Embase, PubMed) were searched from inception until March 2021. We selected randomized controlled trials comparing GLP-1 RAs and SGLT-2 inhibitors with control in adult NAFLD patients with or without T2DM. Network meta-analyses were performed using fixed and random effect models, and the mean difference (MD) with corresponding 95% confidence intervals (CI) were determined. The within-study risk of bias was assessed with the Cochrane collaborative risk assessment tool RoB.

Results: 25 studies with 1595 patients were included in this network meta-analysis. Among them, there were 448 patients, in 6 studies, who were not comorbid with T2DM. Following a mean treatment duration of 28.86 weeks, compared with the control group, GLP-1 RAs decreased the HOMA-IR (MD [95%CI]; -1.573[-2.523 to -0.495]), visceral fat (-0.637[-0.992 to -0.284]), weight (-2.394[-4.625 to -0.164]), fasting blood sugar (-0.662 [-1.377 to -0.021]) and triglyceride (- 0.610[-1.056 to -0.188]). On the basis of existing studies, SGLT-2 inhibitors showed no statistically significant improvement in the above indicators. Compared with SGLT-2 inhibitors, GLP-1 RAs decreased visceral fat (-0.560 [-0.961 to -0.131]) and triglyceride (-0.607[-1.095 to -0.117]) significantly.

Conclusions: GLP-1 RAs effectively improve IR in NAFLD, whereas SGLT-2 inhibitors show no apparent effect.

Systematic Review Registration: PROSPERO https://www.crd.york.ac.uk/PROSPERO/, CRD42021251704

Keywords: GLP-1 RAs, SGLT-2 inhibitors, nonalcoholic fatty liver disease, insulin resistance, network meta-analysis

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1 INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic liver disease characterized by increased lipid accumulation in hepatocytes but is not caused by clear causes related to alcohol consumption. NAFLD is often associated with central obesity, insulin resistance (IR) and in general with some symptoms of metabolic syndrome (1, 2).The global prevalence rate of NAFLD is 25%, and it is one of the most common chronic liver diseases in the world (3). Its clinical features are liver triglyceride (TG) accumulation and IR. TG in the liver is synthesized from fatty acyl-CoA. The concentration of fatty acyl-CoA is determined by the balance between the formation of fatty acids (circulating free fatty acids, *de novo* lipogenesis, TG decomposition) and utilization (lipid synthesis, β-oxidation) (4, 5). When IR occurs, the lipolysis of white lipids increases, and the synthesis of lipids decreases (6). At the same time, with the decrease in glucose utilization by skeletal muscle, more fatty acyl-CoA produced by glucose metabolism turns to *de novo* lipogenesis (7), which increases the accumulation of liver TG and even transforms into lipotoxic substances such as long-chain fatty acids, ceramides, and diacylglycerols, resulting in inflammation, endoplasmic reticulum stress, liver fibrosis and hepatocyte apoptosis (5). In short, IR increases the accumulation of lipids in the liver, leading to NAFLD occurrence and development.

For IR, GLP-1 RAs and SGLT-2 inhibitors show satisfactory efficacy in patients with type 2 diabetes mellitus (T2DM), has and have been recommended by experts from many associations (8–10). GLP-1 RAs can reduce oxidative stress (11, 12), inflammation (13), and endoplasmic reticulum stress (14), improve β-cell function (14, 15) and enhance insulin sensitivity (16–18). SGLT-2 inhibitors act on the sodium-glucose cotransporter in renal tubules to inhibit the reabsorption of glucose in renal tubules, reduce blood glucose and alleviate the effects of hyperglycemia on β-cells and IR (19–24). There are a considerable number of studies that have already compared GLP-1 RAs and SGLT-2 inhibitors in T2DM patients on variety outcomes, such as in the PIONEER-2 and SUSTAIN-8 trials, which found that similitude is superior to empagliflozin and canagliflozin in reducing HbA1c and body weight at week 52, respectively (2, 25).

At present, there is no recognized drug treatment for NAFLD (26–29), but as a metabolic disease, GLP-1 RAs and SGLT-2 inhibitors should have significant effects on IR and seem to be appropriate choices. Several studies have used GLP-1 RAs and SGLT-2 inhibitors in the treatment of NAFLD, but no study have directly compared their effects. Therefore, we conducted this systematic review and network meta-analysis to comprehensively evaluate and compare the abilities of GLP-1 RAs and SGLT-2 inhibitors to ameliorate IR in patients with NAFLD.

2 METHODS

2.1 Agreement to Register

We registered the protocol for this system review at PROSPERO (CRD42021251704).

2.2 Search Strategy

The study team co-designed a literature search strategy to search for randomized controlled trials (RCTs) published up to March 01, 2021, in Embase, Medline, and PubMed with language limited to English (**Appendix 1**). In addition, we screened references in the included articles to look for other potential studies.

2.3 Study Selection

Two reviewers, working independently, screened citations and evaluated the full text of eligible studies. A third reviewer resolved disagreements by consensus.

2.3.1 Eligibility Criteria

Inclusion criteria were defined using the 'Patients, interventions, comparators, outcomes, study designs, timeframe' (PICOST) framework, as follows:

2.3.1.1 Patients

NAFLD Patients with or without T2DM, age ≥ 18.

2.3.1.2 Interventions

Antidiabetic drugs, including GLP-1 RAs, SGLT-2 inhibitors, thiazolidinediones (TZDs), dipeptidyl peptidase (DPP-4), sulfonylureas (SUs), and metformin.

2.3.1.3 Comparators

Control group including Placebo, standard care or another antidiabetic mentioned in interventions. All treatments should be given alone and not in combination with any other antidiabetic drugs mentioned in interventions.

2.3.1.4 Outcomes

The main results of this review are based on IR-related indicators that show the degree of IR (direct indicators of IR) or influence IR (indirect indicators of IR): 1) the direct indicator of IR was the homoeostasis model assessment of insulin resistance (HOMA-IR) index; 2) the indirect indicators were adipose tissue, such as subcutaneous fat (SAT), visceral fat (VAT), weight and body mass index (BMI), and adipokines, including leptin and adiponectin. Secondary outcomes were IR-related laboratory measurements, including: 1) glycolipid metabolism, such as fasting blood sugar (FBS), total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL); 2) systolic blood pressure (SBP) and diastolic blood pressure (DBP); and 3) liver enzymes aspartate aminotransferase (AST) and alanine transaminase (ALT).

2.3.1.5 Study Design

RCTs reporting the mean and standard deviation of outcome indicators after interventions.

2.3.1.6 Timeframe

The duration of treatment should be longer than two months.

2.3.2 Other Limitation

First, the language of the publications was limited to English. Second, for studies whose results were reported in multiple publications, we excluded publications presenting duplicate

data and included the publications reporting the most complete data from any study. Third, studies under the risk of low-quality (retracted, terminated and impact factor less than 1 point) were excluded. Finally, studies were excluded if the data could not be extracted.

2.4 Data Extraction

For each eligible study, two reviewers independently extracted the following: study characteristics (study registration number, year of publication, country or countries, funding, duration), population (setting, sample size, patient demographics, whether subjects had coexisting T2DM), intervention description (drug class, name, dose, presence or absence of lifestyle intervention, and specific type of lifestyle intervention) and results. For outcome indicators, the mean and standard deviation after intervention of each study were extracted. Reviewers resolved disagreements by discussion or, if necessary, consultation with a third reviewer.

2.5 Risk of Bias Assessment

The risk of bias was assessed by two reviewers independently using the Cochrane collaborative risk assessment tool RoB (30). The tool is used to determine the risk of bias in randomized trials, including seven dimensions of sources, six types of bias risk: selection bias (random sequence generation and allocation concealment), performance bias (blinding of participants and personnel), detection bias (blinding of outcome assessment), attrition bias (incomplete outcome data), reporting bias (selective reporting) and other bias (funding sources, etc.) (30). Each risk of bias evaluation dimension had three classifications: low, unclear, or high.

If the random sequence was generated correctly and hidden, the risk of selection bias was considered to be low. The risk of performance bias was deemed to be low if participants were blinded as well as those administering the treatment. If the outcome evaluator was blinded, or the outcome indicators were not influenced by evaluator subjectivity, the risk of detection bias was considered to be low. The risk of attrition bias was considered to be low if there was no missing data, or the number and cause of missing data were similar between groups, the missing data was not sufficient to affect the effect size of treatment, and the missing values are handled properly. The risk of reporting bias was required to determine whether an outcome was selectively reported by comparison of protocols and research reports.

2.6 Statistical Analysis

A network meta-analysis was conducted within a Bayesian framework to assess the relative effects of GLP-1 RAs and SGLT-2 inhibitors. ADDIS1.16.6 and R-3.6.2 software were used for data analysis, STATA.16 software was used to draw the network evidence graph, and risk of bias graphs were drawn by RevMan 5.3 software.

Because the outcome index was continuous variables, the mean difference (MD) and associated 95% confidence interval (95% CI) was used as the index for effect size of treatment. In this study, a network meta-analysis was conducted within a Bayesian framework to compare six hypoglycemic agents, especially to assess the relative effectiveness of GLP-1 RAs and SGLT-2 inhibitors for NAFLD.

All outcomes were analyzed by using the consistency model and the inconsistency model, the overall heterogeneity was compared based on the differences in deviance information criteria and I^2. If the difference of deviance information criteria between the two models was ≥ 5, the inconsistency model was used. Both a fixed effect (FE) model and a random effect (RE) model were run for each result, and a more appropriate model based on the deviance information criteria, mean posterior residences, and I^2 was chosen.

The Markov Chain Monte Carlo method was used to estimate the posterior densities of all unknown parameters in each model. Four Markov chains were initially set for simulation with 50,000 iterations, and the first 10,000 anneals were used for eliminating the effects of the initial values. The potential scale reduction factor (PSRF) was calculated to diagnose the degree of the model's convergence. A PSRF ≥ 1.2 would indicate that the current simulation times were insufficient to achieve good convergence and more iterations were needed, a PSRF < 1.2 would indicate that convergence has been achieved, and a PSRF value close to 1 would indicate the model achieved good convergence.

The included studies were tested for consistency and inconsistency. We used node splitting approaches to assess the agreement between direct and indirect estimates in every closed loop of evidence, and a $P > 0.05$ was considered to indicate good consistency, whereas a $P \leq 0.05$ was considered to indicate inconsistency. If there was evidence of material inconsistency, the specific reasons were identified by reviewing the corresponding study with further analysis.

The rank probability of each treatment was estimated by the surface under the cumulative sorting curve (SUCRA) (31). SUCRA is a percentage interpreted as the probability of a treatment that is the most effective without uncertainty on the outcome, which is equal to 1 or 0 when the treatment is certain to be the best or the worst, respectively.

3 RESULTS

3.1 Description of the Included Studies

The electronic search yielded 586 unique records. Screening and full-text article analysis identified 25 trials with 1595 patients (**Figure 1**) (**Appendix 2**) comparing the effects of 6 glucose-lowering drugs (GLP-1 RAs, DPP-4, SGLT-2 inhibitors, TZDs, SUs, and metformin) with placebo or standard care on IR in patients with NAFLD. The median trial mean age was 52 years, the median baseline FBS was 7.66mmol/L and the mean treatment duration was 28.86 weeks. **Figure 2** shows the treatment comparison network from the included studies. The sample sizes ranged from 12 to 162. Of the 25 studies, 13 studies indicated active lifestyle interventions, 1 showed no lifestyle intervention, and the other 11 studies did not specify whether or not they had a lifestyle intervention. In addition, 6 studies had patients with NAFLD alone without T2DM, 16 studies had patients with NAFLD and T2DM, 1 study had T2DM or

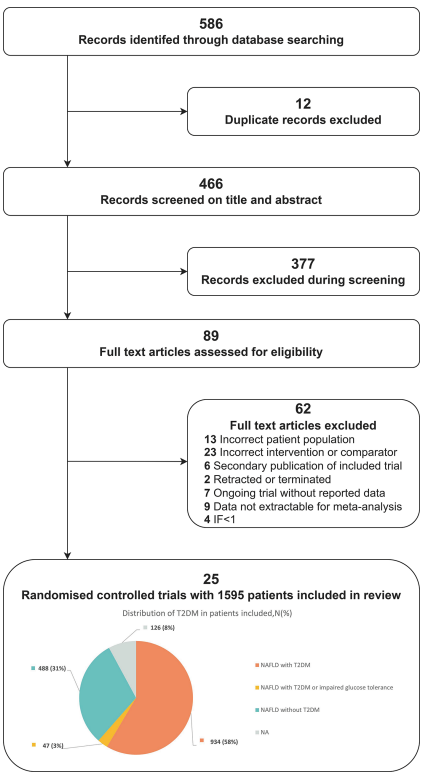

FIGURE 1 | Flow diagram for the study selection.

impaired glucose tolerance and 2 studies did not report whether or not their patients had comorbid T2DM (**Figure 1**).

3.2 Risk of Bias

Appendix 3 presents the risk of bias and the reasons for its determination in each trial. The key limitation was low levels of reported blinding of participants and personnel because the GLP-1 RAs were mainly administered by injection and could not be blinded. Of the 25 trials, for selection bias, 17 trials (68%) were at low risk of bias in random sequence generation, 14 trials (56%) were at low risk of bias in allocation concealment, and 11 trials (44%) were at low risk in performance bias. The outcome indicators in this analysis were all objective and were not influenced by evaluators, so the 25 trials (100%) were at low risk for detection bias. 16 trials (64%) were adjudicated as being at low risk of attrition bias, 17 trials (68%) were at low risk for reporting bias, and 21 trials (84%) were judged to have a low risk of other bias (**Figures 3**, **4**).

3.3 Outcomes

Appendix 4 presents the network plot for each outcome indicator. **Appendix 8** gives a network estimate for each drug comparison for all outcomes.

3.3.1 HOMA-IR

HOMA-IR was reported in 14 trials with 1153 patients (**Appendix 4; Supplementary Figure 1**). Compared with the control group, GLP-1 RAs reduced the HOMA-IR (MD [95% CI]; -1.573[-2.523 to -0.495]), whereas SGLT-2 inhibitors had no statistically significant effect (MD -0.342 [-1.156 to 0.218]) (**Figure 5A**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 97% and 23%, respectively (**Figure 6A**). Compare with SGLT-2 inhibitors, GLP-1 RAs showed no difference in the effect on the HOMA-IR (MD -1.217 [-2.210 to 0.087]) (**Figure 7**).

3.3.2 Adipose Tissue and Adipokines

3.3.2.1 VAT and SAT

The VAT was reported in 8 trials with 561 patients (**Appendix 4; Supplementary Figure 2**). Compared with the controls, GLP-1 RAs decreased VAT (MD -0.637 [-0.992 to -0.284]), whereas SGLT-2 inhibitors had no statistically significant effect (MD -0.078 [-0.308 to 0.120) (**Figure 5C**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 99% and 49%, respectively (**Figure 6B**).

The SAT was reported in 4 trials with 235 patients (**Appendix 4; Supplementary Figure 3**). Compared with the control group, both GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on SAT (MD -0.176 [-0.758 to 0.403] and -0.360 [-0.979 to 0.260], respectively) (**Figure 5C**). The SUCRA chart shows that the probabilities of GLP-1 RAs and

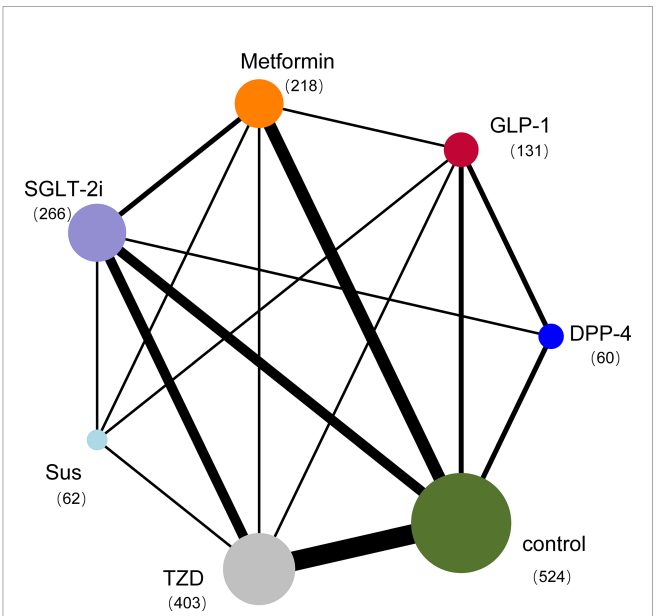

FIGURE 2 | Network plot of trials evaluating glucose-lowering drugs for NAFLD. The network shows the number of participants assigned to each glucose-lowering class, and the size of each circle is proportional to the number of participants randomly assigned to treatment (sample size per drug in parentheses). The thickness of the line is proportional to the number of trials between the corresponding drugs. Compared with placebo, the most commonly compared drugs were TZDs. DPP-4=Dipeptidyl peptidase-4 inhibitors; GLP-1 RAs=Glucagon-like peptide-1 receptor agonists; SGLT-2 inhibitors=Sodium-glucose cotransporter-2 inhibitors; SUs=Sulfonylureas; TZDs=Thiazolidinediones.

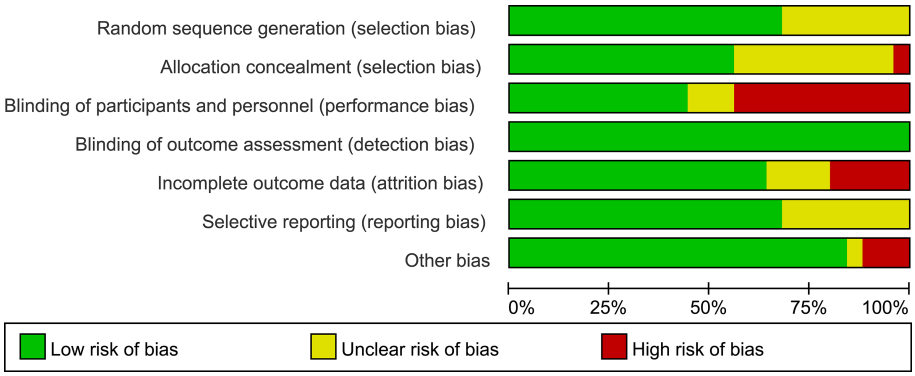

FIGURE 3 | Risk of bias graph. Review of authors' judgements about each risk of bias presented as percentages across all included studies.

SGLT-2 inhibitors being among the top three most effective drugs were 73% and 89%, respectively (**Figure 6C**).

Compared with SGLT-2 inhibitors, GLP-1 RAs had a higher probability of reducing VAT (MD -0.560 [-0.961 to -0.131]), whereas they did not have different effects on SAT (MD 0.184 [-0.669 to 1.030]) (**Figure 7**).

3.3.2.2 BMI and Weight

BMI was reported in 18 trials with 1006 patients (**Appendix 4; Supplementary Figure 4**). Compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on BMI (MD -1.262 [-2.933 to 0.218] and -0.964 [-2.385 to 0.423], respectively) (**Figure 5D**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 87% and 67%, respectively (**Figure 6D**).

Weight was reported in 19 trials with 1143 patients (**Appendix 4; Supplementary Figure 5**). As shown in **Figure 5D**, compared with the control group, GLP-1 RAs significantly reduced body weight (MD -2.394 [-4.625 to -0.164]), whereas SGLT-2 inhibitors had no effect (MD -1.059 [-3.056 to 0.931]). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 98% and 66%, respectively (**Figure 6E**).

Compared with SGLT-2 inhibitors, GLP-1 RAs showed no difference in the effects on BMI or weight (MD 0.501 [-1.582 to 2.434] and 0.5796 [-4.127 to 5.034], respectively) (**Figure 7**).

3.3.2.3 Leptin and Adiponectin

Leptin was reported in 4 trials with 158 patients (**Appendix 4; Supplementary Figure 6**). None of the studies reported the effect of GLP-1 RAs on leptin. Compared with the control group, SGLT-2 inhibitors had no statistically significant effect on leptin (MD -6.479 [-17.4 to 3.127]) (**Figure 5E**). The SUCRA chart shows that the probabilities of SGLT-2 inhibitors being among the top three most effective drugs is 92% (**Figure 6F**).

7 trials, including 345 patients, reported adiponectin (**Appendix 4; Supplementary Figure 7**). Compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on adiponectin (MD 7.007 [-5.033 to 18.850] and 3.402 [-7.910 to 14.670], respectively) (**Figure 5F**). The SUCRA chart shows that the probabilities of GLP-1 RAs being among the top three most effective drugs was 77%, while SGLT-2 inhibitors' was only 31% (**Figure 6G**).

There was no difference between GLP-1 RAs and SGLT-2 inhibitors in the effect on adiponectin (MD 3.575 [-8.045 to 15.340]) (**Figure 7**).

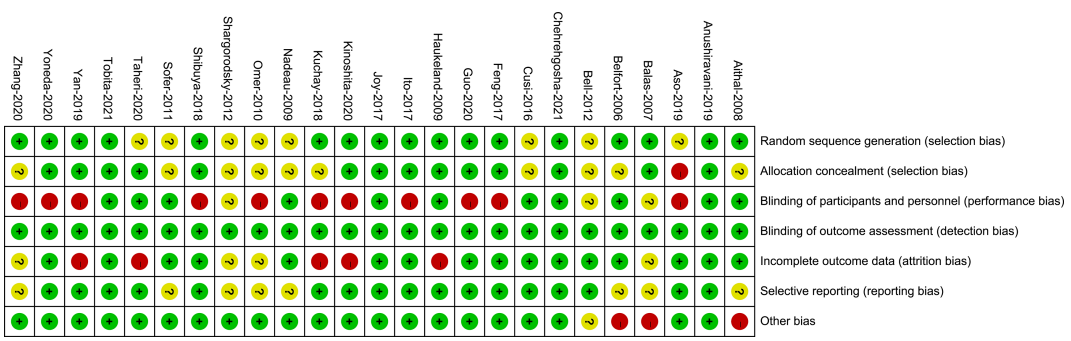

FIGURE 4 | Risk of bias summary. Review of authors' judgements about each risk of bias for each included study.

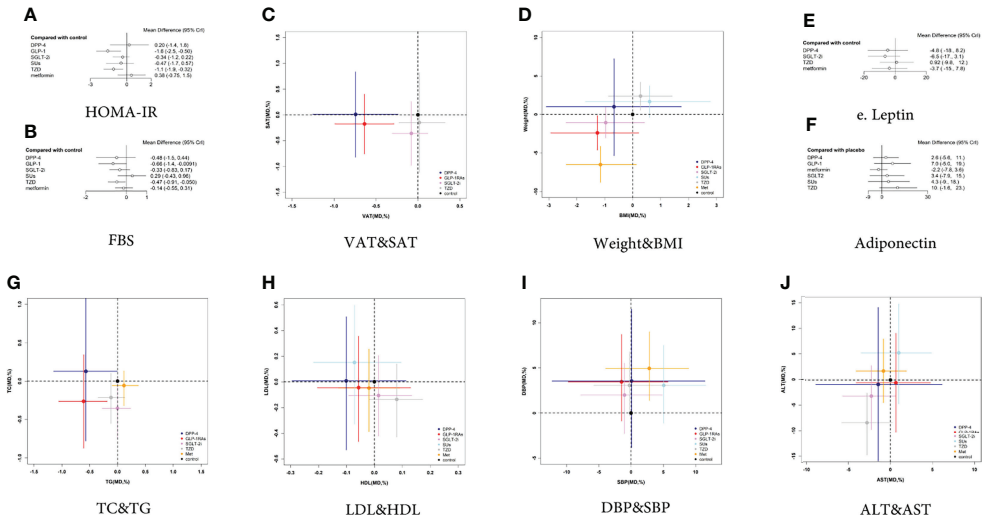

FIGURE 5 | Two-dimensional graphs and forest plots for different outcome indicators. **(A)** HOMA-IR, **(B)** FBS, **(C)** VAT and SAT, **(D)** Weight and BMI, **(E)** Leptin, **(F)** Adiponectin, **(G)** TC and TG, **(H)** LDL and HDL, **(I)** DBP and SBP, **(J)** ALT and AST.

3.3.3 Glucose and Lipid Metabolism

3.3.3.1 FBS

FBS was reported in 20 trials with 1216 patients (**Appendix 4; Supplementary Figure 8**). Compared with the control group, GLP-1 RAs decreased the FBS (MD -0.663 [-1.377 to -0.021]), whereas SGLT-2 inhibitors had no statistically significant effect (MD -0.330 [-0.832 to 0.170]) (**Figure 5B**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 89% and 47%, respectively (**Figure 6H**). GLP-1 RAs and SGLT-2 inhibitors showed no difference in the effect on FBS (MD -0.333 [-1.106 to 0.371]) (**Figure 7**).

3.3.3.2 TG and TC

TG was reported in 17 trials with 986 patients (**Appendix 4; Supplementary Figure 9**). Compared with the control group, GLP-1 RAs decreased TG (MD -0.608 [-1.056 to -0.188]), whereas SGLT-2 inhibitors had no statistically significant effect (MD -0.003 [-0.279 to 0.234]) (**Figure 5G**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 99% and 12%, respectively (**Figure 6I**).

TC was reported in 12 trials with 741 patients (**Appendix 4; Supplementary Figure 10**). Compared with the control group, neither GLP-1 RAs nor SGLT-2 inhibitors had any statistically significant effect on TC (MD -0.263 [-0.872 to 0.344] and -0.354 [-0.754 to 0.035], respectively) (**Figure 5G**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 75% and 92%, respectively (**Figure 6J**).

Compared with SGLT-2 inhibitors, GLP-1 RAs had a higher probability of decreasing TG (MD -0.607 [-1.095 to -0.116]). However, there was no difference between GLP-1 RAs and SGLT-2 inhibitors in effect on TC (MD 0.090 [-0.568 to 0.750]) (**Figure 7**).

3.3.3.3 HDL and LDL

HDL was reported in 19 trials with 1171 patients (**Appendix 4; Supplementary Figure 11**). Compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on HDL (MD -0.056 [-0.204 to 0.129]) and 0.015 [-0.092 to 0.133]) (**Figure 5H**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 21% and 67%, respectively (**Figure 6K**).

LDL was reported in 19 trials with 1171 patients (**Appendix 4; Supplementary Figure 12**). Compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on LDL (MD -0.045 [-0.466 to 0.355] and -0.107 [-0.421 to 0.205], respectively) (**Figure 5H**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 44% and 64%, respectively (**Figure 6L**).

Compared with SGLT-2 inhibitors, GLP-1 RAs showed no difference in effects on HDL or LDL (MD -0.072 [-0.228 to 0.119] and 0.061 [-0.404 to 0.512], respectively) (**Figure 7**).

3.3.4 Blood Pressure: SBP and DBP

SBP was reported in 9 trials with 604 patients (**Appendix 4; Supplementary Figure 13**). As shown in **Figure 5I**, compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on SBP (MD -1.486 [- 9.753 to 5.709] and -1.029 [- 7.830 to 4.853], respectively). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 77% and 67%, respectively (**Figure 6M**).

DBP was reported in 9 trials with 604 patients (**Appendix 4; Supplementary Figure 14**). Compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on DBP (MD 3.457 [-0.877 to 8.709) and 1.990 [-2.272 to

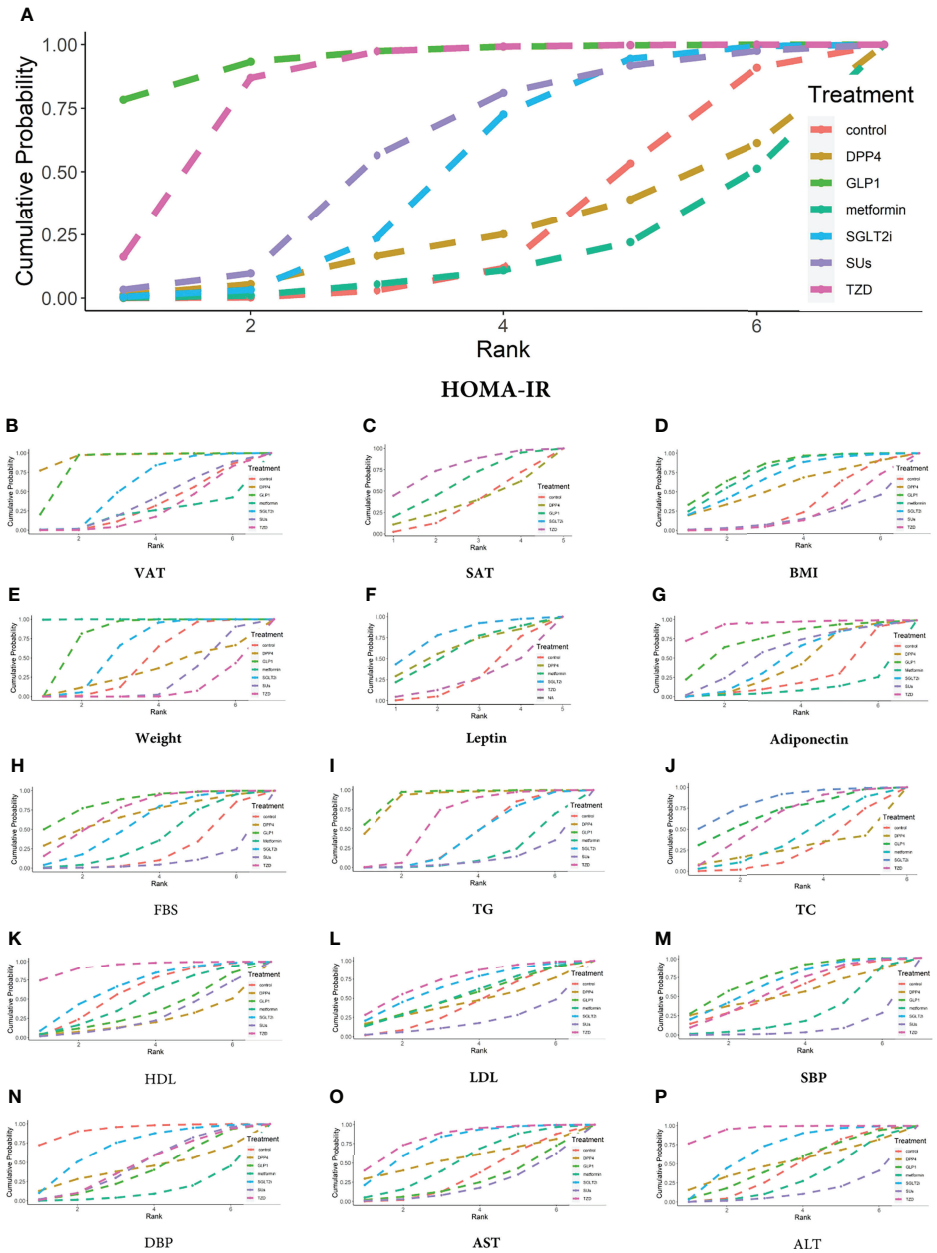

FIGURE 6 | Ranking probabilities of different hypoglycemic agents for different outcome indicators. **(A)** HOMA-IR, **(B)** VAT, **(C)** SAT, **(D)** BMI, **(E)** Weight, **(F)** Leptin, **(G)** Adiponectin, **(H)** FBS, **(I)** TG, **(J)** TC, **(K)** HDL, **(L)** LDL, **(M)** SBP, **(N)** DBP, **(O)** AST, **(P)** ALT.

5.526], respectively) (**Figure 5I**). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 22% and 75%, respectively (**Figure 6N**).

Compared with SGLT-2 inhibitors, GLP-1 RAs showed no difference in effects on SBP or DBP (MD 0.460 [-8.146 to 7.142] and 1.311 [-2.683 to 7.328], respectively) (**Figure 7**).

3.3.5 Liver Function: AST and ALT

AST was reported in 20 trials with 1206 patients (**Appendix 4; Supplementary Figure 15**). As shown in **Figure 5J**, compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on AST (MD 0.643 [-4.097 to 4.777]and -2.274 [-5.712 to 0.588], respectively). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2

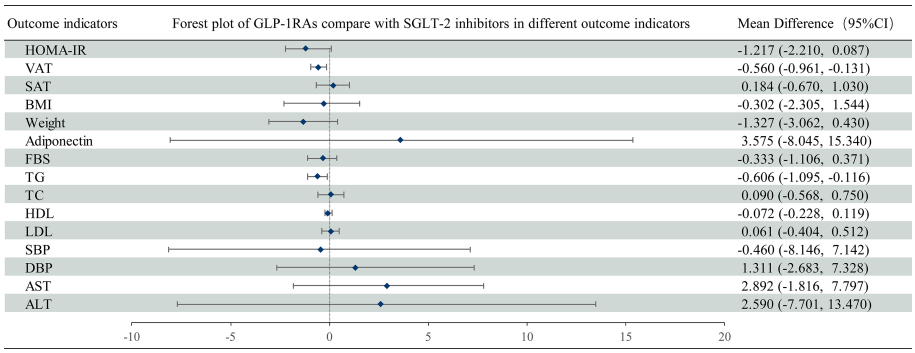

FIGURE 7 | Mean difference of GLP-1 RAs compared with SGLT-2 inhibitors on different outcome indicators in NAFLD patients. Mean difference and 95% confidence intervals were derived with the use of network meta-analysis. GLP-1 RAs, Glucagon-like peptide-1 receptor agonists; SGLT-2 inhibitors, Sodium-glucose cotransporter-2 inhibitors; CI, confidence interval.

inhibitors being among the top three most effective drugs were 13% and 84%, respectively (**Figure 6O**).

ALT was reported in 22 trials with 1312 patients (**Appendix 4; Supplementary Figure 16**). As shown in **Figure 5J**, compared with the control group, GLP-1 RAs and SGLT-2 inhibitors had no statistically significant effect on ALT (MD -0.534 [-10.180 to 9.163] and -3.136 [-9.704 to 2.860], respectively). The SUCRA chart shows that the probabilities of GLP-1 RAs and SGLT-2 inhibitors being among the top three most effective drugs were 39% and 73%, respectively (**Figure 6O**).

There was no difference between GLP-1 RAs and SGLT-2 inhibitors on AST or ALT (MD 2.892 [-1.816 to 7.797] and 2.590 [-7.701 to 13.470], respectively) (**Figure 7**).

3.4 Heterogeneity and Inconsistency Test

The difference value in deviance information criteria between the consistency and inconsistency models was less than 5, indicating the data have met the premise of consistency. In terms of deviance information criteria and mean posterior residuals, the RE model provided a better fit than the FE model in the analysis of all outcome indicators except for SAT and weight (**Appendix 5**). The node splitting method based on a Monte Carlo Markov Chain simulation was used to evaluate the network inconsistency of different outcome indicators, considering random-effect models, normal priors for treatment fixed effects, and uniform priors for the variances of the random effects. Supplementary materials (**Appendix 6**) show evidence of overall network inconsistencies or heterogeneity with no severe concerns of incoherence between direct and indirect evidence, and there were no local inconsistencies except for the following: (1) BMI of TZDs versus GLP-1 RAs, and TZDs versus metformin (P = 0.049 and 0.006, respectively); (2) FBS between TZDs and metformin (P = 0.046); (3) HDL level between TZDs and GLP-1 RAs, and metformin and GLP-1 RAs (P = 0.006 and 0.032, respectively); (4) AST level between metformin versus TZDs (P= 0.008); and (5) ALT level between metformin versus TZDs (P= 0.006). Convergence analysis shows that each Monte Carlo Markov chain achieved stable fusion from the initial part, and it could be visually analyzed in the subsequent calculation. Single chain fluctuations could not be recognized, which means the degree of convergence was high (**Appendix 7; Supplementary Figures 1–16**).

4 DISCUSSION

To our knowledge, this is the first systematic review and network meta-analysis to directly compare the effects of GLP-1 RAs and SGLT-2 inhibitors on IR levels in patients with NAFLD. NAFLD is a chronic metabolic liver disease, with the main clinical manifestation being increased lipid accumulation in the liver without a clear link to alcohol consumption and is a clinical manifestation of metabolic syndrome in the liver (32). In 2020, two articles proposed that NAFLD should be renamed MAFLD (metabolic associated fatty liver disease), and experts have agreed that compared with NAFLD, MAFLD more accurately reflects the mechanism of NAFLD (32, 33).

Given the increasingly defined metabolic nature of the disease, treatments targeting metabolism will be very promising. GLP-1 RAs and SGLT-2 inhibitors are two types of drugs that treat NAFLD through metabolic targeting. We evaluated the effect of these two drugs on the degree of IR, in patients with NAFLD, by applying Bayesian network meta-analysis and showing that, compared with the control group, GLP-1 RAs can reduce HOMA-IR value, weight, VAT, FBS, and TG, whereas SGLT-2 inhibitors had no significant effect on those outcomes. In addition, in the absence of head-to-head comparisons between GLP-1 RAs and SGLT-2 inhibitors, we also found significant differences between them. Importantly, GLP-1 RAs reduced VAT content and TG levels to a greater extent than SGLT-2 inhibitors. Our results provide both direct and indirect evidence that GLP-1 RAs improves IR and has certain advantages over SGLT-2 inhibitors in ameliorating IR in NAFLD patients.

GLP-1 RAs are incretin hormones secreted by intestinal L-cells following meal ingestion, and have various metabolic functions, including: 1) inducing β-cell proliferation and reducing lipotoxic β-cell apoptosis; 2) enhancing both insulin

synthesis and glucose-stimulated insulin secretion; 3) inhibiting glucagon secretion in a glucose-dependent manner; 4) reducing IR and improving peripheral insulin sensitivity through promoting weight loss caused by delayed gastric emptying and appetite suppression; and 5) increasing liver and muscle glucose uptake, followed by lowering of free fatty acid levels (34–37). A recent meta-analysis, concerning the use of GLP-1 RAs in patients with NAFLD (12 studies involving 780 patients), found significant improvements in FBS levels and HOMA-IR when the trial lasted longer than 24 weeks in subgroup analysis (38), similar to the results of our analysis. However, few studies have focused on the improvement in IR. In another RCT, GLP-1 RAs also reduced VAT in patients with polycystic ovary syndrome (39). In addition, low activity of brown adipose tissue has been associated to NAFLD (40), but none of 25 included RCTs have involved data of brown adipose tissue between groups, suggesting the need for NAFLD drug therapy studies focusing on brown adipose tissue. Mechanistically, GLP-1 RAs reduce hepatic steatosis and increases insulin sensitivity of hepatocytes through AMP-activated protein kinase, which exert an influence on insulin signaling pathways (41). At the same time, GLP-1 RAs may also reduce the expression of genes related to fatty acid synthesis, TG level or *de novo* synthesis, and the accumulation of liver and ectopic fat (42), which is consistent with the results obtained in this paper. We speculate that GLP-1 RAs improve IR and further reduce FBS and TG, as well as improve glucose and lipid metabolism by reducing VAT. This would suggest that GLP-1 RAs should be applied in NAFLD patients with IR and obesity (especially abdominal obesity), and glucose or lipid metabolic disorders. Moreover, GLP-1 RAs tended to reduce BMI, TC, SAT and LDL levels, and increase HDL and adiponectin, but these improvements were not statistically significant. In the included studies, the mean duration of medication in all 25 studies was 28.86 weeks, but for those studies using GLP-1 RAs, medication was collected after taken for only 20.4 weeks in average. The average duration of treatment with GLP-1 RAs was less than the average intervention duration of all 25 studies, which may have reduced efficacy.

SGLT-2 inhibitors are a new class of antidiabetic drugs that reduce blood sugar by inhibiting the kidney's reabsorption of glucose and allowing excess glucose to be excreted in the urine. In short, its mechanism of action is the direct excretion of glucose instead of insulin sensitization to promote glucose transport. Its principle is similar to the dam principle, only promoting the excretion of excess glucose, which also makes the risk of hypoglycemia low. In animal studies, SGLT-2 inhibitors have reduced new fat generation and increased lipoprotein decomposition (43, 44). Based on the existing literature, SGLT-2 inhibitors have been suggested to reduce HOMA-IR, weight, BMI, SAT, VAT, FBS, TC, LDL, AST, ALT, SBP, and also increase HDL, but these improvements were not statistically significant. According to SUCRA, SGLT-2 inhibitors have more advantages than GLP-1 RAs in improving HDL, LDL, TC, AST, ALT, and DBP in NAFLD patients. Considering that some of our patients with NAFLD did not have T2DM comorbidity, their median FBS was 7.66 mmol/L, indicating glucose toxicity was not severe. In this case, due to the normal levels of glucose, the ability of SGLT-2 inhibitors to improve IR, i.e. by excreting excess glucose, would not be activated. No trial has been reported on SGLT-2 inhibitors in pure NAFLD patients without T2DM diabetes. A study on the "Effect of Empagliflozin on Liver Fat in Non-diabetic Patients" (NCT04642261) has been registered in Clinical Trials and is expected to be completed by December 31, 2022. In addition, the average duration of SGLT-2 inhibitor medication for all studies was 25.09 weeks, shorter than the average duration of intervention in the included studies overall, which may be one of the reasons why SGLT-2 inhibitors have no significant effect on IR in NAFLD patients.

The advantages of this systematic review and network meta-analysis are as follows. First, we grasp the nature of NAFLD as a metabolic disease and focus our analysis on IR as a metabolic marker. Second, a network meta-analysis is used to comprehensively measure the effects of GLP-1 RAs and SGLT-2 inhibitors on various indicators that are related to IR in patients with NAFLD, making up for the lack of direct comparison between them. Third, a network meta-analysis is used to enlarge the sample size and correct the results obtained with smaller sample size. In addition, the emergence of new studies on these two classes of drugs has created a need for updated analysis, and this article meets this need (45–55).

There are also some limitations in this study. First, there is some heterogeneity in the clinical environment of each trial. For example, due to the small number of related studies in this field, we did not limit whether the included patients had diabetes, which may lead to some heterogeneity. Still, the consistency of the results was acceptable. We also run both the RE and the FE models, choosing the appropriate model to obtain more reliable results. Second, the measurement of insulin resistance in our included trails were HOMA-IR instead of hyper-insulinemic-euglycemic clamp technology, which is internationally recognized as the gold standard. Hyper-insulinemic-euglycemic clamp technology can be applied to all study groups, but at the same time it is a complex operation and requires repeated blood puncture. HOMA-IR is suitable for large-scale evaluation of IR in research with large sample sizes (56). However, the sample size of some included trials was relatively small and the application of HOMA-IR to evaluate IR may have some defects. Therefore, we selected other indicators that are highly correlated with the degree of IR, such as SAT, VAT, BMI, TG, and adipocytokines (57–60) to assist judgment of IR and make up for this deficiency. Finally, the average duration of treatment was not balanced. For example, the average duration of treatment with GLP-1 RAs and SGLT-2 inhibitors was lower than the average duration of all included studies, which suggests that larger and longer RCTs are needed to verify our results.

5 CONCLUSION

In conclusion, this network meta-analysis provides evidence for the effect of GLP-1 RAs and SGLT-2 inhibitors on reducing IR in patients with NAFLD. This study suggests that GLP-1 RAs can improve the metabolism of NAFLD, and in this regard, the effect

of SGLT-2 inhibitors still needs to be determined using rigorous long-term and large-scale RCTs.

6 PROSPECTS

As one of the most prevalent chronic diseases in the world, the public health and economic impact of NAFLD has been gradually given increasing attention by patients, regulatory agencies, and biopharmaceutical organizations. Although the cure for NAFLD is still unknown, drug research and development for each link of its mechanism is underway. Due to the close relationship between NAFLD and metabolic syndrome, especially IR, this review indicates that GLP-1 RAs, but not SGLT-2 inhibitors can be used for treating NAFLD patients, based on obesity especially abdominal obesity, a high-HOMA-IR index and glucose or lipid metabolic disorder. More clinical studies targeting IR are needed to provide more evidence for improving IR and reduce the risk of chronic complications in patients with 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

HY designed the study, collected the data, contributed to the statistical analysis, and served as the primary author of the manuscript. WL co-designed the study and contributed to the writing of the manuscript and provided critical feedback to shape the manuscript. CH and XS contributed to the statistical analysis and assisted with the writing of the manuscript. JL and SZ contributed to the data collection. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.923606/full#supplementary-material

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Association between use of liraglutide and liver fibrosis in patients with type 2 diabetes

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Objective: Patients with type 2 diabetes have a high risk of non-alcoholic fatty liver disease (NAFLD) and related liver fibrosis. Glucagon-like peptide-1 receptor agonists (GLP-1RAs) have demonstrated efficacy in improving NAFLD, while their effectiveness on liver fibrosis is limited in type 2 diabetic patients.

Materials/Methods: A prospective cohort study was performed in type 2 diabetic patients. The study subjects were divided into two groups based on the use of liraglutide or not, and propensity score matching (PSM) was also conducted. After 12 months follow-up, liver fibrosis was assessed by NAFLD fibrosis score (NFS) fibrosis-4 (FIB-4), and liver stiffness measurement (LSM). The association between liraglutide use and liver fibrosis was analyzed by multivariable linear regression.

Results: In the current study, a total of 1,765 type 2 diabetic patients were enrolled. 262 patients were liraglutide user and 1,503 were nouser. After 12 months follow-up, liraglutide use tended to be associated with reduced prevalence of advanced fibrosis (3.1% vs. 6.1%, P = 0.218). After adjustment for confounding factors, multivariable linear regression revealed that liraglutide use was negatively associated with decreased NFS (β= -0.34, P = 0.043), FIB4 (β= -0.26, P = 0.044) and LSM (β= -4.95, P = 0.007) in type 2 diabetics. The results after PSM were similar to those before PSM.

Conclusions: Liraglutide treatment is associated with decreased liver fibrosis in type 2 diabetic subjects.

KEYWORDS

nonalcoholic fatty liver disease, type 2 diabetes mellitus, liver fibrosis, obesity, liraglutide

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disorder worldwide, which includes a range of pathological conditions from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis and even hepatocellular carcinoma (HCC) (1). The incidence of NAFLD is rising along with obesity and type 2 diabetes mellitus (T2DM). NAFLD is estimated to affect up to 25% of the general population and 70%–80% of people with T2DM (1–3). T2DM further promotes the progression of NAFLD from simple steatosis to NASH and fibrosis (3).

During the last decade, it has grown increasingly evident that hepatic fibrosis is the strongest predictor of NAFLD-related morbidity and mortality (4, 5). The presence of clinically relevant liver fibrosis (F2 to F4) can occur in up to 15% of those with NAFLD and T2DM (6). Early recognition and treatment of NAFLD and liver fibrosis in people with T2DM are crucial. Although liver biopsy remains the gold-standard diagnosis of fibrosis, several non-invasive indices including NAFLD fibrosis score (NFS) and fibrosis-4 (FIB-4) can be used to estimate the prevalence and extent of fibrosis (7, 8). With regard to the treatment of NAFLD, up to date the pharmacological therapy of NAFLD and related liver fibrosis is still rare.

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are subcutaneous antidiabetic drugs approved for the treatment of T2DM. They are also effective in reducing both body weight and visceral adipose tissue, and have beneficial effects on the risk of cardiovascular and renal outcomes (9–13). In addition, recent evidence has shown that GLP-1RAs also improve hepatic histological components of NAFLD (14–16). Liraglutide and semaglutide consistently resolved NASH histologically in 40% to 60% of patients (17, 18). However, their effects on fibrosis in NAFLD were inconsistent (17, 18). Thus, it remains to be determined whether GLP-1RAs have ameliorative effects on NAFLD related liver fibrosis.

The present study was therefore conceived to explore the association between liraglutide use and liver fibrosis related to NAFLD in an unselected sample of adults with T2DM.

Methods

Subjects

All subjects were enrolled from the department of Endocrinology and Metabolism at Shanghai General Hospital from May 2017 to June 2021. Diagnosis of type 2 diabetes was based on the 1999 World Health Organization criteria. A standard questionnaire was distributed to all participants, which asked questions about present and past illnesses and medical treatment, and subjects with an alcohol intake >140 grams per week for men and 70 grams per week for women, with hepatitis, auto-immune hepatitis, or any other chronic liver disease, and with the treatment of pioglitazone and other GLP-1RAs rather than liraglutide were excluded from the study. The subjects were followed for 12 months and data was collected at baseline and 12 months later. In the end, 1,765 type 2 diabetic patients were included in the final analysis. The Institutional Review Board of Shanghai General Hospital affiliated to Shanghai Jiao Tong University School of Medicine approved this study, which was performed in accordance with the principle of the Helsinki Declaration II. Written informed consent was obtained from all subjects.

Anthropometric and biochemical measurements

Body weight, height, systolic and diastolic blood pressure (SBP, DBP) were measured after overnight fasting for at least 8 hours. BMI was calculated by dividing the body weight by the square of height in meters.

A nurse with extensive experience collected blood samples. Biochemical parameters including serum triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum creatinine (Scr) and serum uric acid (SUA) were measured using an autoanalyzer (Beckman, Palo Alto, CA). Blood glucose were measured with glucose oxidase method and HbA1c was evaluated by high-performance liquid chromatography.

Non-invasive assessments of liver fibrosis

NFS was calculated according previous study: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+ 1.13 \times$ IFG/diabetes (yes = 1, no = 0) $+ 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet ($\times 10^9$/L) $- 0.66 \times$ albumin (g/dL) (7). As all subjects in the present study were diabetic, so NFS = $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+ 1.13 + 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet ($\times 10^9$/L) $- 0.66 \times$ albumin (g/dL). FIB-4 was calculated as follow: (age (years) $\times$ AST (U/L))/(platelet count ($\times 10^9$/L) $\times$ ALT (U/L)1/2) (8). In addition, liver stiffness measurement (LSM) was performed using Fibroscan (Echosens®, Paris, France).

Statistical analysis

All statistical analyses were performed using SPSS 13.0 (Chicago, IL). Continuous variables were presented as means ± SD or median (interquartile range). Differences among groups were tested by t test for continuous variables and x^2 test for categorical variables. Multivariate linear regression model was performed to evaluate the independent association between

liraglutide use and liver fibrosis assessed by NFS. $P < 0.05$ was considered statistically significant.

Results

Baseline clinical characteristics of the subjects before and after matching

Among the 1,765 type 2 diabetic patients, 262 were taking liraglutide users and 1,503 were nousers. Clinical characteristics of the subjects according to the use of liraglutide were summarized in Table 1. Proportion of female, BMI, DBP, duration of diabetes, ALT, AST, UA, HbA1C was significantly higher, while age, HDL-C was lower in the user of liraglutide when compared with the nousers (all $P < 0.05$). There was no significant difference in SBP, FBG, Scr, TC, LDL-C and NFS between the two groups.

Furthermore, the subjects were propensity score matching (PSM) (1:1) according to the age, sex and BMI of the subjects. Clinical characteristics of the matched population was exhibited in Table 2, liraglutide users and nousers had similar age and sex proportion, and the difference of BMI was decreased when compared with the difference before PSM.

Effects of liraglutide on clinical data and the prevalence of advanced liver fibrosis

After a 12-month follow-up, liraglutide users showed a significant reduction in body weight and BMI compared to the control group (all $P < 0.05$). In contrast, there was no significant differences in HbA1c and ALT (Figures 1A–D). After PSM, similar results were observed in the cohort (Figures 1E–H).

Next, liver fibrosis was evaluated by NFS and the overall prevalence of advanced liver fibrosis (NFS > 0.676) was 5.0%. At baseline, the prevalence of advanced liver fibrosis in the control and liraglutide group was 4.4% and 8.3%, respectively ($P < 0.01$). After 12 months treatment, the prevalence of advanced liver fibrosis in the two group was comparable (3.4% vs. 3.1%, $P > 0.05$) (Figure 2A). In the cohort after PSM, the prevalence of advanced liver fibrosis in the control and liraglutide group at baseline was 5.3% and 8.1%, respectively ($P > 0.05$). After 1 year treatment, the prevalence of advanced liver fibrosis was 6.1%, while that in the liraglutide group was decreased to 3.1%, though the difference was not significant probably due to the limit of sample size ($P = 0.218$) (Figure 2B).

Association of liraglutide use with liver fibrosis

Using multivariate linear regression, we further studied the independent association between liraglutide use and noninvasive liver fibrosis markers including NFS and FIB-4. As shown in Table 3, liraglutide use was negatively associated with NFS after adjustment for age, sex, BMI, SBP, DBP, smoking, drinking and duration of diabetes (model 1). After further adjustment for FBG, HbA1c, TG, TC, LDL-C and HDL-C (model 2), liraglutide use remained significantly correlated with NFS. Finally, additional adjustment of the use of other antidiabetic medicines including metformin, SGLT2i, sulfonylurea, DPP-4i and insulin also did not significantly change the association between liraglutide use and NFS (model 3). The association between liraglutide and NFS was further analyzed after PSM, and the results were consistent with those before PSM (Table 3). Consistently, use of liraglutide was also negatively associated with FIB-4 before and after PSM (Table 4).

Furthermore, we investigated the effect of liraglutide on LSM performed by transient elastography. As expected, treatment of liraglutide was associated with reduced LSM after adjustment of confounders (β: -4.95; 95%CI:-8.43, -1.47; P=0.007).

Discussion

NAFLD related liver fibrosis affects a large proportion of individuals with T2DM. Nonetheless, to date, no medicine has been approved for the treatment NAFLD and liver fibrosis. In the present study, we explored the association of liraglutide use and liver fibrosis in T2DM patients. It was found that liraglutide use was negatively associated with liver fibrosis in patients with T2DM.

NAFLD and related liver fibrosis is common in patients with T2DM. In one recent study, the prevalence of NAFLD in T2DM patients was 70%, while advanced liver fibrosis is 9% (6). In another meta-analysis, the prevalence of NAFLD in patients with T2DM was 55.5%, while the prevalence of advanced fibrosis is 17.0%. In our study, NFS was used as a marker of liver fibrosis and the prevalence of advanced liver fibrosis in T2DM was 5.0%. Different populations and methods to assess liver fibrosis may be responsible for the inconsistency in the above studies. In fact, T2DM has been recognized a promoter of liver fibrosis. Though the underlying mechanisms remain largely unknown, insulin resistance and hyperglycemia may contribute to liver fibrosis in T2DM patients (19, 20).

GLP-1RAs are widely used in the treatment of T2DM and are effective in lowering blood glucose level and body weight. In recent years, the role of GLP-1RAs in NAFLD has also been investigated. The role of GLP-1RAs in hepatic steatosis and NASH is demonstrated in the previous studies (15, 16, 18). In contrast, its role in liver fibrosis in NAFLD is still controversial. In the Liraglutide Efficacy and Action in Non-alcoholic steatohepatitis (LEAN) randomized phase 2 trial, liraglutide improved NASH as well as reduced fibrosis progression both in diabetics and non-diabetics (15). More recently, a phase 2 study of semaglutide, a longer-acting GLP-1RA has also shown

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TABLE 1 Baseline data in the population according the use of liraglutide before PSM.

Characteristics	Liraglutide nonusers	Liraglutide users	*P* value
Number	1503	262	
Age (years)	50.7 ± 11.9	47.8 ± 12.9	<0.001
DBP (mmHg)	77.1 ± 10.8	79.0 ± 10.1	0.007
SBP (mmHg)	129.3 ± 17.3	128.8 ± 16.5	0.677
BMI (kg/m^2)	25.2 ± 3.3	29.3 ± 3.4	<0.001
Duration (months)	53.1 ± 73.0	80.4 ± 85.2	<0.001
FBG (mmol/L)	8.3 ± 3.1	8.1 ± 2.8	0.351
HbA1c (%)	8.5 ± 2.2	8.8 ± 2.0	0.045
ALT (IU/L)	30.2 ± 30.8	36.6 ± 28.6	0.002
AST (IU/L)	23.9 ± 19.1	27.4 ± 19.9	0.007
Scr (μmol/L)	68.6 ± 205.0	64.1 ± 27.6	0.727
UA (μmol/L)	339.5 ± 100.7	361.2 ± 99.7	0.001
TG (mmol/L)	2.2 ± 3.2	2.6 ± 2.7	0.05
TC (mmol/L)	4.8 ± 1.4	4.8 ± 1.5	0.555
HDL-C (mmol/L)	1.0 ± 0.3	0.9 ± 0.2	<0.001
LDL-C (mmol/L)	2.8 ± 1.0	2.7 ± 1.0	0.14
NFS	-1.2 ± 1.2	-1.2 ± 1.3	0.689
Sex (n,%)			0.002
Female	471 (31.3%)	108 (41.2%)	
Male	1032 (68.7%)	154 (58.8%)	
Current smoking (n,%)			0.028
0	787 (69.8%)	166 (77.2%)	
1	341 (30.2%)	49 (22.8%)	
Current drinking (n,%)			0.787
0	701 (51.7%)	128 (52.7%)	
1	654 (48.3%)	115 (47.3%)	
Metformin (n,%)			<0.001
0	506 (33.7%)	41 (15.6%)	
1	997 (66.3%)	221 (84.4%)	
Akabose (n,%)			<0.001
0	1011 (67.3%)	119 (45.4%)	
1	492 (32.7%)	143 (54.6%)	
DPP-4i (n,%)			<0.001
0	537 (35.7%)	262 (100.0%)	
1	966 (64.3%)	0 (0.0%)	
SGLT-2i (n,%)			<0.001
0	1282 (85.3%)	165 (63.0%)	
1	221 (14.7%)	97 (37.0%)	
Sulfonylurea (n,%)			<0.001
0	1203 (80.0%)	245 (93.5%)	
1	300 (20.0%)	17 (6.5%)	
Insulin (n,%)			<0.001
0	1100 (73.2%)	162 (61.8%)	
1	403 (26.8%)	100 (38.2%)	

DPP-4i, dipeptidyl peptidase-4 inhibitor; GLP1-RAs, glucagon-like peptide 1 receptor agonists; HbA1c, hemoglobin A1c; SGLT-2i, sodium glucose co-transporters 2 inhibitor; TZD, thiazolidinedione.

to effectively reduce liver enzymes and amealioate NASH after 72 weeks of therapy. Nevertheless, the study failed to show any significant improvement in fibrosis stage (18). In another real-world study, GLP-1RAs use was shown to improve markers of liver fibrosis in T2DM (21). Consistently, we observed decreased prevalence of advance liver fibrosis in T2DM individuals treated with liraglutide compared with nousers. In addition, liraglutide use was negatively associated with NFS, FIB-4 and LSM, three

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TABLE 2 Baseline data in the population according the use of GLP-1RAs after PSM.

Characteristics	Liraglutide nonusers	Liraglutide users	*P* value
Number	254	254	
Age (years)	49.0 ± 13.2	47.8 ± 12.9	0.313
BMI (kg/m^2)	27.8 ± 4.3	29.1 ± 3.3	<0.001
DBP (mmHg)	78.8 ± 11.5	79.1 ± 10.0	0.783
SBP (mmHg)	133.0 ± 17.1	128.8 ± 16.6	0.006
Duration (months)	40.8 ± 58.5	81.0 ± 85.9	<0.001
FBG (mmol/L)	8.6 ± 3.2	8.2 ± 2.8	0.107
HbA1c (%)	8.6 ± 2.2	8.8 ± 2.0	0.314
ALT (IU/L)	37.0 ± 58.0	36.9 ± 28.9	0.978
AST (IU/L)	28.6 ± 38.6	27.4 ± 20.0	0.654
Scr (μmol/L)	61.2 ± 15.5	64.1 ± 27.6	0.148
UA (μmol/L)	350.5 ± 94.6	361.3 ± 99.3	0.212
TC (mmol/L)	4.9 ± 1.4	4.8 ± 1.5	0.311
TG (mmol/L)	2.2 ± 1.8	2.6 ± 2.7	0.071
HDL-C (mmol/L)	1.0 ± 0.2	0.9 ± 0.2	<0.001
LDL-C (mmol/L)	2.9 ± 0.9	2.7 ± 1.0	0.037
NFS	-1.1 ± 1.3	-1.2 ± 1.3	0.523
FIB-4	1.2 ± 0.8	1.1 ± 0.7	0.042
Sex (n,%)			0.716
0	98 (38.6%)	102 (40.2%)	
1	156 (61.4%)	152 (59.8%)	
Current smoking (n,%)			0.614
0	184 (72.7%)	189 (74.7%)	
1	69 (27.3%)	64 (25.3%)	
Current drinking (n,%)			0.428
0	173 (68.9%)	166 (65.6%)	
1	78 (31.1%)	87 (34.4%)	
Metformin (n,%)			<0.001
0	72 (28.3%)	40 (15.7%)	
1	182 (71.7%)	214 (84.3%)	
Akarbose (n,%)			<0.001
0	202 (79.5%)	115 (45.3%)	
1	52 (20.5%)	139 (54.7%)	
DPP-4i (n,%)			<0.001
0	106 (41.7%)	254 (100.0%)	
1	148 (58.3%)	0 (0.0%)	
SGLT-2i (n,%)			<0.001
0	209 (82.3%)	161 (63.4%)	
1	45 (17.7%)	93 (36.6%)	
Sulfonylurea (n,%)			<0.001
0	210 (82.7%)	237 (93.3%)	
1	44 (17.3%)	19 (6.7%)	
Insulin (n,%)			<0.001
0	202 (79.5%)	156 (61.4%)	
1	52 (20.5%)	98 (38.6%)	

DPP-4i, dipeptidyl peptidase-4 inhibitor; GLP1-RAs, glucagon-like peptide 1 receptor agonists; HbA1c, hemoglobin A1c; SGLT-2i, sodium glucose co-transporters 2 inhibitor; TZD, thiazolidinedione.

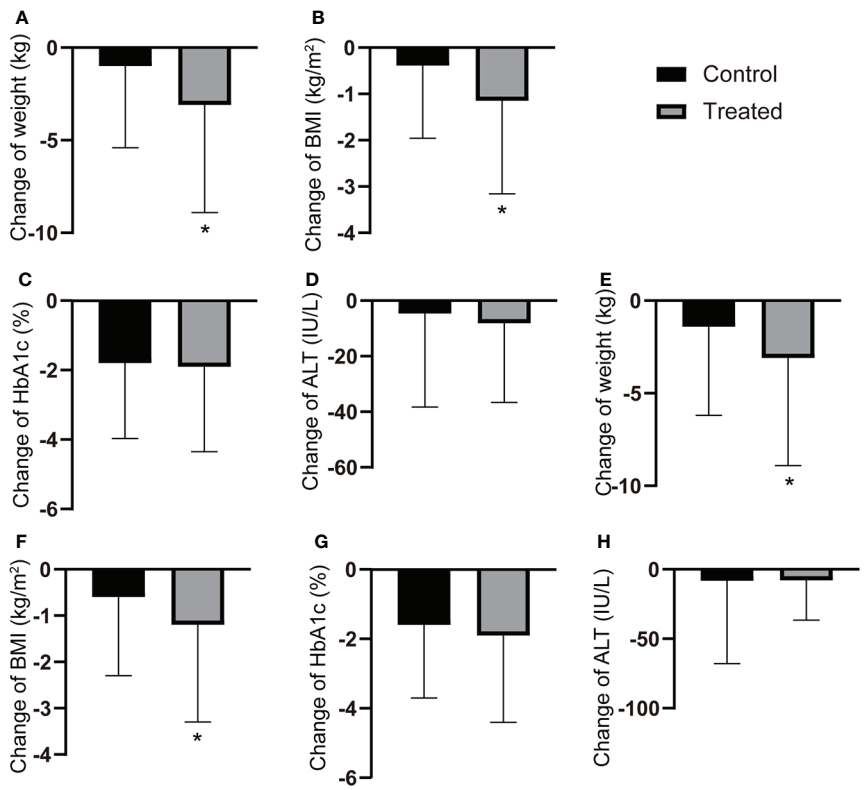

FIGURE 1
Changes of the clinical characteristics after 12-months follow-up. **(A-D)** The changes of body weight, BMI, HbA1c and ALT after 12-month follow-up in all T2DM patients with or without GLP-1RAs use. **(E-H)** The changes of body weight, BMI, HbA1c and ALT after 12-month flow-up in T2DM patients with or without GLP-1RAs use after PSM. *P < 0.05.

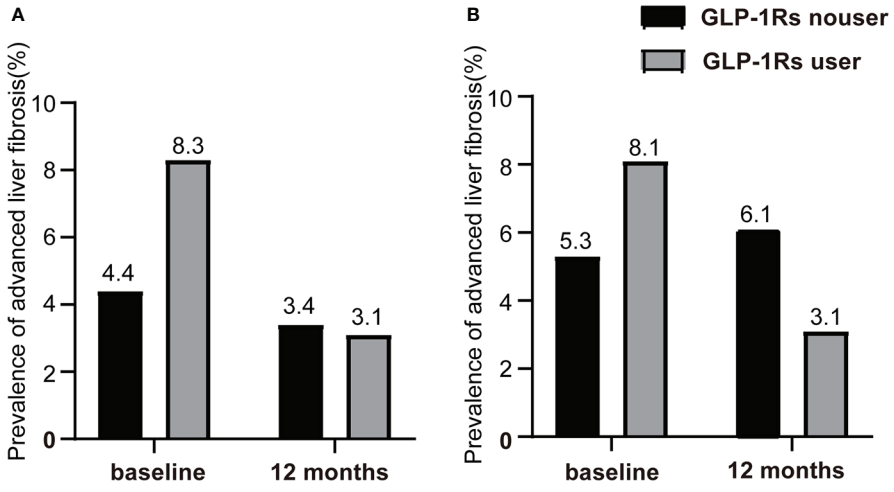

FIGURE 2
Change of the prevalence of advanced liver fibrosis after 12-month follow-up. **(A)** The prevalence of advanced liver fibrosis at baseline and 12-month follow-up in all T2DM patients with or without GLP-1RAs use. **(B)** The prevalence of advanced liver fibrosis at baseline and 12-month follow-up in T2DM patients with or without GLP-1RAs use after PSM.

TABLE 3 Association between GLP-1RAs use and NFS before and PSM.

Before PSM	β	95%CI	*P*
Model 1	-0.2	-0.45, 0.04	0.104
Model 2	-0.32	-0.62, -0.02	0.037
Model 3	-0.34	-0.67, -0.01	0.043
After PSM			
Model 1	-0.3	-0.54, -0.07	0.012
Model 2	-0.3	-0.55, -0.05	0.018
Model 3	-0.27	-0.54, -0.01	0.045

TABLE 4 Association between GLP-1RAs use and FIB-4 before and after PSM.

Before PSM	β	95%CI	*P*
Model 1	-0.17	-0.34, -0.01	0.048
Model 2	-0.24	-0.48, -0.01	0.045
Model 3	-0.26	-0.51, -0.01	0.044
After PSM			
Model 1	-0.23	-0.42, -0.03	0.022
Model 2	-0.23	-0.43, -0.04	0.021
Model 3	-0.25	-0.46, -0.04	0.021

noninvasive assessments of liver fibrosis. Thus, our study provides additional clinical evidence of a possible role of GLP-1RAs in the treatment of liver fibrosis in T2DM patients.

The mechanism of GLP-1RAs' role on NAFLD is still not well illustrated. Reduction in body weight is one of the reason responsible for the favorable effect of GLP-1RAs on NAFLD. Besides, GLP-1RAs have anti-inflammatory and antioxidant properties and contributed to significant reductions in biomarkers of inflammation and oxidative stress in clinical trials (22). In animal models, GLP-1RAs treatment could alleviate inflammation in liver (especially M1 pro-inflammatory macrophages accumulation) (23). These properties of GLP-1RAs could confer its protection against liver fibrosis.

The present study has several limitations that need to be considered. First, the follow up period was relative short, which may affect the association between liraglutide use and liver fibrosis. Second, this study did not include a diagnosis of NAFLD, which precluded stratifying by the presence or absence of NAFLD. Third, fibrosis was not evaluated by liver biopsy. Nevertheless, NFS, FIB-4 and LSM has become widely used detect liver fibrosis.

Conclusion

In conclusion, the present study showed that GLP-1RA use was negatively correlated with liver fibrosis in type 2 diabetic patients. GLP-1RAs may be a therapy to ameliorate liver fibrosis in type 2 diabetic patients.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Board of Shanghai General Hospital affiliated to Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study design: NF and YP; Collection and assembly of data: YT and QZ; Data analysis and interpretation: All authors; Manuscript writing: All authors; Final approval of manuscript: All authors.

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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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Effects of sitagliptin on intrahepatic lipid content in patients with non-alcoholic fatty liver disease

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Purpose: Dipeptidyl peptidase-4 inhibitors (DPP-4I), key regulators of the actions of incretin hormones, exert anti-hyperglycemic effects in type 2 diabetes mellitus (T2DM) patients. A major unanswered question concerns the potential ability of DPP-4I to improve intrahepatic lipid (IHL) content in nonalcoholic fatty liver disease (NAFLD) patients. The aim of this study was to evaluate the effects of sitagliptin on IHL in NAFLD patients.

Methods: A prospective, 24-week, single-center, open-label, comparative study enrolled 68 Chinese NAFLD patients with T2DM. Subjects were randomly divided into 4 groups: control group who did not take medicine (14 patients); sitagliptin group who received sitagliptin treatment (100mg per day) (17 patients); metformin group who received metformin (500mg three times per day) (17 patients); and sitagliptin plus metformin group who received sitagliptin (100mg per day) and metformin (500 mg three times per day) (20 patients). IHL, physical examination (waist circumstances, WC; body mass index, BMI), glucose-lipid metabolism (fasting plasma glucose, FPG; hemoglobin A1c, Hb1A1c; triglycerides; cholesterol; alanine aminotransferase, ALT; aspartate aminotransferase, AST) were measured at baseline and at 24 weeks.

Results: 1) WC and BMI were decreased significantly in all groups except control group (all $P<0.05$). 2) There was no statistically significant difference in IHL among the sitagliptin, metformin, and sitagliptin plus metformin groups before and after treatment(all $P>0.05$). Only the metformin group showed a statistically significant difference in IHL before and after treatment($P<0.05$). 3) Sitagliptin treatment led to a significant decrease in FBG and HbA1c when compared with the control group (all $P<0.01$). Additionally, HhA1c was significant decreased in the sitagliptin group when compared with the metformin group ($P< 0.05$). 4) HbA1c and FBG were decreased by 0.8% and 0.7 mmol/l respectively and the percentage of patients with HbA1c less than 7% was 65% with sitagliptin treatment.

Conclusion: Sitagliptin improves abnormalities in glucose metabolism, but not reduces the IHL in T2DM with NAFLD, indicating that sitagliptin might be a therapeutic option for treatment of NAFLD indirectly while not directly on IHL. Clinical Trial Registration: https://clinicaltrials.gov/, identifier CTR# NCT05480007.

KEYWORDS

intrahepatic lipid, nonalcoholic fatty liver disease, sitagliptin, metabolism, glucose

Introduction

Non-alcoholic fatty liver disease (NAFLD) is highly associated with several components of metabolic syndrome (MS), particularly obesity, increased plasma lipid levels, insulin resistance, and type 2 diabetes mellitus (T2DM) (1–3). The proportion of NAFLD patients with concomitant obesity and T2DM is in the range of 25%-75% (4, 5). Diabetic patients with NAFLD may be existing insulin resistance and impaired lipid metabolism, and the insulin resistance may further increase fat deposition in the liver (6, 7), thereby exacerbating both conditions.

Lifestyle adjustments leading to weight loss are effective in the treatment of NAFLD and can also improve insulin sensitivity (8–10). The present therapeutic options used for treatment of NAFLD patients with diabetes include insulin sensitizing agents such as metformin and thiazolidinediones (11, 12). Metformin maintains glucose homeostasis by increasing the utilization of glycogen and inhibiting hepatic glucose output, and reduces the accumulation of fat mass in liver (11, 13). However, the effects metformin on liver histology is not clear and metformin is currently not recommended in treatment of NASH (non-alcoholic steatohepatitis). By activation of the peroxisome proliferator activated receptor (PPAR), thiazolidinediones increase insulin sensitivity, regulate lipid metabolism, alleviate liver damage, and decrease fat accumulation in liver (14, 15). However, they have not been able to improve the histological appearance of liver damage. No effective drug therapy for NAFLD has been established. Thus, given the difficulty in sustaining lifestyle modification, effective pharmacological options are necessary and incretin based therapies may represent an effective option.

Glucose dependent insulinotropic polypeptide (GIP) and glucagon like peptide 1 (GLP-1) are known incretine hormones. However, the metabolic effects of GIP are blunted in T2DM and only GLP-1 remains of interest in the treatment of T2DM and related disorders (16). Circulating GLP-1 is readily degraded by the enzyme dipeptidyl peptidase-4(DPP-4) (17–19). Therefore, DPP-4 inhibitors (DPP-4I) have been developed which can inhibit the degradation of GLP-1, thereby increasing incretin levels, stimulating insulin secretion, increasing sensitivity of beta cells to incretins, and increasing beta cell proliferation (20). DPP-4I can reduce fasting and postprandial plasma glucose levels (21, 22) and reduce HbA1c, although to a greater extent in Asian patients compared with Caucasians (23). Thus, these agents may be particularly useful for Chinese patients. Sitagliptin is a DPP-4 inhibitor which can increase insulin release and decrease glucagon levels by increasing the levels of active incretin (24–26).

The GLP-1 receptor (GLP-1R) has been found on human hepatocytes (27). Sitagliptin treatment has been shown to be safe, well tolerated, and have anti-diabetic effects. A previous study has shown that the change in HbA1c after stigliptin treatment for 8 and 12 weeks were significantly greater in T2DM patients with NAFLD than in T2DM patients without NAFLD (28). Forty-one patients with biopsy-proven NAFLD with T2DM were treated with sitagliptin (50 mg/day) for 12 months and showed a 0.7% reduction in HbA1c (29). Another study showed that HbA1c and fasting plasma glucose (FPG) in 20 NAFLD patients with T2DM who were treated with sitagliptin group were significantly decreased compared with a control group treated with diet and exercise (30). Four months of treatment with sitagliptin in NAFLD patients with T2DM diagnosed by ultrasonography led to significant decreased plasma glucose, HbA1c, aspertate aminotransferase (AST), and alanine transaminase (ALT) (31). However, body weight did not change in NAFLD patients with T2DM treated with sitagliptin (32). Animal experiments have shown a decrease in intrahepatic lipid content (IHL) after treatment with DPP-4I (33, 34). However, the effect of DPP-4I on hepatic fat accumulation has not been fully evaluated in NAFLD patients with T2DM. Therefore, the aim of this study was to investigate the effect of sitgliptin on IHL and glucose-lipid metabolism in Chinese NAFLD patients with T2DM.

Methods

Subjects

A total of 68 Chinese subjects were recruited from the outpatient department of Shanghai Tenth People's Hospital. Informed consent was obtained from each participant after being informed of the aims of the study and potential adverse effects of the study drugs. This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital. Inclusion criteria were as follows: 1) age 30-70 years old, 2) fulfillment of the diagnostic criteria for T2DM by WHO in 1999 (HbA1c ranged from 7% to 10%, FPG< 11 mol/l, 2-hour blood glucose postprandial< 20mmol/l), 3) Fulfillment of the diagnostic criteria for NAFLD according to the guidelines of the Chinese Medical Association in 2010. The IHL content was measured by using 1H-magnetic resonance spectroscopy (1H-MRS) quantitative detection, and the liver fat content of the enrolled subjects was more than 5.5%. The subjects included in this study had either no history of alcohol consumption or their alcohol intake was less than 70 g/week in males and less than 140 g/week in females. Liver transaminase and serum creatinine were less than two times the upper limit of normal. The exclusion criteria were as following: 1) T2DM complicated with ketoacidosis, hyperosmolarity, acute and chronic infection, 2) serious heart, liver, kidney, lung disease, and liver damage, 3) alcoholic fatty liver, 4) drug use that influences glucose metabolism such as thiazide diuretics and hormones within three months, 5) Hypertension ≥ 180/110 mmHg, 6) gastrointestinal disease or absorption dysfunction, 7) recent trauma, surgery, or other conditions resulting in an increased stress response within the past three months. The clinical trial is NCT02118376.

Anthropometric data measurement

Weight (Wt), height, waist circumference (WC), and calculated to body mass index (BMI, according to the formula: BMI (kg/m^2) = body weight (kg)/height (m) ^{2}) were measured for each subject at baseline as well as at 24 weeks after starting the trial.

Lab testing

Venous blood was collected and serum was prepared after 8 hours of fasting before and after treatment. Total cholesterol (TCH), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), ALT, AST, and alkaline phosphatase (AKP) were measured using an automatic biochemistry analyzer (model) at baseline and 24 weeks. FPG was measured using the glucose oxidase method. HbA1c was measured using the BIO-RAD VARIANTII.

Measurement of liver fat content

To evaluate the liver fat content, HD magnetic resonance imaging (1.5 Tesla, GE) was performed at baseline and 24 weeks. The peak value of water proton peak, the area under the water proton peak (AUC-water), the peak value of fat peak and the area under the fat peak (AUC-IHL) were determined. The relative content of IHL was calculated by the following formula: IHL= AUC-IHL/(AUC-water+ AUC-IHL) ×100%.

Grouping

Subjects were randomly divided into 4 groups using blocked randomization according to the drug treatment:

Group A: control group (14 patients, average age:53.4 ± 5.2 years old). The subjects received nothing at all. They were under diet and exercise control.

Group B: sitagliptin group (17 patients, mean age:54.4 ± 9.4 years old). These subjects received sitagliptin 100 mg per day.

Group C: metformin group (17 patients, mean age: 55.6 ± 10.9 years old). These subjects took metformin 500 mg three times per day.

Group D: sitagliptin plus metformin group (20 patients, mean age 54.5 ± 5.6 years old). These subjects took sitagliptin 100 mg per day and metformin 500 mg three times per day.

The treatment period was 24 weeks. All subjects were given health education regarding eating a diabetic diet and performing routine exercise during the treatment period. Adverse reactions and side effects of drug were monitored throughout the study. The primary endpoint was liver fat content and secondary endpoints including indicators of lipid (TCH, TG and LDL-C) (HDL-C), liver enzymes (ALT, AST and AKP) and glucose(FPG and HbA1c).

Statistical analysis

All data are presented as mean ± standard deviation($\overline{X}$±s). SPSS17.0 software was used for statistical analysis. Comparison of mean values was performed using a paired or unpaired *t*-test. Variance analysis (ANOVA) was utilized when comparing the difference among groups. LSD (Least-Significant Difference) was

what we used for *post-hoc* tests in ANOVA. The statistical difference was considered significant for $P< 0.05$.

Results

The general clinical data

Self-pair comparison showed that BMI was significantly decreased with sitagliptin treatment($P< 0.05$). Additional comparisons between the sitagliptin group and control group are displayed in Table 1. WC and BMI showed a statistically significant decrease comparing baseline with 24 weeks after treatment with sitagliptin, metformin, and metformin and sitagliptin (all $P< 0.05$). There was no statistically significant difference in WC and BMI at 24 weeks among the three groups (all $P > 0.05$) as shown in Table1.

Liver fat content

The average IHL content was 61.88%, the highest IHL was 86.67% and the lowest IHL was 22.5% in sitagliptin group at baseline. The average IHL content was 42.11% in the sitagliptin group at 24 weeks which showed a trend decrease compared with baseline without statistical significance ($P > 0.05$). The average IHL was 54.43% at baseline and was 47% at 24 weeks in the control group. There was no significant statistical significance between sitagliptin group and the control group as shown in Table 2. IHL content was significantly decreased in metformin group (from 68.73 ± 14.6 to 32.57 ± 16.7) ($P< 0.05$). Data for liver function tests are shown in Table 2. ALT and AST were not significantly different among the groups before treatment, however after treatment, ALT and AST in the sitagliptin group was significnalty higher compared with the metformin group (all $P< 0.05$).

Glucose-lipid metabolism

Self-pair comparison showed that FBG and HbA1c were significantly decreased after 24 weeks treatment with sitagliptin (all $P< 0.05$). Additionally, FBG and HbA1c were significantly decreased in the sitagliptin group when compared with the control group (all $P< 0.01$) as shown in Table 3. There was a significant decrease in HbA1c in tbe sitagliptin group compared with the metformin group (all $P< 0.05$). However, the sitagliptin group had a higher HbA1c and FBG compared with the sitagliptin plus metformin group after the 24 week treatment period (all $P<0.05$) as shown in Table 3. HbA1c was decreased by 0.8% and FBG was decreased by 0.7mmol/l with sitagliptin treatment while HbA1c was decreased by 0.3% and FBG was decreased by 0.7mmol/l with metformin treatment. Additionally, the percentage of HbA1c less than 7% was 65% and 25% after sitagliptin and metformin treatment, respectively. HbA1c was decreased by 1% and FBG was decreased by 1.3mmol/l, and the percentage of HbA1c level that less than 7% was 72.7% after sitagliptin plus metformin treatment. TCH, TG, HDL-C, LDL-C showed no significant difference among the groups before or after treatment. There was a trend toward a

TABLE 1 Comparison of the clinical characteristics between groups.

Group		At baseline	At 24 weeks
A(control)	Sex	14(male/female:8/6)	14(male/female:8/6)
	Age (years old)	53.40 ± 5.20	53.40 ± 5.20
	BMI (kg/m^2)	24.06 ± 2.85	23.85 ± 2.74
	Waistline(cm)	83.0 ± 6.5	81 ± 6.87
B(sitagliptin)	Sex	17(male/female:10/7)	17(male/female:10/7)
	Age (years old)	54.40 ± 9.0	54.40 ± 9.40
	BMI (kg/m2)	25.41 ± 3.45	24.39 ± 3.02※
	Waistline(cm)	86.9 ± 9.8	84.88 ± 8.19※
C(metformin)	Sex	17(male/female:9/8)	17(male/female:9/8)
	Age (years old)	55.63 ± 10.9	55.63 ± 10.9
	BMI (kg/m^2)	26.46 ± 2.86	25.75 ± 3.55※
	Waistline(cm)	88.50 ± 8.00	88.67 ± 8.80※
D(Sitagliptin+ metformin)	Sex	20(male/female:9/11)	20(male/female:9/11)
	Age (years old)	54.55 ± 6.6	54.55 ± 6.6
	BMI (kg/m^2)	26.07 ± 3.24	25.53 ± 3.02※
	Waistline(cm)	90.0 ± 8.20	86.8 ± 6※

Self-pair comparison before and after treatment, ※P<0.05.
Data are number of patients or mean ± standard deviation.
BMI, body mass index.

TABLE 2 Comparison of the intrahepatic lipid and liver enzymes between groups.

Group		At baseline	At week 24
A(Control)	The maximum fat content(%)	84.74%	67.2%
	The minimum fat content(%)	6%	6.8%
	The average fat content(%)	54.43%	47.7%
	Fat content of male(%)	56.4%	49.6%
	Fat content of female(%)	52.4%	45.6%
B(Sitagliptin)	The maximum fat content(%)	86.67%	91.20%
	The minimum fat content(%)	22.5%	7.10%
	The average fat content(%)	61.88%	42.11%
	Fat content of male(%)	60%	40.18%
	Fat content of female(%)	64%	41.97%
	ALT(U/L)	26.81 ± 12.68	26.23 ± 17.33 △
	AST(U/L)	21.76 ± 5.02	22.00 ± 8.24 △
	AKP(U/L)	75.62 ± 22.49△	77.15 ± 37.94
C(metformin)	The average fat content(%)	68.73 ± 14.6	32.57 ± 16.7※
	ALT(U/L)	24.00 ± 9.53	22.00 ± 12.57※
	AST(U/L)	19.25 ± 3.62	21.20 ± 8.50
	AKP(U/L)	90.75 ± 14.87	71.20 ± 32.38
D(sitagliptin+ metformin)	The average fat content(%)	65.88 ± 16.4	42.7 ± 22.5
	ALT(U/L)	25.45 ± 14.45	25.14 ± 15.99
	AST(U/L)	22.00 ± 8.75	21.14 ± 7.27
	AKP(U/L)	64.18 ± 19.59△	59.00 ± 20.39

Self-pair comparison before and after treatment, ※P<0.05;vs Group C, △P<0.05.
ALT, alanine transaminase; AST, aspertate aminotransferase; AKP, alkaline phosphatase.

decrease in TG and HDL in the metformin group after treatment but this did not reach statistical significance (all $P>0.05$).

Discussion

The prevalence of NAFLD is increasing with the rapid development of modern economies which permit lifestyles contributing to the development of numerous metabolic disorders and making NAFLD one of the most serious chronic diseases globally (35). NAFLD has been associated with vascular endothelial dysfunction, which is an indicator of the early stages of arteriosclerosis (36). The thickness of the carotid artery intima-media and the plaque formation rate in NAFLD patients were higher compared with controls (37). It was further shown that, the risk of cardiovascular disease in NAFLD patients is 5.3 times higher than in controls, and the incidence of diabetes, hypertension, systemic inflammatory response and metabolic syndrome are 15 times higher than that of the control group (38). Therefore, an effective treatment is needed to improve the underlying disease process of patients with NAFLD. A previous study has shown that the effects of dietary intervention using a carbohydrate (CH)-restricted low energy diet (CH: 10% of total energy) for 48 hours lowered IHL content in obese subjects compared with a high CH diet (CH: 65% of total energy) (39). The effect of diet on reducing IHL content can also be found in T2DM patients or obese subjects with impaired glucose tolerance (IGT) (40–42). In this study, we explored the effects of sitagliptin on IHC lipid content and metabolic profile in NAFLD patients with T2DM.

Liver fat content measured by magnetic resonance 1H-MRS has been shown to be reliable and has been accurately replicated. The diagnosis of fatty liver is based on the area under lipid peak/AUC-IHL + AUC-water≥ 5.5% or area under lipid peak/area under water peak≥20% (43, 44). The liver fat content was about 65% and ranged from 6% to 89.7% in all these subjects. Many of the subjects in this study had never been shown to have fatty liver by abdominal ultrasound prior to entry into the study. 1H-MRS can detect mild fatty liver with a much higher sensitively than conventional ultrasonography. Utilizing 1H-MRS in our study, the average fat content of all subjects was about 65%, which classifies most patients as having moderate or severe fatty liver.

The mechanism of occurrence and development of NAFLD is not completely understood. One commonly proposed theory suggesting a “two hit” mechanism: too much triglycerides stored in the liver cells constitutes the first hit, and this contributes to the second hit which may be a variety insults such as insulin resistance, oxidative stress, inflammatory reaction, lipid peroxidation, etc (45). Logistic regression analysis also found that insulin resistance (HOMA-IR), waist to hip ratio, total cholesterol, and triglycerides are independent risk factors for

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TABLE 3 Comparison of the glucose-lipid data between groups.

Group		At baseline	At week 24s
A(control)	FPG(mmol/l)	9.45 ± 1.83	9.96 ± 2.55
	HbA1c(%)	8.08 ± 1.13	8.16 ± 1.37
	TCH(mmol/l)	4.81 ± 0.95	4.56 ± 0.55
	HDL(mmol/l)	1.22 ± 0.17	1.20 ± 0.24
	TG(mmol/l)	1.73 ± 0.93	1.59 ± 1.04
	LDL(mmol/l)	2.92 ± 1.02	2.92 ± 1.02
B(sitagliptin)	FPG(mmol/l)	8.39 ± 1.89	7.60 ± 1.55※*
	HbA1c(%)	7.93 ± 0.91	6.95 ± 0.86※*△
	TC(mmol/l)	5.22 ± 0.92	5.19 ± 0.95
	HDL(mmol/l)	1.31 ± 0.38	1.31 ± 0.50
	TG(mmol/l)	1.70 ± 0.82	1.67 ± 1.08
	LDL(mmol/l)	3.02 ± 0.97	3.02 ± 0.97
C(metformin)	FPG(mmol/l)	10.40 ± 2.46	9.71 ± 2.16
	HbA1c(%)	8.60 ± 1.17	8.38 ± 0.61
	TCH(mmol/l)	4.88 ± 0.64	4.84 ± 1.06
	HDL(mmol/l)	1.18 ± 0.31	1.20 ± 0.28
	TG(mmol/l)	1.94 ± 0.97	1.69 ± 0.83
	LDL(mmol/l)	2.83 ± 0.65	2. 90 ± 0.26
D(sitagliptin+ metformin)	FPG(mmol/l)	9.60 ± 2.03	8.33 ± 1.63△
	HbA1c(%)	7.83 ± 0.58	7.04 ± 0.48△
	TCH(mmol/l)	4.46 ± 1.03	4.53 ± 1.02
	HDL(mmol/l)	1.34 ± 0.27	1.37 ± 0.27
	TG(mmol/l)	1.26 ± 0.62	1.35 ± 0.51
	LDL(mmol/l)	2.54 ± 1.01	2.54 ± 0.90

Self-pair comparison before and after treatment, ※P<0.05;vs Group A, *P<0.05; vs Group B, #P<0.05; vs Group C, △P<0.05: vs Group D, ※P<0.05.
FBG, fasting plasma glucose; HbA1c, hemoglobin A1c; TCH, total cholesterol; HDL, high density lipoprotein cholesterol; TG, triglyceride; LDL, low density lipoprotein cholesterol.

NAFLD (46). Insulin resistance is thought to be one of the key initial pathogenic mechanisms contributing to the development of NAFLD as high insulin levels can result in liver fat deposition by stimulating the oxidation of surrounding adipose tissue resulting in increased levels of serum free fatty acids (FFA), increased removal of serum FFA through increased hepatic storage, and increasing endogenous synthesis of FFA in the liver. Furthermore, deposition of FFA in the liver also exacerbates insulin resistance through increase fatty acid oxidation and free radical production leading to a vicious cycle of worsening liver fat accumulation and insulin resistance (47). Some adipocytokines such as adiponectin, leptin and resistin play important roles in the development of NAFLD by acting on liver by producing increased inflammation reaction and increasing insulin resistance (48, 49).

It is believed that both insulin resistance and hypertriglyceridemia play important roles in the development of diabetes and NAFLD. Currently, the treatment of patients with diabetes and NAFLD includes the use of insulin sensitizing agents such as metformin and thiazolidinediones (11, 12). Metformin improves glucose homeostasis by increasing the utilization of glycogen and inhibiting glucose output. Additionally, metformin reduces the accumulation of fat in the liver (11, 13). A previous study has shown that metformin (750 mg/day) reduced IHL content by 25% in obese patients with T2DM (50). However, metformin has no significant effect on liver histology and is not recommended for the treatment of NASH. A recent meta-analysis suggested that the use of thiazolidinediones may improve hepatic steatosis both in non-diabetic and diabetic patients (51). However, it exerts limit actions in histological liver injury. Thus, we studied the effects of DPP-4 inhibitors on intrahepatic lipid content and the metabolic profile in NAFLD patients with T2DM. There was a trend toward a reduction in liver fat content with sitagliptin treatment. The average liver fat content was decreased from 61.88% to 42.11% after sitagliptin treatment, although this did not reach statistical significance. The lack of statistical significance may be due to the small sample size and short treatment period among other factors. Additionally, the peak plasma level of active GLP-1 was 15-20 pmol/L after treatment with sitaglitin while the GLP-1 receptor agonist lirglutide led to higher levels (4000-8000 pmmol/L) (52, 53). Thus, the lack of statistical significance observed in the reduction of IHL content may be due to the relatively lower plasma concentrations of GLP-1 achieved with sitagliptin. Metformin treatment resulted in a significant

reduction in liver fat content, which is likely due to activation of hepatic AMP-activated protein kinase (AMPK) by metformin which suppresses hepatic TG production and increases hepatic catabolic metabolism (54). Previous studies have also suggested that AMPK may be able to be directly activated by GLP-1 (55).

The GLP-1 receptor belongs to the G protein coupled glucagon receptor family and is widely distributed throughout the body and can be found in the pancreas, gastrointestinal tract and other tissues (56). Studies in animal models have shown that DPP-4 inhibitors can inhibit DPP-4 activity by 90% and increase plasma concentrations of exogenous GLP-1 thereby decreasing postprandial blood glucose (56, 57). Previous studies have shown that HbA1c was decreased by 0.65 percentage points with sitagliptin treatment after 24 weeks poorly controlled T2DM patients (24–26). In this study, HbA1c was decreased by 0.8 percentage points, and FBG was decreased by 0.7 mmol/l in the sitagliptin group while HbA1c was decreased by 1 percentage point and FBG was decreased by 1.3 mmol/l in the sitagliptin plus metformin group. HbA1c significantly decreased in the sitagliptin group was significant compared with the metformin group. The percentage of HbA1c level less than 7% was 65%, 25%, and 72.7% after sitagliptin, metformin and sitagliptin plus metformin treatments, respectively. These results suggest that sitagliptin effectively decreases blood glucose levels. There were no adverse reactions of hypoglycemia during the treatment period. Each participant demonstrated good compliance, and the treatment was well tolerated. Animal studies have confirmed that DPP-4 inhibitors can reduce blood glucose and triglyceride levels significantly in high fat diet (HFD) fed diabetic rats (58). However, in our study, the effects of sitaglipin and metformin on lipid metabolism were not obvious. This may be due to relatively short duration of treatment used in this study.

The present study also had some limitations. Firstly, the number of patients included in this study was relatively small and the study period also was relatively brief. Secondly, the change of total fat mass and distribution of fat was not measured in this study. Therefore, we cannot estimate the effects of sitagliptin on fat redistribution. A large-scale, longer-term study clinical trial is warranted to verify our findings.

In conclusion, our study showed that 24 weeks of treatment with sitagliptin was safe and well tolerated. It improves glycemic control and can slightly reduce liver fat content in NAFLD patients with T2DM. Therefore, sitagliptin may have therapeutic potential for NAFLD patients was well as those with other metabolic disorders indirectly while not directly on IHL.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Shanghai Tenth People' s Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XCW and BFZ performed the experiment and draft the manuscript. HS involved in review and revised the manuscript. HY participated in the data collection. SQ designed the study in the revised manuscript.

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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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Associations of lipid parameters with non-alcoholic fatty liver disease in type 2 diabetic patients according to obesity status and metabolic goal achievement

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Aims: Non-obese non-alcoholic fatty liver disease (NAFLD) phenotype has sparked interest and frequently occurred in type 2 diabetes mellitus (T2DM). Information on associations between lipid parameters and NAFLD in non-obese patients with diabetes has been lacking. We aimed to investigate the relationships between lipid parameters and NAFLD according to obesity status and metabolic goal achievement in T2DM patients.

Methods: A total of 1,913 T2DM patients who were hospitalized between June 2018 and May 2021 were cross-sectionally assessed. We used logistic regression models to estimate the associations of lipid parameters with NAFLD risk according to obesity and metabolic goal achievement status.

Results: Higher triglycerides, non-HDL-cholesterol, and all lipid ratios including (total cholesterol/HDL-cholesterol, triglyceride/HDL-cholesterol, LDL-cholesterol/HDL-cholesterol, non-HDL-cholesterol/HDL-cholesterol), and lower HDL-cholesterol were associated with NAFLD risk in both non-obese and obese patients. The associations were stronger in non-obese patients than in obese patients. Further, the inverse associations of total cholesterol and LDL-cholesterol with NAFLD risk were only detected in non-obese patients. Triglycerides, HDL-cholesterol, and all lipid ratios studied were significantly associated with NAFLD risk, irrespective of whether the patients achieved their HbA1c, blood pressure, and LDL-cholesterol goal. The presence of poor lipids and lipid ratios were more strongly associated with NAFLD in patients who attained the HbA1c, blood pressure, and/or LDL-cholesterol goal than in those who did not achieve the goal attainment.

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Conclusions: The associations of lipids and lipid ratios with NAFLD risk were stronger in T2DM patients who were non-obese and achieved the HbA1c, blood pressure, and/or LDL-cholesterol goal attainment.

KEYWORDS

non-alcoholic fatty liver disease, diabetes mellitus, obesity, blood pressure, lipids

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become one of the major liver diseases worldwide, affecting around 25.2% of the global population (1). It may surpass alcohol as the leading cause for liver transplantation (1). The NAFLD epidemic has paralleled that of the diabetes epidemic. Approximately 60-70% of patients with type 2 diabetes mellitus (T2DM) suffered from NAFLD (2). T2DM is an aggravating factor for NAFLD. For example, it was reported that T2DM patients were at 2 to 4-fold risk for developing advanced liver fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma compared to those without T2DM (3); Vice versa, patients with NAFLD are more commonly progress toward diabetic micro- and macro-vascular complications (4).

Dyslipidemia plays a central role in the pathogenesis of NAFLD (5, 6). Accumulating evidence showed that lipid profile was significantly associated with an increased risk of NAFLD in the general population (7–9). Insulin resistance (IR), well known in T2DM and the main physio-pathological link between NAFLD and T2DM (10–12), triggers an increase in free fatty acids from peripheral adipose tissue and favoring the development of dyslipidemia. However, whether lipids can affect NAFLD independent of IR in T2DM is less well-defined. Additionally, despite NAFLD is predominantly seen with overweight or obesity, this entity can occur in non-obese individuals (13). It was reported that the global prevalence of non-obese NAFLD was above 40% among the NAFLD population and nearly 20% in non-obese population (14). Non-obese NAFLD can develop IR and the full spectrum of metabolic comorbidities and liver damage that occurs in obese NAFLD (13, 15) and may have as severe consequences as obese NAFLD (16). Previous studies conducted in general population have shown that the association between dyslipidemia and NAFLD was more pronounced in non-obese persons than in obese persons (17). It is unclear whether lipid parameters play a role in non-obese T2DM patients and whether the associations between lipid parameters and NAFLD differ between non-obese and obese patients with diabetes. Further, NAFLD is more frequent in patients with poor "ABCs" (parameters usually followed by clinicians for diabetes control, including glycated hemoglobin [HbA1c] [A], blood pressure [BP] [B], and low-density lipoprotein cholesterol [LDL-C] [C]) metabolic treatment goals. It remains unclear whether lipid parameters are associated with different risks of NAFLD in distinct populations defined by glycated hemoglobin (HbA1c), blood pressure (BP), and low-density lipoprotein cholesterol (LDL-C) levels. Therefore, we aimed to investigate the relationships between lipid variables and NAFLD according to obesity and metabolic treatment goal status in T2DM.

Methods

Study design and population

This cross-sectional study included 2,946 T2DM patients hospitalized in the Department of Endocrinology, Tongji Hospital, Tongji medical college, Huazhong University of Science and Technology (Wuhan, China) between June 2018 and May 2021. T2DM was diagnosed according to the 2022 American Diabetes Association criteria (18). The exclusion criteria included a history of alcohol abuse (alcohol consumption >140 g/week for male or >70 g/week for female), other causes of hepatic diseases including viral hepatitis, autoimmune liver disease and cirrhosis, current diagnosis of life-threatening cancer, severe psychiatric disturbance, pregnancy or lactation. We excluded 516 with alcohol abuse, 145 with viral hepatitis, and 1 with hepatic cirrhosis; 127 with missing data on blood lipids and liver ultrasound. In addition, to avoid the effects of lipid-lowering on all lipid parameters, 244 participants with lipid-lowering medication use were excluded. The remaining 1,913 subjects were included in our data analyses According to the Private Information Protection Law, information that might identify subjects was safeguarded by the Computer Center. This study was approved by the institutional review board of Tongji Hospital. Because we only retrospectively accessed a de-identified database for purposes of analyses, informed consent requirement was exempted by the institutional review board.

Clinical measurements

Patients' data including age, sex, height, weight, current and previous illness histories, and medical treatments were obtained from medical records. Weight was measured with participants wearing light clothing on a calibrated beam scale. Height was measured without shoes. Waist circumference (WC) was measured with an inelastic tape at a midpoint between the bottom of the rib cage and the top of the iliac crest at the end of exhalation. Seated systolic/diastolic BP was measured in triplicate after a 10-min rest, using mercury manometers. The means of the last two readings was used in data analyses. Body mass index (BMI) was calculated as weight (in kilograms) divided by height in square meters.

Blood was collected from the antecubital vein of each individual after an at least 8-hour overnight fast. Measurements were done soon after the blood samples had been collected, and no samples were stored and reused. Glycated hemoglobin (HbA1c) was measured using high performance liquid chromatography (D-10^{TM}; Bio-Rad Laboratories, Hercules, CA, USA). Fasting plasma glucose (FPG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), uric acid, and creatinine were measured on an autoanalyzer (Cobas C8000, Roche, Mannheim, Germany). Hepatitis viral antigens/antibodies were detected with corresponding Architect reagents (Architect i2000, Abbott Diagnostics, Abbott Park, IL). Non-HDL-C was calculated as TC minus HDL-C. HOMA-IR was calculated as fasting insulin (μU/mL) × FPG (mmol/L)/22.5.

Definitions

According to the China Obesity Working Group (19), obesity was defined as BMI≥28kg/m^2.

Ultrasound tests were performed by certified sonographers using a high-resolution, real-time scanner (model SSD-2000; Aloka Co., Ltd., Tokyo Japan). Certified radiologists used standard criteria in evaluating the presence or absence of hepatic fat. Generally, liver steatosis was defined as the presence of stronger echoes in the hepatic parenchyma compared with echoes in the kidney or spleen parenchyma (20). The presence of advanced liver fibrosis was defined as the presence of the high probability for advanced fibrosis calculated by NAFLD fibrosis score (NFS) or BARD score. NFS was calculated as -1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m^2) + 1.13 × IFG/diabetes (yes 1, no 0) + 0.99 × AST/ALT ratio - 0.013 × platelet (10^9/L) - 0.66 × albumin (g/dl) (21). The presence of advanced liver fibrosis was confirmed when the score was greater than 0.676. BARD score: BMI > 28 = 1 point, AAR (Aspartate transaminase/alanine animo-transferase [AST/ALT] ratio) of > 0.8 = 2 points, DM = 1 point. A score of ≥ 2 was associated with advanced fibrosis (22).

Statistical analyses

All statistical analyses were conducted using SPSS software (version 24.0 for mac; SPSS, Chicago, IL, USA). Continuous variables were presented as means (minimum to maximum) or medians (IQRs) depending on their distribution. Categorical variables were presented as percentages. Differences in continuous variables between groups were tested with one-way ANOVA or Kruskal-Wallis test. Differences in categorical variables were tested with χ^2 test. Logistic regression models were used to estimate the associations (odds ratios [ORs], with 95% confidence Intervals [CIs]) between each lipid parameter and risk of NAFLD. Four models were fitted. Model 1 was adjusted for age, smoking status, family history of diabetes. Model 2 was additionally adjusted for body mass index, systolic blood pressure, glycated hemoglobin, use of anti-hypertensive drug. Model 3 was additionally adjusted for HOMA-IR. Model 4 was additionally adjusted for anti-diabetic drug use. A receiver operating characteristic (ROC) curve analysis was performed for each lipid parameter to compare the abilities of these measures to discriminate NAFLD correctly. The overall diagnostic accuracy was quantified using the area under the ROC curve (AUC). Significance was accepted at a two-tailed P <0.05.

Results

Baseline characteristics of study subjects

Of the 1,913 T2DM patients included in the present analyses, the mean age was 52.1 (13.3) years, 55.2% were men, the mean BMI value was 24.9 (3.8) kg/m^2. The overall prevalence of NAFLD was 48.5%. 73.49% diabetic patients with NAFLD were non-obese. T2DM patients with obese NAFLD phenotype have a mean BMI value of 31.14 (3.33) kg/m^2 and a mean HbA1c value of 9.86% (2.36%). The corresponding figures were 22.92 (2.68) kg/m^2 and 9.14% (2.57%), respectively, for T2DM patients with non-obese NAFLD phenotype. As seen in Table 1, NAFLD patients were younger, had higher BMI, WC, HbA1c, AST, ALT and adverse lipids and lipid ratios than patients without NAFLD (all P value <0.001). Moreover, NAFLD patients were less likely to have the care goal achievement (all P value <0.001).

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TABLE 1 Characteristics of participants according to non-alcoholic fatty liver disease status.

	Without NAFLD	With NAFLD	P value
n	985	928	
Male, %	55.20	60.23	0.011
Smoking, %	17.25	19.94	0.084
Age, years	55.55 (14-85)	50.23 (14-89)	<0.001
Weight, kg	63.98 (30-105)	73.62 (45-159.3)	<0.001
BMI, kg/m^2	23.60 (15.31-37.46)	26.38 (18.75-49.63)	<0.001
Obesity, %	16.94	26.51	<0.001
WC, cm	89.20 (62-129)	95.86 (74-188)	<0.001
SBP, mmHg	130.70 (70-216)	132.08 (76-215)	0.106
DBP, mmHg	80.09 (49-137)	84.22 (46-133)	<0.001
HbA1c, %	9.05 (4.30-18.10)	9.74 (5.20-18.70)	<0.001
ALT, U/L	21.27 (5-450)	32.47 (5-393)	<0.001
AST, U/L	20.01 (5-212)	25.15 (5-317)	<0.001
TC, mmol/L	4.38 (1.78-13.70)	4.76 (1.56-14.10)	<0.001
TG, mmol/L	2.15 (0.36-22.06)	3.60 (0.21-45.21)	<0.001
HDL-C, mmol/L	1.12 (0.23-2.82)	0.97 (0.38-2.03)	<0.001
LDL-C, mmol/L	2.68 (0.61-7.14)	2.82 (0.20-6.23)	<0.001
TC/HDL-C	4.15 (1.59-18.92)	5.16 (1.89-32.05)	<0.001
TG/HDL-C	2.27 (0.21-36.77)	4.30 (0.24-88.65)	<0.001
LDL/HDL-C	2.53 (0.48-7.61)	2.98 (0.38-10.02)	<0.001
non-HDL-C, mmol/L	3.26 (0.94-12.56)	3.79 (1.00-13.66)	<0.001
nonH-DL-C/HDL-C	3.15 (0.59-17.92)	4.16 (0.89-31.05)	<0.001
Anti-hypertensive drug use, %	32.28	31.79	0.816
Anti-diabetic drug use			
Sulfonylureas use, %	14.98	17.97	0.078
Non-sulfonylureas use, %	1.94	3.15	0.095
Metformin use, %	30.82	32.59	0.589
Glucosidase inhibitor use, %	18.43	28.73	<0.001
Thiazolidinediones use, %	5.17	7.92	0.016
DPP4i use, %	6.25	7.72	0.209
SGLT2i use, %	3.02	3.45	0.592
Insulin use, %	22.31	39.70	<0.001
GLP-1 RA use, %	1.72	0.81	0.073
'A' attained, %	14.72	5.93	<0.001
'B' attained, %	35.63	25.54	<0.001
'C' attained, %	46.80	37.82	<0.001

Values are proportions, and means (minimum to maximum)
BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; ALT, alanine animo-transferase; AST, aspartate transaminase; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; DPP4i, Dipeptidyl peptidase-4 inhibitor; SGLT2i, Sodium-glucose cotransporter 2 inhibitor; GLP-1 RA, glucagon-like peptide 1 receptor agonists; 'A' attained, HbA1c <6.5%; 'B' attained, blood pressure < 130/80mmHg; 'C' attained, LDL-C <2.6mmol/L.

ROC analysis of lipids and lipid ratios for identifying NAFLD in patients with diabetes

AUCs for all lipid parameters studied indicated that all lipid parameters could effectively discriminate NAFLD (all AUC > 0.5). In addition, AUCs derived from lipid ratios were in general significantly greater than from single lipids (Figure 1).

Associations of lipid parameters with NAFLD according to obesity status

The prevalence of NAFLD increased from the first to the fourth quartiles of the serum TG levels and each lipid ratio and decreased from the first to fourth quartiles of the serum HDL-C levels (all P <0.001) (Figure 2).

The associations of lipid parameters with NAFLD according to obesity status were shown in Table 2. After controlling for

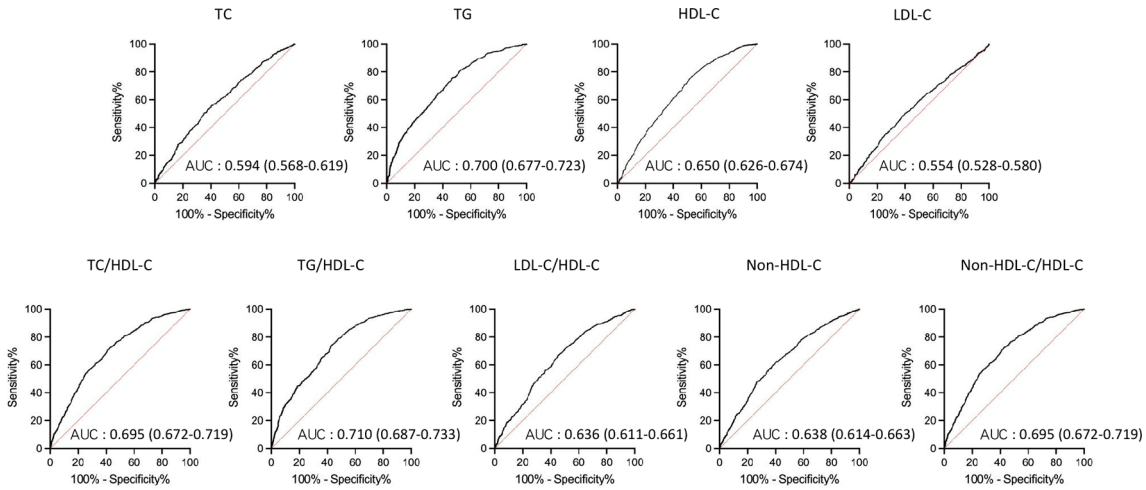

FIGURE 1
Receiver operating characteristic (ROC) curves of lipid parameters for detecting non-alcoholic fatty liver disease in T2DM patients. AUC, area under the curve; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; T2DM, type 2 diabetes mellitus.

potential intermediate variables including HOMA-IR and anti-diabetic medication use, all lipid parameters studied, except LDL-C, were significantly associated with NAFLD in non-obese T2DM patients. Among obese T2DM subjects, TG and each lipid ratio were positively associated with NAFLD, while HDL-C was negatively associated with NAFLD. In both obese and non-obese T2DM patients, lipid ratios were more closely associated with NAFLD than any of the individual variables used alone.

The odds ratios (ORs) and 95% confidence intervals (CIs) of quartiles of each lipid parameter for NAFLD were presented in Table 3. Among both non-obese and obese patients, after controlling for potential intermediate variables including HOMA-IR and anti-diabetic medication use, higher TG, TC/HDL-C, TG/HDL-C, and non-HDL-C/HDL-C, and lower HDL-C were significantly associated with NAFLD risk. In non-obese subjects, higher TC, LDL-C, non-HDL-C, and LDL-C/HDL-C levels were also significantly associated with NAFLD risk.

Odds ratios of lipid parameters for NAFLD according to diabetes control parameters

The associations of lipid parameters with NAFLD in different T2DM control parameters, namely HbA1c (A), BP (B), and LDL-C (C) were shown in Table 4. After adjusting for potential confounding variables, TG, HDL-C, and all lipid ratios studied

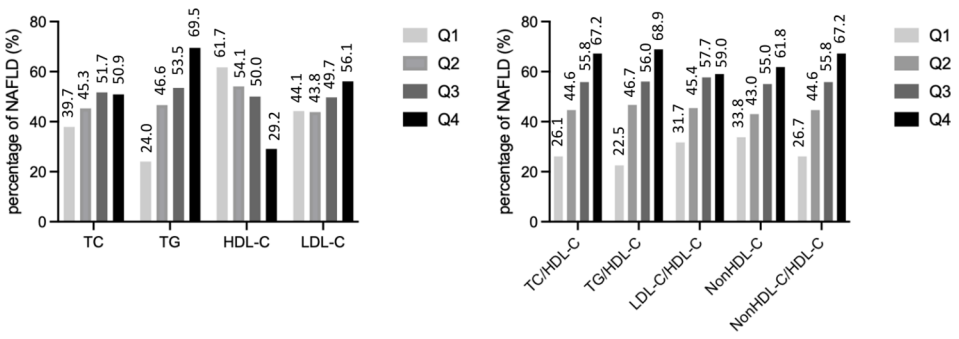

FIGURE 2
The prevalence of non-alcoholic fatty liver disease by quartiles of lipid parameters. TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

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TABLE 2 Odds ratios and 95% confidence intervals of lipid parameters for non-alcoholic fatty liver disease according to obesity status.

	Model	Total	Obese	Non-obese
TC	1	1.28 (1.18-1.38)*	1.23 (0.96-1.57)	1.27 (1.17-1.39)*
	2	1.22 (1.12-1.33)*	1.11 (0.86-1.42)	1.28 (1.16-1.41)*
	3	1.23 (1.12-1.35)*	1.13 (0.88-1.47)	1.28 (1.15-1.43)*
	4	1.22 (1.11-1.34)*	1.13 (0.87-1.46)	1.25 (1.12-1.39)*
TG	1	1.29 (1.22-1.36)*	1.26 (1.06-1.48)#	1.27 (1.20-1.35)*
	2	1.22 (1.15-1.28)*	1.22 (1.02-1.45)#	1.21 (1.14-1.29)*
	3	1.21 (1.15-1.28)*	1.26 (1.06-1.51)#	1.19 (1.13-1.27)*
	4	1.21 (1.15-1.28)*	1.23 (1.04-1.46)#	1.19 (1.12-1.26)*
HDL-C	1	0.15 (0.11-0.22)*	0.07 (0.02-0.25)*	0.19 (0.13-0.28)*
	2	0.24 (0.16-0.36)*	0.10 (0.03-0.33)*	0.34 (0.22-0.52)*
	3	0.22 (0.14-0.34)*	0.08 (0.02-0.31)*	0.30 (0.19-0.49)*
	4	0.23 (0.15-0.36)*	0.10 (0.02-0.38)*	0.28 (0.17-0.46)*
LDL-C	1	1.18 (1.07-1.30)#	1.19 (0.89-1.58)	1.20 (1.07-1.33)*
	2	1.13 (1.01-1.26)#	1.05 (0.76-1.44)	1.16 (1.03-1.31)#
	3	1.12 (1.00-1.27)	1.05 (0.76-1.47)	1.15 (1.01-1.31)#
	4	1.08 (0.96-1.22)	1.04 (0.75-1.45)	1.09 (0.95-1.25)
TC/HDL-C	1	1.52 (1.41-1.64)*	1.58 (1.26-1.99)*	1.48 (1.36-1.60)*
	2	1.37 (1.27-1.49)*	1.37 (1.09-1.73)#	1.34 (1.23-1.47)*
	3	1.39 (1.27-1.51)*	1.47 (1.16-1.86)*	1.38 (1.26-1.51)*
	4	1.39 (1.28-1.51)*	1.41 (1.11-1.79)*	1.32 (1.20-1.45)*
TG/HDL-C	1	1.20 (1.15-1.25)*	1.22 (1.02-1.42)#	1.18 (1.13-1.23)*
	2	1.14 (1.10-1.19)*	1.18 (1.02-1.35)#	1.13 (1.08-1.18)*
	3	1.14 (1.09-1.19)*	1.22 (1.05-1.41)#	1.12 (1.07-1.17)*
	4	1.14 (1.10-1.19)*	1.20 (1.04-1.40)#	1.12 (1.07-1.17)*
LDL-C/HDL-C	1	1.52 (1.37-1.67)*	1.64 (1.23-2.22)*	1.49 (1.34-1.65)*
	2	1.36 (1.22-1.51)*	1.40 (1.03-1.91)#	1.31 (1.17-1.47)*
	3	1.37 (1.22-1.53)*	1.40 (1.03-1.92)#	1.31 (1.16-1.48)*
	4	1.34 (1.20-1.51)*	1.38 (1.01-1.81)#	1.27 (1.12-1.44)*
Non-HDL-C	1	1.45 (1.32-1.58)*	1.40 (1.08-1.82)#	1.43 (1.30-1.58)*
	2	1.34 (1.22-1.47)*	1.21 (0.92-1.57)	1.40 (1.25-1.55)*
	3	1.35 (1.22-1.50)*	1.24 (0.94-1.63)	1.40 (1.25-1.57)*
	4	1.34 (1.21-1.48)*	1.23 (0.93-1.61)	1.36 (1.21-1.52)*
Non-HDL-C/HDL-C	1	1.52 (1.41-1.64)*	1.58 (1.26-1.99)*	1.48 (1.36-1.60)*
	2	1.37 (1.27-1.49)*	1.36 (1.07-1.74)#	1.34 (1.23-1.47)*
	3	1.39 (1.27-1.51)*	1.47 (1.16-1.86)*	1.38 (1.26-1.51)*
	4	1.39 (1.28-1.51)*	1.41 (1.11-1.79)*	1.32 (1.20-1.45)*

Model 1 was adjusted for age, sex, smoking status, family history of diabetes mellitus.
Model 2 was adjusted for all the variables in model 1 plus SBP, BMI, HbA1c and use of anti-hypertensive drugs for total; In obesity and non-obesity subgroup, BMI was replaced by waist circumference.
Model 3 was adjusted for all the variables in model 2 plus HOMA-IR.
Model 4 was adjusted for all the variables in model 3 plus use of anti-diabetic drugs.
TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; BMI, body mass index; HbA1c, glycated hemoglobin.
*P < 0.001, #P < 0.05.

were significantly associated with NAFLD risk, irrespective of A, B, and C status. The associations of lipid parameters with NAFLD were stronger in patients who achieved the A, B, and/or C goal. Moreover, lipid ratios were more closely associated with NAFLD risk than any of the individual variables used alone, regardless of whether the patients reached their care goal attainment.

Association of lipid parameters and advanced liver fibrosis

The ORs of quartiles of each lipid parameters for advanced fibrosis, defined by two non-invasive advanced fibrosis predict scores: NFS and BARD, were shown in Table 5. Lower HDL-C

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TABLE 3 Odds raitos and 95% confidence intervals of lipid parameters in terms of the quartiles for non-alcoholic fatty liver disease according to obesity status.

		Total		Obese		Non-obese	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
TC	Q1	ref.		ref.		ref.	
	Q2	1.10 (0.80-1.51)	0.572	0.84 (0.37-0.93)	0.683	1.09 (0.76-1.57)	0.639
	Q3	1.51 (1.09-2.10)	0.013	1.88 (0.74.4.61)	0.170	1.50 (1.05-2.16)	0.028
	Q4	1.78 (1.24-2.48)	0.001	1.62 (0.64-4.06)	0.473	1.73 (1.20-2.50)	0.004
TG	Q1	ref.		ref.		ref.	
	Q2	2.08 (1.50-2.89)	<0.001	1.64 (0.73-3.66)	0.228	2.61 (1.78-3.84)	<0.001
	Q3	2.64 (1.90-3.68)	<0.001	4.67 (1.89-10.50)	0.001	2.89 (1.97-4.26)	<0.001
	Q4	4.73 (3.35-6.68)	<0.001	4.68 (1.66-13.22)	0.004	4.99 (3.37-7.39)	<0.001
HDL-C	Q1	ref.		ref.		ref.	
	Q2	0.69 (0.50-0.96)	0.028	1.29 (0.42-3.91)	0.656	0.73 (0.52-1.04)	0.081
	Q3	0.60 (0.43-0.82)	0.002	0.72 (0.27-1.90)	0.503	0.69 (0.49-0.98)	0.038
	Q4	0.32 (0.23-0.46)	<0.001	0.41 (0.15-1.08)	0.071	0.39 (0.27-0.58)	<0.001
LDL-C	Q1	ref.		ref.		ref.	
	Q2	1.16 (0.84-1.59)	0.374	1.16 (0.50-2.71)	0.735	1.07 (0.75-1.53)	0.697
	Q3	1.14 (.083-1.56)	0.427	1.40 (0.57-3.42)	0.464	1.11(0.78-1.57)	0.572
	Q4	1.38 (1.00-1.90)	0.050	1.30 (0.54-3.15)	0.557	0.41 (0.99-2.02)	0.058
TC/HDL-C	Q1	ref.		ref.		ref.	
	Q2	1.84 (1.33-2.55)	0.002	3.93 (1.72-8.99)	0.001	1.60 (1.09-2.34)	0.016
	Q3	2.55 (1.83-3.57)	<0.001	3.64 (1.44-9.22)	0.006	2.47 (1.70-3.59)	<0.001
	Q4	3.23 (2.28-4.57)	<0.001	3.33 (1.26-8.78)	0.015	3.36 (2.28-4.94)	<0.001
TG/HDL-C	Q1	ref.		ref.		ref.	
	Q2	2.67 (1.92-3.72)	<0.001	1.52 (0.69-3.36)	0.297	3.02 (2.04-4.46)	<0.001
	Q3	2.76 (1.98-3.85)	<0.001	4.93 (1.95-12.44)	0.001	2.94 (1.99-4.35)	<0.001
	Q4	5.25 (3.69-7.50)	<0.001	5.12 (1.65-15.88)	0.005	5.14 (3.45-7.68)	<0.001
LDL-C/HDL-C	Q1	ref.		ref.		ref.	
	Q2	1.74 (1.26-2.40)	0.001	2.20 (0.94-5.15)	0.069	1.74 (1.20-2.51)	0.003
	Q3	2.15 (1.55-2.96)	<0.001	2.37 (1.01-5.53)	0.047	2.14 (1.49-3.06)	<0.001
	Q4	1.97 (1.41-2.75)	<0.001	1.35 (0.54-3.38)	0.518	1.83 (1.26-2.66)	0.001
Non-HDL-C	Q1	ref.		ref.		ref.	
	Q2	1.42 (1.02-1.97)	0.037	0.94 (0.42-2.13)	0.886	1.29 (0.89-1.87)	0.176
	Q3	2.26 (1.62-3.12)	<0.001	2.07 (0.84-5.15)	0.116	1.90 (1.32-2.74)	0.001
	Q4	2.44 (1.75-3.41)	<0.001	1.96 (0.69-4.48)	0.236	2.27 (1.57-3.29)	<0.001
Non-HDL/HDL-C	Q1	ref.		ref.		ref.	
	Q2	1.69 (1.21-2.35)	0.002	3.93 (1.72-8.99)	0.001	1.60 (1.09-2.34)	0.016
	Q3	2.55 (1.83-3.57)	<0.001	3.64 (1.44-9.22)	0.006	2.47 (1.70-3.59)	<0.001
	Q4	3.23 (2.28-4.57)	<0.001	3.33 (1.26-8.78)	0.015	3.36 (2.28-4.94)	<0.001

Odds ratios were adjusted for age, sex, smoking status, family history of diabetes mellitus, SBP, BMI, HbA1c, HOMA-IR, use of anti-hypertensive drugs, and anti-diabetic drugs.
TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; BMI, body mass index; HbA1c, glycated hemoglobin.

was significantly associated with advanced fibrosis risk defined by NFS.

Sensitivity analysis

Since metformin and glucagon-like peptide 1 receptor agonists (GLP-1RAs) are two of the few anti-diabetic medications preventing weight gain or even favoring weight loss in T2DM patients (23, 24), to avoid the effects of these medications use on results, we did the above analysis after excluding patients taking these two drugs. The results were essentially the same (Supplementary Tables 1, 2) except in the obese subgroup, in whom the associations were no longer significant. However, estimates in this subgroup should be interpreted with caution due to limited sample size and inadequate statistical power.

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TABLE 4 Odds ratios 95% confidence intervals of lipid parameters for non-alcoholic fatty liver disease according to metabolic goal attainment status.

	HbA1c ≥ 6.5%	HbA1c < 6.5%	BP ≥ 130/80mmHg	BP <130/80mmHg	LDL-C ≥ 2.6mmol/L	LDL-C < 2.6mmol/L
TC	1.19 (1.07-1.33)*	1.46 (0.85-2.49)	1.22 (1.08-1.37)*	1.20 (1.03-1.41)#	1.19 (1.02-1.38)#	1.29 (1.10-1.52)*
TG	1.23 (1.15-1.31)*	1.53 (1.09-2.15)#	1.31 (1.21-1.42)*	1.11 (1.04-1.19)#	1.34 (1.22-1.51)*	1.18 (1.11-1.25)*
HDL-C	0.25 (0.15-0.41)*	0.02 (0.002-0.15)*	0.18 (0.10-0.31)*	0.36 (0.16-0.80)#	0.29 (0.15-0.53)*	0.10 (0.05-0.21)*
LDL-C	1.07 (0.93-1.23)	2.14 (1.02-4.48)#	1.02 (0.88-1.18)	1.22 (0.97-1.53)	1.10 (0.88-1.37)	0.98 (0.67-1.45)
TC/HDL-C	1.37 (1.24-1.51)*	2.62 (1.58-4.36)*	1.49 (1.34-1.65)*	1.24 (1.10-1.41)*	1.37 (1.21-1.55)*	1.39 (1.24-1.57)*
TG/HDL-C	1.16 (1.10-1.22)*	1.69 (1.23-1.33)*	1.22 (1.15-1.30)*	1.08 (1.02-1.13)*	1.31 (1.20-1.44)*	1.12 (1.08-1.17)*
LDL-C/HDL-C	1.29 (1.11-1.47)*	3.90 (1.88-8.10)*	1.32 (1.15-1.52)*	1.32 (1.08-1.63)#	1.33 (1.13-1.57)*	1.62 (1.27-2.07)*
Non-HDL-C	1.29 (1.15-1.45)*	1.65 (1.00-2.71)#	1.36 (1.20-1.55)*	1.26 (1.07-1.48)*	1.30 (1.11-1.53)*	1.48 (1.23-1.78)*
Non-HDL-C/HDL-C	1.37 (1.24-1.51)*	2.62 (1.58-4.36)*	1.49 (1.34-1.65)*	1.24 (1.10-1.41)*	1.37 (1.21-1.55)*	1.39 (1.24-1.57)*

Model for HbA1c subgroup was adjusted for age, sex, smoking status, family history of diabetes mellitus, SBP, BMI, RBG(random blood glucose), HOMA-IR, use of anti-hypertensive drugs, and anti-diabetic drugs;
Model for blood pressure subgroup was adjusted for age, sex, smoking status, family history of diabetes mellitus, BMI, HbA1c, HOMA-IR, use of anti-hypertensive drugs, and anti-diabetic drugs;
Model for LDL-cholesterol subgroup was adjusted for age, sex, smoking status, family history of diabetes mellitus, SBP, BMI, HbA1c, HOMA-IR, use of anti-hypertensive drugs, and anti-diabetic drugs.
TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; BMI, body mass index; HbA1c, glycated hemoglobin; BP, blood pressure.
*P < 0.001, #P < 0.05.

Discussion

This is, as far as we are aware, the first report to describe the associations of lipids and lipid ratios with NAFLD in T2DM patients according to obesity status and metabolic goal achievement status. We found that in patients with T2DM, adverse lipids and lipid ratios were significantly associated with NAFLD risk, regardless of obesity status and metabolic goal attainment status. The associations were stronger in patients who were non-obese and had the A, B, and/or C goal attainment. Moreover, lipid ratios have a stronger association with NAFLD risk than any of the individual variables used alone.

The associations of lipids and lipid ratios with NAFLD have been established in the general population (7–9, 25). However, in patients with T2DM, the associations between lipid parameters and NAFLD risk remain less clear. Here, we verified the significant associations of lipids and lipid ratios with NAFLD risk in T2DM patients. In consistent with previous studies conducted in the general population (7, 8, 25), we also noted that lipid ratios were more effective than single measures of lipids in detecting NAFLD. This may be explained by that lipid ratios taken account of the proportion between the pro-atherogenic and anti-antherogenic fractions (26, 27).

The relatively low BMI in diabetic patients with NAFLD may be due to the following reasons: 1) Chinese individuals are

TABLE 5 Odds raitos and 95% confidence intervals of lipid parameters in terms of the quartiles for advanced liver fibrosis.

	NFS			BARD		
	≤ 0.676	> 0.676	P value	< 2	≥ 2	P value
TC	ref.	0.95 (0.39-2.28)	0.904	ref.	0.92 (0.62-1.36)	0.668
TG	ref.	0.85 (0.35-2.07)	0.715	ref.	0.89 (0.60-1.32)	0.550
HDL	ref.	4.98 (2.17-11.40)	<0.001	ref.	1.25 (0.82-1.90)	0.296
LDL	ref.	0.96 (0.43-2.15)	0.919	ref.	0.91 (0.62-1.34)	0.629
TC/HDL-C	ref.	0.74 (0.28-1.93)	0.534	ref.	1.25 (0.83-1.88)	0.283
TG/HDL-C	ref.	0.58 (0.25-1.36)	0.208	ref.	1.10 (0.74-1.64)	0.637
LDL-C/HDL-C	ref.	0.32 (0.51-3.47)	0.568	ref.	1.04 (0.69-1.55)	0.862
non-HDL	ref.	0.82 (0.34-1.94)	0.648	ref.	1.07 (0.72-1.60)	0.733
non-HDL/HDL-C	ref.	0.43 (0.28-1.93)	0.534	ref.	1.25 (0.83-1.88)	0.283

Odds ratios were adjusted for age, sex, smoking status, family history of diabetes mellitus, SBP, BMI, HbA1c, HOMA-IR, use of anti-hypertensive drugs, and anti-diabetic drugs.
TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; BMI, body mass index; HbA1c, glycated hemoglobin.

characterized by a greater amount of visceral or ectopic adipose tissue than Europeans at a given BMI (28); 2) Non-obese NAFLD phenotype was more frequent in patients with T2DM. The non-obese NAFLD phenotype has sparked interest because of its high prevalence (6, 7, 29), and unanswered questions regarding whether stratifying NAFLD patients based on their obesity status could prioritize allocation of clinical resources for those most at risk of poor outcomes (30). Reports convinced that non-obese NAFLD subjects had severe impaired glucose tolerance and dyslipidemia that were identical or even worse than obese NAFLD subjects (15, 16). This evidence from general population-based analyses supports that non-obese NAFLD may represent a distinct entity in the disease spectrum of NAFLD. To date, analysis of the association of lipids and lipid ratios with non-obese NAFLD has not been reported in patients with diabetes, in whom NAFLD and dyslipidemia commonly occur (2, 3, 31).

We addressed this fundamental knowledge gap in the present study. We found that more severe dyslipidemias in T2DM, including higher TG, all lipid ratios studied, and lower HDL-C were associated with NAFLD risk in both non-obese and obese patients. The associations were stronger in non-obese patients than in obese patients. Further, the inverse associations of TC and LDL-C levels with NAFLD risk were only detected in non-obese patients. One possible explanation for these results may be due to a decreased capacity for storing fat in adipose tissue in non-obese NAFLD patients (13, 32, 33). According to the overflow hypothesis, adipose tissue acts as a reservoir of free fatty acids and prevents their overflow into insulin-sensitive tissues including liver. Alterations in fatty acid trafficking lead to abnormalities in lipid storage and consequent dyslipidemia and ectopic fat deposition (33). Further, obesity is a well-defined risk factor for NAFLD (34–36). Thus, obesity attenuates the relationship between lipids and lipid ratios and NAFLD. Although the percentage of metformin and/or GLP-1RAs use, which were used for a dual approach of treating both diabetes and NAFLD (23, 24), were similar in T2DM patients with and without NAFLD, to avoid the effects of these medications use on NAFLD, we have adjusted the anti-diabetic medication use. Moreover, we did sensitivity analysis after excluding patients taking these two drugs. The results were essentially the same. This suggested that dyslipidemia in subjects with diabetes, even if they were not obese, might be identified as an indicator of the presence of NAFLD.

Since diabetic control parameters have strong effects on NAFLD and lipid profile (3, 34, 35), we also investigated whether the relationships between lipids and lipid ratios and NAFLD differed by HbA1c, BP, and LDL-C status. We found that in patients with T2DM, TG, HDL-C, and all lipid ratios studied were significantly associated with NAFLD risk, irrespective of A, B, and C status. When further adjusting for the use of anti-diabetic drugs, the results were essentially the same. The presence of poor lipids and lipid ratios were more strongly associated with NAFLD in patients who attained the A, B, and/or C goal than in those who did not achieve the goal attainment. Further, the inverse association of LDL-C levels with NAFLD risk was only detected in patients who achieved the A, B, and/or C goal. One possible explanation for these results may be due to the independent associations of increased HbA1c, BP, and/or LDL-C levels with NAFLD (37–40). The significant and independent associations of lipids and lipid ratios with NAFLD in those who achieved the A, B, and/or C goal attainment highlight that lipids and lipid ratios predispose to increased NAFLD risk, regardless of care goal attainment status. However, estimates across subgroups should be interpreted with caution because of limited sample size and inadequate statistical power.

In the present study, when using NFS and BARD to indicate advanced fibrosis, we found lower HDL-C was significantly associated with advanced fibrosis risk, defined by NFS. The relations between lipid parameters and advanced fibrosis is still controversial (41, 42). Hegazy M,et,al. found that lipid ratios, particularly TG/HDL-C, are associated with advanced fibrosis (43). While other studies showed that the advanced fibrosis risk did not differ by lipid status (44). Further studies are warranted to explore the associations between lipid parameters and advanced fibrosis in T2DM patients.

Our findings have important clinical implications. With the diabetes epidemics in China, the incidence of NAFLD is expected to be even more prevalent in patients with diabetes in the near future. The increased prevalence of NAFLD suggests that more patients with diabetes are predisposed to an increased cardiovascular disease risk. The established insulin resistance (IR) in T2DM plays a key role in the development of NAFLD by increasing the accumulation of free fatty acids in the liver and inhibiting adipose tissue lipolysis (10–12). The current study demonstrates the important impacts of adverse lipids and lipid ratios on NAFLD independent of HOMA-IR in both obese and non-obese T2DM patients. Therefore, NAFLD cannot be explained by IR alone, as other factors such as genetic and epigenetic factors, lipotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress, microbiota, chronic low-grade inflammation and oxidative stress, dysfunction of adipose tissue, and nutritional factors and lifestyle are also involved in the development of the disease (45). Taken together, management of dyslipidemia in patients with T2DM, regardless of obesity status and care goal achievement status, may be therefore of importance for the prevention and reduction of NAFLD and cardiovascular disease risk.

The main strength of this study is the large number of T2DM patients included from an academic hospital. Further, we can get access to clinical, laboratory, and imaging data in medical records, which provided more in-depth clinical information that are not usually available in large epidemiological surveys.

There are several limitations. First, NAFLD was diagnosed by ultrasonography rather than liver histopathology, which may lead to an inaccurate diagnosis. Nevertheless, liver ultrasonography has been confirmed as an accurate and

reliable tool for detecting fatty liver. Due to the relatively low cost and lack of radiation exposure, ultrasonography is widely used for identifying fatty liver in clinical settings and population studies. Second, although we adjusted for multiple potential confounding variables, residual and unmeasured confounding might not be fully addressed. Third, our study population were mainly based on inpatients suffering from T2DM, whose health conditions might be severer than those of outpatients. Thus, our findings could not be generalized to outpatients with T2DM. Fourth, the cross-sectional study design makes it difficult to infer causality between the lipid parameters and NAFLD risk. At last, some anti-diabetic drug use in T2DM patients including metformin and/or GLP-RA, can affect weight and liver fat content (23, 24).

In conclusion, in patients with T2DM, lipids and lipid ratios were significantly associated with NAFLD risk, independent of HOMA-IR, irrespective of obesity status and metabolic goal attainment status. The associations of lipids and lipid ratios with NAFLD risk were stronger in T2DM patients who were non-obese and achieved the HbA1c, blood pressure, and/or LDL-cholesterol goal attainment.

Data availability statement

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

Ethics statement

This study was approved by the institutional review board of Tongji Hospital. Because we only retrospectively accessed a de-identified database for purposes of analysis, informed consent was not required.

Author contributions

ZZ and NY, study design, statistical analyses, acquisition and interpreting of data, and drafting of manuscript. HF, acquisition of data. GY and YC, critical revision of the manuscript. TD and XZ, study design, interpreting data, and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1002099/full#supplementary-material

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Correlation between long-term use of metformin and incidence of NAFLD among patients with type 2 diabetes mellitus: A real-world cohort study

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Background and aims: Studies have demonstrated that the short-term use of metformin benefits liver function among patients with type 2 diabetes mellitus (T2DM). However, few studies have reported on the effects of long-term metformin treatment on liver function or liver histology. This study investigated the correlation between metformin use and the incidence of nonalcoholic fatty liver disease (NAFLD) among patients with T2DM.

Methods: This population-based study investigated the risk of NAFLD among patients with T2DM who received metformin treatment between 2001-2018. Metformin users and metformin nonusers were enrolled and matched to compare the risk of NAFLD.

Results: After 3 years, the patients who received <300 cDDD of metformin and those with metformin use intensity of <10 and 10–25 DDD/month had odds ratios (ORs) of 1.11 (95% confidence interval [CI] = 1.06–1.16), 1.08 (95% CI = 1.02–1.13), and 1.18 (95% CI = 1.11–1.26) for NAFLD, respectively. Moreover, metformin users who scored high on the Diabetes Complications and Severity Index (DCSI) were at high risk of NAFLD. Patients with comorbid hyperlipidemia, hyperuricemia, obesity, and hepatitis C were also at high risk of NAFLD.

Conclusion: Patients with T2DM who received metformin of <300 cDDD or used metformin at an intensity of <10 and 10–25 DDD/month were at a high risk of developing NAFLD. The results of this study also indicated that patients with T2DM receiving metformin and with high scores on the DCSI were at a high risk of developing NAFLD.

KEYWORDS

nonalcoholic fatty liver disease, metformin, type 2 diabetes mellitus, cumulative defined daily dose, NHIRD

Highlights

1. According to results from 3-year follow-up, metformin users with type 2 diabetes had an increased risk for NAFLD, with odds ratio of 1.11 (95% confidence interval [CI] = 1.06–1.16).
2. Metformin users who scored high on the Diabetes Complications and Severity Index (DCSI) were at high risk of NAFLD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a major public health concern worldwide because of its high prevalence. NAFLD is characterized by increased hepatic triglycerides in patients who do not consume alcohol excessively (1). NAFLD is typically classified into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH); NASH is characterized by liver inflammation and hepatocyte damage due to the development of NAFLD (2). The accumulation of triglycerides within the cytoplasm of hepatocytes is a distinguishing characteristic of NAFLD (1).

The correlation between NAFLD and type 2 diabetes mellitus (T2DM) is indicated by insulin resistance (IR) and the progression of compensatory hyperinsulinemia leading to defective lipid metabolism and hepatic triglyceride accumulation (3). NAFLD is highly prevalent among patients with T2DM, accompanied by frequent incidences of obesity and IR (4). Hepatic fat accumulation among patients with T2DM is more likely to progress to NASH and fibrosis than among patients without T2DM (5). Patients with T2DM exhibit more than a twofold increase in the prevalence of NAFLD, regardless of the diagnostic method used (6).

Recent studies have reported that metformin can improve IR and hyperinsulinemia and may aid in the treatment of NAFLD (7). Evidence from animal and human studies has indicated that metformin may attenuate the onset and progression of NAFLD (8–11). Several studies have attributed the alleviating effects of metformin on NAFLD to the anti-inflammatory effects of metformin (12, 13),. However, metformin is not used for treating NAFLD because of a lack of evidence that metformin significantly improves liver histology (2, 14).

Few epidemiological studies have reported the effects of long-term metformin use on the risk of NAFLD among patients with T2DM. Therefore, we investigated whether long-term metformin use is associated with the risk of NAFLD by using the patient population in Taiwan's National Health Insurance Research Database (NHIRD).

Material and methods

Data source

Secondary data analysis was performed in this study by using the Longitudinal Health Insurance Database (LHID; a subset of the NHIRD) from 2001 to 2018 released by the Health and Welfare Data Science Center, Ministry of Health and Welfare (HWDC, MOHW). The LHID is prepared from claims from Taiwan's National Health Insurance (NHI) program that enrolls up to 99% of Taiwanese citizens. Hence, the database is a nationally representative health database for Taiwan. The data in the LHID, including detailed clinical data of outpatient visits, hospitalizations, diagnostic results, and prescriptions, have demonstrated high concordance between NHI claims records and patient self-reports (15). Therefore, the LHID was used to analyze the risk of NAFLD among patients with DM receiving metformin. The data in the LHID are anonymized, and the HWDC assigns scrambled random identification numbers to insured patients to protect their privacy. The requirement of informed consent was waived.

Ethics approval

This study was conducted in compliance with the Declaration of Helsinki. Data used in the analysis were anonymized and released by the HWDC, MOHW, Taiwan. The HWDC assigns scrambled random identification numbers to insured patients to protect their privacy. The study was approved by the Central Regional Research Ethics Committee of China Medical University, Taiwan, as meeting all ethical criteria (No. CRREC-109-011).

Study participants

Patients with new-onset DM who were aged above 20 years were enrolled in this study to investigate the effects of metformin on incident NAFLD from 2002 to 2013. The criterion for DM was three diagnoses in a year according to the *International Classification of Diseases, Ninth Revision, Clinical Modification* (ICD-9-CM; code 250). The criterion for metformin use was based on Anatomical Therapeutic Chemical (ATC) code A10BA02. To reduce study bias, we excluded patients with type 1 DM diagnosed with NAFLD before the onset of DM, those diagnosed with NAFLD in the first year after the onset of DM, and those hospitalized within one year after the onset of DM. The patients were divided into two groups: a case group and a comparison group. The case group included patients who had received metformin in the first year after the onset of DM, and the comparison group included patients who had not received any metformin. A total of 1,000,080 patients with new-onset DM were included from 2002 to 2013; of them, 459,064 patients had not received any metformin, and 541,016 patients had received metformin in the first year after the onset of DM. Figure 1 illustrates the process of selecting study participants.

Study design

This study had a cross-sectional design and investigated the risk of NAFLD among patients with DM who received metformin for 3 or 5 years. The defined daily dose (DDD) is used as a standard unit for measuring drug utilization and drug exposure in a population. The World Health Organization defines DDD as the estimated average maintenance dose per day of a drug used to treat a condition in adults. The DDD does not necessarily reflect the recommended or prescribed daily dose (16). Each patient was observed for one year after the diagnosis of DM to assess the use of metformin. The DDD of metformin used to evaluate the medication was 2 g (17). The cumulative

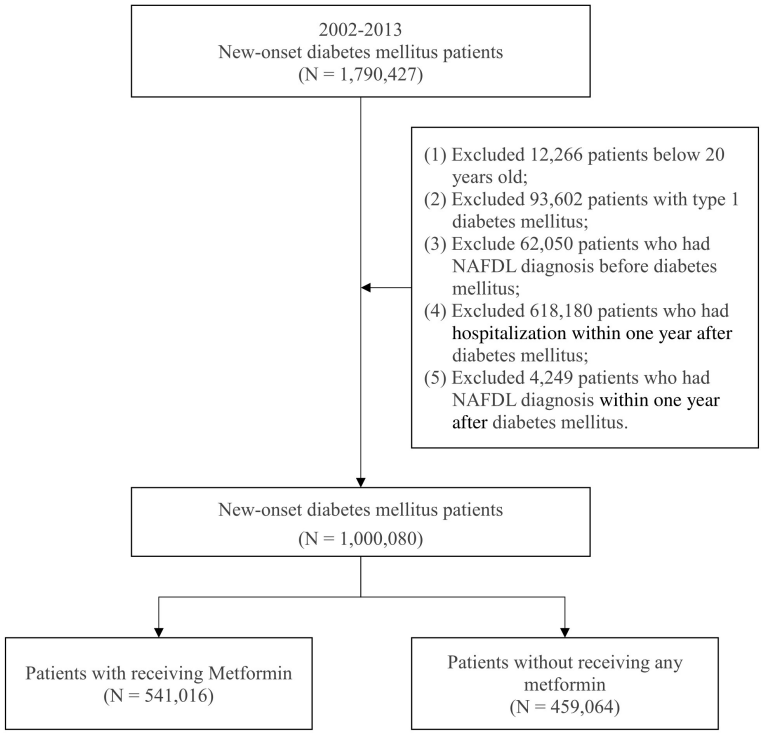

FIGURE 1
Patient selection process.

DDD (cDDD) of metformin use in the first year was calculated and categorized into five groups for dose–response analysis: nonusers, <300 cDDD, 300–500 cDDD, and above 500 cDDD. Furthermore, we calculated and categorized the average monthly DDD into four groups to investigate the association of metformin use intensity with NAFLD incidence: nonusers, <10 DDD, 10–25 DDD, and above 25 DDD. All patients were observed for 3 and 5 years to analyze the association between metformin use and NAFLD incidence. The criterion for NAFLD in this study was three or more diagnoses within one year, according to ICD-9-CM code 571.8 and ICD-10-CM codes K75.81 and K76.0. The control variables included diabetes severity and related comorbidities. We used the Diabetes Complications Severity Index (DCSI) to adjust the diabetes severity. The DCSI was used to assess the DM patients' risks of adverse outcomes calculated by the information from the seven diabetes complication categories (retinopathy, nephropathy, neuropathy, cerebrovascular, cardiovascular, peripheral vascular disease, and metabolic) (18, 19). The assessed comorbidities included hypertension (ICD-9-CM 401–405), hyperlipidemia (ICD-9-CM 272.0–272.4), hyperuricemia (ICD-9-CM 790.6), chronic kidney disease (CKD; ICD-9-CM 585), obesity (ICD-9-CM 278.00), *Helicobacter pylori* infection (ICD-9-CM 041.86), psoriasis (ICD-9-CM 696.1), rheumatoid arthritis (RA ICD-9-CM 714), hypothyroidism (ICD-9-CM 244.9), polycystic ovary syndrome (ICD-9-CM 256.4), and hepatitis C virus (HCV; ICD-9-CM 070.4, 070.5, 070.70).

Statistical analysis

All analyses in the study were performed using SAS version 9.4. The chi-square test was used to evaluate the distribution of the baseline characteristics between metformin users and nonusers. Differences in the incidence of NAFLD between metformin users and nonusers were estimated through multiple logistic regression with the adjustment of the relevant variables, and the results are presented as odds ratios (ORs) with 95% confidence intervals (CI). Two adjusted models were developed to estimate the incidence of NAFLD among metformin users; the estimation involved calculating the cDDD and intensity of metformin use (DDD/month). Statistical significance in this study was indicated by p-values <0.05.

Results

Characteristic distribution of study participants

Table 1 displays the baseline characteristics of the patients. The average age of all the patients was 56.37 ± 12.49 years. Among the selected patients, 47.39% were female and 52.61% were male. Further, 29.12% of the patients were aged 20–49 years, 16.38% were aged 50–54 years, 15.77% were aged 55–59 years, 12.62% were aged 60–64 years, and 26.12% were aged ≥65 years.

Among metformin users, the average age was 55.06 ± 12.16 years. Among the selected patients, 194,590 patients (35.97%) had hypertension, 80,546 patients (14.89%) had hyperlipidemia, 3,507 patients (0.65%) had hyperuricemia, 1,656 patients (0.31%) had CKD, 3,338 (0.62%) patients had obesity, 839 patients (0.16%) had *H. pylori* infection, 2,052 patients (0.38%) had psoriasis, 3,372 patients (0.62%) had RA, 1,553 patients (0.29%) had hypothyroidism, 1,279 patients (0.24%) had polycystic ovary syndrome, and 1,573 patients (0.29%) had HCV. Furthermore, the difference in the distribution of each comorbid disease between metformin users and nonusers was statistically significant.

Incident NAFLD in patients with new-onset dm receiving metformin medication

Table S1 displays the distribution of incident NAFLD among patients with T2DM. Table 2 displays the data on NAFLD incidence obtained through 3-year follow-up; 7,451 patients (0.75%) developed NAFLD within 3 years after the diagnosis of DM. The incidence rate of NAFLD among metformin nonusers was 0.70%, and those among the metformin users were 0.79% for cDDD <300, 0.81% for cDDD 300–500, and 0.88% for cDDD ≥500. In terms of metformin use intensity, the incidence rate of NAFLD was 0.76% for <10 DDD/month, 0.85% for 10–25 DDD/month, and 0.81% for ≥25 DDD/month. After 3-year follow-up, the ORs for the incidence of NAFLD among patients with DM receiving cDDD <300, 300–500, and >500 were 1.11 (95% CI = 1.06–1.16), 1.08 (95% CI = 0.85–1.37), and 1.19 (95% CI = 0.39–3.70), respectively. In terms of metformin use intensity, the ORs for the incidence of NAFLD among patients with DM receiving <10, 10–25, and ≥25 DDD/month were 1.08 (95% CI = 1.02–1.13), 1.18 (95% CI = 1.11–1.26), and 1.09 (95% CI = 0.86–1.37), respectively. In terms of risk factors, the ORs for dementia among patients with DM scoring 1 and ≥2 on the DCSI were 1.07 (95% CI = 1.01–1.14) and 1.16 (95% CI = 1.09–1.24), respectively. Furthermore, the patients with DM comorbid with hyperlipidemia (OR = 1.30, 95% CI = 1.22–1.38) and obesity (OR = 1.78, 95% CI = 1.44–2.21) were at high risk of developing NAFLD. Patients with DM comorbid with hypertension (OR = 0.94, 95% CI = 0.89–0.99) and CKD (OR = 0.66, 95% CI = 0.47–0.94) were at low risk of developing NAFLD. By contrast, patients with comorbid hyperuricemia, *H. pylori* infection, psoriasis, RA, hypothyroidism, polycystic ovary syndrome, and HCV were not at risk of developing dementia.

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TABLE 1 Baseline characteristics of patients with new-onset diabetes mellitus.

Variables	Total		Metformin				p-value
			Non-users		Users		
	N	%	N	%	N	%	
Total	1,000,080	100.00	459,064	45.90	541,016	54.10	
Gender							<0.001
Female	473,966	47.39	226,960	49.44	247,006	45.66	
Male	526,114	52.61	232,104	50.56	294,010	54.34	
Age (year) (Mean ± SD)	56.37 ± 12.49		57.92 ± 12.69		55.06 ± 12.16		<0.001
20-49	291,184	29.12	116,329	25.34	174,855	32.32	
50-54	163,773	16.38	70,456	15.35	93,317	17.25	
55-59	157,692	15.77	70,933	15.45	86,759	16.04	
60-64	126,193	12.62	60,554	13.19	65,639	12.13	
≥65	261,238	26.12	140,792	30.67	120,446	22.26	
Income level (NTD) [a]							<0.001
≤21,000	516,216	51.62	240,725	52.44	275,491	50.92	
21,001-33,000	232,549	23.25	100,298	21.85	132,251	24.44	
≥33,001	251,315	25.13	118,041	25.71	133,274	24.63	
Urbanization [b]							<0.001
Level 1	274,537	27.45	133,435	29.07	141,102	26.08	
Level 2	328,483	32.85	149,758	32.62	178,725	33.04	
Level 3	162,209	16.22	70,392	15.33	91,817	16.97	
Level 4	136,631	13.66	61,759	13.45	74,872	13.84	
Level 5	21,690	2.17	10,215	2.23	11,475	2.12	
Level 6	39,780	3.98	17,472	3.81	22,308	4.12	
Level 7	36,750	3.67	16,033	3.49	20,717	3.83	
DCSI score [c]							<0.001
0	650,315	65.03	290,957	63.38	359,358	66.42	
1	195,134	19.51	90,537	19.72	104,597	19.33	
≥2	154,631	15.46	77,570	16.90	77,061	14.24	
Hypertension							<0.001
No	618,217	61.82	271,791	59.21	346,426	64.03	
Yes	381,863	38.18	187,273	40.79	194,590	35.97	
Hyperlipidemia							<0.001
No	818,863	81.88	358,393	78.07	460,470	85.11	
Yes	181,217	18.12	100,671	21.93	80,546	14.89	
Hyperuricemia							<0.001
No	992,413	99.23	454,904	99.09	537,509	99.35	
Yes	7,667	0.77	4,160	0.91	3,507	0.65	
CKD [c]							<0.001
No	993,567	99.35	454,207	98.94	539,360	99.69	
Yes	6,513	0.65	4,857	1.06	1,656	0.31	
Obesity							<0.001
No	994,396	99.43	456,718	99.49	537,678	99.38	
Yes	5,684	0.57	2,346	0.51	3,338	0.62	
Helicobacter pylori							<0.001
No	998,245	99.82	458,068	99.78	540,177	99.84	
Yes	1,835	0.18	996	0.22	839	0.16	
Psoriasis							0.012
No	996,427	99.63	457,463	99.65	538,964	99.62	

(Continued)

TABLE 1 Continued

Variables	Total		Metformin				
			Non-users		Users		p-value
	N	%	N	%	N	%	
Yes	3,653	0.37	1,601	0.35	2,052	0.38	
RA [c]							<0.001
No	992,795	99.27	455,151	99.15	537,644	99.38	
Yes	7,285	0.73	3,913	0.85	3,372	0.62	
Hypothyroidism							<0.001
No	995,976	99.59	456,513	99.44	539,463	99.71	
Yes	4,104	0.41	2,551	0.56	1,553	0.29	
Polycystic ovary syndrome							<0.001
No	998,493	99.84	458,756	99.93	539,737	99.76	
Yes	1,587	0.16	308	0.07	1,279	0.24	
HCV [c]							<0.001
No	996,636	99.66	457,193	99.59	539,443	99.71	
Yes	3,444	0.34	1,871	0.41	1,573	0.29	

[a]The premium-based salary of the patient which is according to the payroll bracket table of the National Health Insurance Administration Taiwan. NTD is New Taiwan Dollar. NTD 1 ≈ USD 0.034).
[b]Level 1 denoted the highest degree of urbanization, whereas level 7 denoted the lowest degree of urbanization.
[c]DCSI, diabetes complications severity index; CKD, chronic kidney disease; RA, rheumatoid arthritis; HCV, hepatitis C virus.

Table 3 displays the 5-year follow-up data on NAFLD incidence. After adjusting the related variables, we discovered that the ORs for NAFLD incidence among patients with DM receiving cDDD <300, 300–500, and ≥500 were 1.06 (95% CI = 1.02–1.09), 0.96 (95% CI = 0.80–1.15), and 1.02 (95% CI = 0.43–2.46), respectively. In terms of the intensity of metformin use, the ORs for NAFLD incidence among patients receiving <10, 10–25, and >25 DDD/month were 1.04 (95% CI = 1.00–1.08), 1.11 (95% CI = 1.06–1.16), and 0.96 (95% CI = 0.80–1.15). Adjusted model 1 also indicated that the ORs for NAFLD incidence among patients with DM who scored 1 and ≥2 on the DCSI were 1.08 (95% CI = 1.04–1.13) and 1.14 (95% CI = 1.09–1.20), respectively. In terms of risk factors, patients with DM having hyperlipidemia (OR = 1.33, 95% CI = 1.28–1.39), hyperuricemia (OR = 1.23, 95% CI = 1.04–1.45), obesity (OR = 1.62, 95% CI = 1.38–1.91), and HCV (OR = 1.46, 95% CI = 1.15–1.86) were at high risk of developing NAFLD. Patients with comorbid hypertension (OR = 0.93, 95% CI = 0.90–0.97) and CKD (OR = 0.72, 95% CI = 0.57–0.92) were at low risk of developing NAFLD.

Discussion

To the best of our knowledge, few large-scale epidemiological studies have evaluated the risk of NAFLD incidence among patients with T2DM receiving metformin. The results obtained after 3-year and 5-year follow-up indicated that patients with T2DM receiving metformin in cDDD <300 or at intensities of <10 and 10–25 DDD/month were at high risk for developing NAFLD. However, in patients with T2DM receiving metformin in cDDD of 300–500 and >500 or at intensities of >25 DDD/month, metformin exhibited no protective effects against NAFLD. In addition, metformin users who scored high on the DCSI had high ORs for NAFLD incidence among patients with T2DM; patients with comorbid hyperlipidemia, hyperuricemia, obesity and HCV were also at high risk of NAFLD.

Several studies have reported that patients with T2DM and fatty liver disease exhibited improved aminotransferase levels and IR after metformin therapy (20–23). Therefore, metformin may aid the treatment of NAFLD (8, 20, 24). Animal and physiological studies have proposed various possible mechanisms to explain the relationship between metformin use and the risk of NAFLD incidence. Metformin is considered an activator of AMP-activated protein kinase (AMPK), which is a major cellular regulator of glucose and lipid metabolism. This serves as a key mechanism through which metformin treatment aids glucose metabolism and alleviates diabetes-related complications (25). Metformin decreases triglyceride accumulation in hepatocytes due to high-fat diets *in vivo* and *in vitro* (26). Moreover, metformin can activate intracellular AMPK and stimulate NO synthesis in human aortic endothelial cells (27). The beneficial effects of metformin extend beyond glycemic control and include the improvement of hepatocyte lipid metabolism and the suppression of hepatocyte and macrophage inflammatory responses (13).

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TABLE 2 Three-year follow-up of incident non-alcoholic fatty liver disease in new-onset diabetes mellitus patients with metformin medication.

Variables	Three-year follow-up of incident non-alcoholic fatty liver disease											
	Events		p-value	Model 1					Model 2			
	N	%		OR	95% CI			p-value	OR	95% CI		p-value
Total	7,451	0.75										
cDDD of metformin use			<0.001									
Non-users	3,195	0.70		1					–	–		–
DDD <300	4,185	0.79		1.11	1.06	–	1.16	<0.001	–	–		–
DDD 300-500	68	0.81		1.08	0.85	–	1.37	0.535	–	–		–
DDD ≥500	3	0.88		1.19	0.39	–	3.70	0.759	–	–		–
Intensity of metformin use			<0.001									
Non-users	3,195	0.70							1			
<10	2,914	0.76		–		–		–	1.08	1.02	–	1.13	0.004
10-25	1,271	0.85		–		–		–	1.18	1.11	–	1.26	<0.001
≥25	71	0.81		–		–		–	1.09	0.86	–	1.37	0.500
Gender			<0.001									
Female	3,362	0.71		1					1			
Male	4,089	0.78		1.03	0.98	–	1.08	0.207	1.03	0.98	–	1.08	0.219
Age (year)			<0.001									
20-49	2,776	0.95		1					1			
50-54	1,232	0.75		0.79	0.73	–	0.84	<0.001	0.79	0.73	–	0.84	<0.001
55-59	1,150	0.73		0.76	0.70	–	0.81	<0.001	0.76	0.71	–	0.81	<0.001
60-64	823	0.65		0.67	0.62	–	0.73	<0.001	0.68	0.62	–	0.73	<0.001
≥65	1,470	0.56		0.58	0.55	–	0.62	<0.001	0.59	0.55	–	0.63	<0.001
Income level (NTD) [a]			<0.001									
≤21,000	3,747	0.73		1					1			
21,001-33,000	1,646	0.71		0.94	0.89	–	1.00	0.048	0.94	0.89	–	1.00	0.049
≥33,001	2,058	0.82		1.07	1.02	–	1.14	0.012	1.07	1.02	–	1.14	0.013
Urbanization [b]			0.429									
Level 1	2,039	0.74		1					1			
Level 2	2,518	0.77		1.04	0.98	–	1.10	0.240	1.04	0.98	–	1.10	0.237
Level 3	1,172	0.72		0.98	0.91	–	1.05	0.534	0.98	0.91	–	1.05	0.539
Level 4	1,002	0.73		1.04	0.96	–	1.12	0.361	1.04	0.96	–	1.12	0.350
Level 5	147	0.68		1.01	0.86	–	1.20	0.890	1.01	0.86	–	1.20	0.868
Level 6	285	0.72		1.05	0.93	–	1.19	0.453	1.05	0.93	–	1.19	0.438
Level 7	288	0.78		1.13	0.99	–	1.28	0.062	1.13	1.00	–	1.28	0.060
DCSI score [c]			0.601									
0	4,805	0.74		1					1			
1	1,470	0.75		1.07	1.01	–	1.14	0.023	1.07	1.01	–	1.14	0.023
≥2	1,176	0.76		1.16	1.09	–	1.24	<0.001	1.16	1.09	–	1.24	<0.001
Hypertension			<0.001									
No	4,771	0.77		1					1			
Yes	2,680	0.70		0.94	0.89	–	0.99	0.012	0.94	0.89	–	0.99	0.011
Hyperlipidemia			<0.001									
No	5,876	0.72		1					1			
Yes	1,575	0.87		1.30	1.22	–	1.38	<0.001	1.30	1.22	–	1.38	<0.001
Hyperuricemia			0.024									
No	7,377	0.74		1					1			
Yes	74	0.97		1.23	0.97	–	1.54	0.083	1.23	0.98	–	1.55	0.081
CKD [c]			0.017									

(Continued)

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TABLE 2 Continued

Variables	Three-year follow-up of incident non-alcoholic fatty liver disease											
	Events		p-value	Model 1				Model 2				
	N	%		OR	95% CI			p-value	OR	95% CI			p-value
No	7,419	0.75		1					1				
Yes	32	0.49		0.66	0.47	–	0.94	0.022	0.66	0.47	–	0.94	0.022
Obesity			<0.001										
No	7,366	0.74		1					1				
Yes	85	1.50		1.78	1.44	–	2.21	<0.001	1.78	1.44	–	2.21	<0.001
Helicobacter pylori			0.240										
No	7,433	0.74		1					1				
Yes	18	0.98		1.19	0.75	–	1.90	0.455	1.20	0.75	–	1.91	0.452
Psoriasis			0.059										
No	7,414	0.74		1					1				
Yes	37	1.01		1.35	0.97	–	1.86	0.072	1.35	0.97	–	1.86	0.072
RA [c]			0.756										
No	7,399	0.75		1					1				
Yes	52	0.71		1.00	0.76	–	1.32	0.979	1.01	0.77	–	1.32	0.969
Hypothyroidism			0.659										
No	7,418	0.74		1					1				
Yes	33	0.80		1.04	0.74	–	1.47	0.813	1.04	0.74	–	1.47	0.810
Polycystic ovary syndrome			0.264										
No	7,443	0.75		1					1				
Yes	8	0.50		0.50	0.25	–	1.01	0.053	0.50	0.25	–	1.01	0.054
HCV [c]			0.946										
No	7,425	0.75		1					1				
Yes	26	0.75		1.09	0.74	–	1.61	0.649	1.10	0.75	–	1.61	0.645

[a]The premium-based salary of the patient which is according to the payroll bracket table of the National Health Insurance Administration Taiwan. NTD is New Taiwan Dollar. NTD 1 ≈ USD 0.034).
[b]Level 1 denoted the highest degree of urbanization, whereas level 7 denoted the lowest degree of urbanization.
[c]DCSI, diabetes complications severity index; CKD, chronic kidney disease; RA, rheumatoid arthritis; HCV, hepatitis C virus.

Our results indicated that in patients with T2DM receiving metformin in cDDD of 300–500 and >500 or at an intensity of >25 DDD/month, metformin exhibited no protective effects against NAFLD after 3-year and 5-year follow-up periods. Several studies have investigated the effects of metformin therapy on liver aminotransferase levels and liver histology of patients with NASH or NAFLD (10, 22, 23, 28–31). Several small open-label studies have demonstrated decreases in IR and liver aminotransferase levels with metformin use (10, 29, 31), but liver histology was not considerably improved (10, 29). Although histological necroinflammation improved among the metformin treatment group, the improvement was not statistically significant and no difference in liver fibrosis was observed between the metformin user and nonuser groups (29). Other studies have failed to demonstrate significant improvements in insulin sensitivity, aminotransferase level, or liver histology due to metformin treatment (22, 23). A meta-analysis study that included a subanalysis of the effects of metformin on biochemical and histological outcomes among NASH patients demonstrated that metformin did not improve NASH-related outcomes (28). Another meta-analysis study also demonstrated that metformin therapy did not improve liver histology among patients with NASH or NAFLD (28, 32). Therefore, the clinical administration of metformin among patients with NAFLD is limited because of mixed study results, the heterogeneous effects of treatment, and the small number of patients involved in the studies. Preclinical studies on rodents have suggested that metformin may be a useful therapeutic medication for reducing intrahepatic triacylglycerol (IHTAG) content; however, the effectiveness of metformin therapy in reducing IHTAG levels among patients has yet to be confirmed (33). Therefore, owing to a lack of evidence for significant histological improvement of the liver, metformin is not recommended for treating NASH or NAFLD in adult patients (2, 14).

Our results indicated that patients with T2DM receiving metformin in cDDD of <300 or at intensity of <10 and 10–25

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TABLE 3 Five-year follow-up of incident non-alcoholic fatty liver disease in new-onset diabetes mellitus patients with metformin medication.

Variables	Five-year follow-up of incident non-alcoholic fatty liver disease											
	Events		p-value	Model 1					Model 2			
	N	%		OR	95% CI			p-value	OR	95% CI		p-value
Total	14,281	1.43										
cDDD of metformin use			<0.001									
Non-users	6,294	1.37		1					–	–		–
DDD <300	7,864	1.48		1.06	1.02	–	1.09	<0.001	–	–		–
DDD 300-500	118	1.40		0.96	0.80	–	1.15	0.626	–	–		–
DDD ≥500	5	1.47		1.02	0.43	–	2.46	0.961	–	–		–
Intensity of metformin use			<0.001									
Non-users	6,294	1.37							1			
<10	5,530	1.44		–		–		–	1.04	1.00	–	1.08	0.038
10-25	2,334	1.56		–		–		–	1.11	1.06	–	1.16	<0.001
≥25	123	1.40		–		–		–	0.96	0.80	–	1.15	0.644
Gender			0.009									
Female	6,613	1.40		1					1			
Male	7,668	1.46		0.99	0.95	–	1.02	0.441	0.99	0.95	–	1.02	0.423
Age (year)			<0.001									
20-49	5,257	1.81		1					1			
50-54	2,439	1.49		0.81	0.77	–	0.85	<0.001	0.81	0.77	–	0.85	<0.001
55-59	2,213	1.40		0.76	0.72	–	0.80	<0.001	0.76	0.72	–	0.80	<0.001
60-64	1,585	1.26		0.68	0.64	–	0.72	<0.001	0.68	0.64	–	0.72	<0.001
≥65	2,787	1.07		0.58	0.55	–	0.61	<0.001	0.58	0.55	–	0.61	<0.001
Income level (NTD) [a]			<0.001									
≤21,000	6,958	1.35		1					1			
21,001-33,000	3,344	1.44		1.03	0.98	–	1.07	0.245	1.03	0.98	–	1.07	0.239
≥33,001	3,979	1.58		1.11	1.07	–	1.16	<0.001	1.11	1.07	–	1.16	<0.001
Urbanization [b]			0.087									
Level 1	4,013	1.46		1					1			
Level 2	4,760	1.45		1.00	0.96	–	1.04	0.961	1.00	0.96	–	1.04	0.965
Level 3	2,279	1.40		0.97	0.93	–	1.03	0.307	0.97	0.93	–	1.03	0.311
Level 4	1,906	1.39		1.01	0.96	–	1.07	0.709	1.01	0.96	–	1.07	0.695
Level 5	274	1.26		0.97	0.86	–	1.10	0.640	0.97	0.86	–	1.10	0.657
Level 6	539	1.35		1.02	0.93	–	1.12	0.624	1.02	0.94	–	1.12	0.608
Level 7	510	1.39		1.03	0.94	–	1.13	0.561	1.03	0.94	–	1.13	0.551
DCSI score [c]			0.306									
0	9,205	1.42		1					1			
1	2,851	1.46		1.08	1.04	–	1.13	<0.001	1.08	1.04	–	1.13	0.000
≥2	2,225	1.44		1.14	1.09	–	1.20	<0.001	1.14	1.09	–	1.20	<0.001
Hypertension			<0.001									
No	9,123	1.48		1					1			
Yes	5,158	1.35		0.93	0.90	–	0.97	<0.001	0.93	0.90	–	0.97	<0.001
Hyperlipidemia			<0.001									
No	11,178	1.37		1					1			
Yes	3,103	1.71		1.33	1.28	–	1.39	<0.001	1.33	1.28	–	1.39	<0.001
Hyperuricemia			<0.001									
No	14,138	1.42		1					1			
Yes	143	1.87		1.23	1.04	–	1.45	0.015	1.23	1.04	–	1.45	0.015
CKD [c]			0.006									

(Continued)

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TABLE 3 Continued

Variables	Five-year follow-up of incident non-alcoholic fatty liver disease											
	Events		p-value	Model 1				Model 2				
	N	%		OR	95% CI			p-value	OR	95% CI			p-value
No	14,214	1.43		1					1				
Yes	67	1.03		0.72	0.57	–	0.92	0.009	0.72	0.57	–	0.92	0.009
Obesity			<0.001										
No	14,130	1.42		1					1				
Yes	151	2.66		1.62	1.38	–	1.91	<0.001	1.62	1.38	–	1.90	<0.001
Helicobacter pylori			0.083										
No	14,246	1.43		1					1				
Yes	35	1.91		1.21	0.87	–	1.69	0.262	1.21	0.87	–	1.69	0.260
Psoriasis			0.169										
No	14,219	1.43		1					1				
Yes	62	1.70		1.19	0.92	–	1.52	0.182	1.19	0.92	–	1.52	0.182
RA [c]			0.768										
No	14,174	1.43		1					1				
Yes	107	1.47		1.07	0.88	–	1.29	0.515	1.07	0.88	–	1.29	0.507
Hypothyroidism			0.399										
No	14,216	1.43		1					1				
Yes	65	1.58		1.04	0.81	–	1.32	0.774	1.04	0.81	–	1.32	0.772
Polycystic ovary syndrome			0.777										
No	14,257	1.43		1					1				
Yes	24	1.51		0.79	0.53	–	1.19	0.262	0.80	0.53	–	1.19	0.263
HCV [c]			0.010										
No	14,214	1.43		1					1				
Yes	67	1.95		1.46	1.15	–	1.86	0.002	1.46	1.15	–	1.86	0.002

[a]The premium-based salary of the patient which is according to the payroll bracket table of the National Health Insurance Administration Taiwan. NTD is New Taiwan Dollar. NTD 1 ≈ USD 0.034).
[b]Level 1 denoted the highest degree of urbanization, whereas level 7 denoted the lowest degree of urbanization.
[c]DCSI, diabetes complications severity index; CKD, chronic kidney disease; RA, rheumatoid arthritis; HCV, hepatitis C virus.

DDD/month were at high risk of developing NAFLD after 3-year and 5-year follow-up periods. The effectiveness of short-term metformin treatment in reducing lipid levels and preventing lipid accumulation in hepatocytes has been frequently reported (34, 35). Metformin treatment has been reported to cause only transient improvement in liver chemistry. The reduction in insulin sensitivity due to metformin therapy was not sustainable (10). Animal and physiological studies on the effects of long-term metformin treatment have been inconclusive. Furthermore, data regarding the long-term effects of metformin therapy on liver function among patients with NAFLD are controversial. An animal study demonstrated that long-term treatment with metformin had no preventive effects against NAFLD in Zucker diabetic fatty rats (36). Studies on long-term metformin therapy have not demonstrated any histological protective effects in the liver (20–23). Moreover, metformin-induced hepatotoxic effects, including acute hepatitis, liver transaminitis, and intrahepatic cholestasis, have rarely been reported (37–40). Vitamin deficiency has been reported in many causes of chronic liver disease, and has been associated with the development of NAFLD (41). Furthermore, low vitamin B12 serum levels were revealed to be significantly correlated with NAFLD, especially in grade 2 to grade 3 hepato-steatosis (42). Another study also demonstrated that low level of vitamin B12 has been related to NAFLD patients, and the histological severity of NASH (43). Low levels of vitamin B12 have been linked to high levels of homocysteine characterizing hyper-homocysteinemia as an indicator for oxidative stress (44). Subjects with chronic liver disease can benefit from vitamin B, since its antioxidant effect has possessed hepatoprotective activity to ameliorate chronic liver injury (41). A low vitamin B12 serum level is an independent predictor of NASH histological severity and fibrosis grade (43). Serum vitamin B12 levels were significantly lower among patients with NAFLD than in controls, indicating a correlation with a higher grade of steatohepatitis (43). The

prevalence of B12 deficiency was higher in metformin users than non-metformin users (45). Metformin induces vitamin B12 malabsorption may be dose-related which may increase the risk of vitamin B12 deficiency in T2DM patients (46). Several studies demonstrated that vitamin B12 deficiency occurred when patients taken metformin for more than 2-4 years (45, 47).

Metformin use is associated with vitamin B12 deficiency, which is dependent upon the cumulative dose of metformin (48). Due to the clinical benefits of metformin use, its associated side effects such as vitamin B12 deficiency is often overlooked in T2DM patients. However, the diagnosis of metformin-induced vitamin B12 deficiency may be difficult (46). Vitamin B12 deficiency play a pivotal role in the risk of NAFLD development in T2DM patients receiving cumulative dose of metformin treatment over the long term.

In summary, short-term metformin use is effective in treating NAFLD, whereas long-term cumulative dose of metformin use may not alleviate NAFLD but may instead have harmful effects. Vitamin B12 deficiency may increase the risk of NAFLD among patients with T2DM receiving cumulative dose of metformin use over the long term. However, the actual mechanism of the effects of metformin dosage on the risk of NAFLD remains unclear and should be investigated in the future. Randomized-controlled studies are warranted to verify these effects.

Our study revealed that patients with DM receiving metformin and having higher scores on the DCSI were at high risk of developing NAFLD. The prevalence of NAFLD among young adults was significantly higher than among older adults, likely because of the higher prevalence among women and metabolic syndrome among young adults (49). The DCSI is an effective tool for predicting the risk of hospitalization and mortality among patients with T2DM (18). DCSI may also be used as an indicator for estimating the risk of developing NAFLD.

The results of this study indicated that patients with DM receiving metformin with comorbid hyperlipidemia, obesity, hyperuricemia, and HCV were at high risk of developing NAFLD. Studies have demonstrated that NAFLD is a multisystem disease. Evidence indicated a strong correlation between NAFLD and increased risk of hyperlipidemia (50). Obesity is strongly correlated with the development of NAFLD (51). T2DM, IR, and obesity are key factors influencing the development of NAFLD and NASH (52). The risk of NAFLD among patients with hyperuricemia was significantly higher than among patients with normal uric acid levels (53). NAFLD is a predominant outcome of chronic HCV infection (54), which causes impairment of lipid and glucose metabolism (55).

We included data approximately covering the entire Taiwanese population in this study; thus, the sample size was large and highly representative of patients with T2DM at risk of developing NAFLD, and the data obtained were of high quality.

The follow-up period of metformin use in this study was divided into 3 years and 5 years. The cDDD of metformin use was divided into three levels: ≤300, 300–500, and >500. Similarly, the intensity of metformin use was divided into three levels, namely ≤10, 10–25, and >25 DDD/month, to investigate the correlation between T2DM and the risk of developing NAFLD.

We investigated the correlation between the risk factors of comorbidities and the risk of NAFLD incidence among patients with T2DM.

This study has several limitations that should be addressed by future studies. First, the algorithm used to categorize the severity of liver disease could not be validated because of the limitation of the NHIRD (the Child–Pugh–Turcotte score used for the prognosis of chronic liver disease was not available in the NHIRD).

Second, the ICD codes from the NHIRD data did not include detailed computed tomography findings. Third, a few factors, including alcohol consumption behavior, laboratory parameters, and abdominal ultrasonography findings, that influence NAFLD development could not be determined from the LHID, thereby affecting the findings of this study. Fourth, physical activity and eating habit are the leading causes for developing NAFDL in T2DM patients. However, we could not get information of physical activity and eating habit from these patients. Finally, although the LHID includes a large amount of data, it does not include personal information of patients, such as self-pay medical information, which could influence the development of NAFLD.

Conclusions

Patients with T2DM who received metformin of <300 cDDD or used metformin at an intensity of <10 and 10–25 DDD/month were at a high risk of developing NAFLD. Moreover, patients receiving 300–500 and >500 cDDD of metformin or using metformin at an intensity of >25 DDD/month did not exhibit any protective effects against NAFLD.

Data availability statement

The National Health Insurance Database used to support the findings of this study were provided by the Health and Welfare Data Science Center, Ministry of Health and Welfare (HWDC, MOHW) under license and so cannot be made freely available. Requests to access these datasets should be directed to https://dep.mohw.gov.tw/dos/np-2497-113.html.

Ethics statement

The studies involving human participants were reviewed and approved by Central Regional Research Ethics Committee of

China Medical University, Taiwan (No. CRREC-109-011). The ethics committee waived the requirement of written informed consent for participation.

Author contributions

All the authors involved in drafting or revising the article and approved of the submitted version. Study conception and design: K-HH, C-HL, Y-DC, S-YG, T-HT, N-JC and C-YL. Data acquisition: K-HH and C-YL. Data analysis and demonstration: K-HH, T-HT and C-YL. Original draft preparation: K-HH, C-HL, Y-DC, S-YG, T-HT, N-JC and C-YL.

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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/fendo.2022.1027484/full#supplementary-material

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Non-alcoholic fatty liver disease in type 1 diabetes: Prevalence and pathophysiology

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Non-alcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disease in the general population with a global prevalence of 25%. It is often associated with metabolic syndrome and type 2 diabetes, as insulin resistance and hyperinsulinemia are known to be favoring factors. Recent studies have described growing incidence of NAFLD in type 1 diabetes (T1D) as well. Although increasing prevalence of metabolic syndrome in these patients seems to explain part of this increase in NAFLD, other underlying mechanisms may participate in the emergence of NAFLD. Notably, some genetic factors are more associated with fatty liver disease, but their prevalence in T1D has not been evaluated. Moreover, oxidative stress, poor glucose control and long-lasting hyperglycemia, as well as exogenous insulin administration play an important role in intrahepatic fat homeostasis. The main differential diagnosis of NAFLD in T1D is glycogenic hepatopathy, which needs to be considered mostly in T1D patients with poor glycemic control. This article aims to review the prevalence and pathophysiology of NAFLD in T1D and open perspectives for clinicians taking care of T1D patients with potential hepatopathy.

KEYWORDS

NAFLD, type 1 diabetes, glycogenic hepatopathy, prevalence, pathophysiology

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of lipids in the liver, particularly in the absence of high-risk alcohol consumption. It has seen its prevalence increase steadily for several years due to the global epidemic of overweight and obesity (1, 2). Insulin resistance is a pathological process very frequently associated with NAFLD and explains a very strong association of this condition with diabetes (2).

In recent years, NAFLD in patients with type 1 diabetes (T1D) rises a particular interest due to its apparent higher prevalence (3–5). The rising prevalence of metabolic syndrome in T1D due to unhealthy lifestyle is one important explanation of this increase of NAFLD in these individuals (6), but other underlying biologic mechanisms found in T1D tend to favor liver fat accumulation.

By understanding these mechanisms, we can not only have a better comprehension of NAFLD development, but this can also help us find ways to slow, stop and prevent fatty liver disease in patients with T1D.

Methodology

A literature review was realized using PubMed, Google Scholar and Web of Science including several studies which were linked to the association between NAFLD or MAFLD and T1D. Medical Subject Headings terms such as "Non-alcoholic fatty liver disease", "Metabolic-dysfunction associated fatty liver disease", "Glycogenic Hepatopathy", "Liver disease", "NASH", "Steatohepatitis" were associated with "Type 1 Diabetes". The different articles were analyzed and selected according to their abstract relevance. Similar articles suggested by the research sites were also taken into consideration and selected. In total, this review was based on the study of 62 different articles. The articles were all restricted to English language.

NAFLD and diabetes: Definition and generality

NAFLD encompasses several pathologies affecting the liver ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), and subsequently cirrhosis which is the most severe form of NAFLD. Cirrhosis may lead to hepatocellular carcinoma. The differences between these stages of liver damage can be seen on analysis of a histological section after performing a liver biopsy, which remains an invasive procedure associated with potential morbi-mortality (1). Over the past four decades, NAFLD has become the most prevalent chronic liver disease affecting approximately 25% of the adult population worldwide (1). NAFLD prevalence is even higher in type 2 diabetic (T2D) patients, reaching about 55%, and up to 90% in obese patients with a body mass index (BMI) above 40 kg/m^2 (7). Given its increasing prevalence, NAFLD is the most rapidly increasing cause of liver-related mortality (7). There is no specific approved treatment for this disease and its pathophysiological complexity represents a challenge for the development of potential therapeutic targets. Lifestyle changes remain the best way to prevent and treat the disease. NAFLD is usually associated with metabolic syndrome including T2D and obesity (8, 9). Additionally, this disease is also associated with other illnesses and factors such as dyslipidemia, hypertension, genetic and environmental factors, notably lack of exercise and unhealthy food intake (10). Regarding the mortality of patients with NAFLD in the general population, various studies have shown contradictory results with, on one hand, a slight increase in mortality (all causes combined) in patients with NAFLD compared to the general population and, on the other hand, other studies showed no association between mortality and NAFLD (11, 12). Although NAFLD increases the risk of developing cirrhosis or hepatocellular carcinoma, the main cause of death in these patients remains cardiovascular diseases followed by extrahepatic malignancies (13–15).

T1D is an autoimmune disease characterized by the destruction of beta cells resulting in the cessation of insulin production (16). T1D is mainly diagnosed in childhood or adolescence (under the age of 18) but can also be diagnosed in adults. The risk of developing T1D is extremely low in the general population (0.4%) but increases in the presence of risk factors such as a T1D in a first-degree relative (parent or brother/sister) or the presence of self-specific antibodies (17, 18). Typically, the autoantibodies sought in T1D are anti-GAD (glutamic acid decarboxylase), IA2 (islet antigen 2), ZnT8 (Zinc transporter protein member 8) and Islets of Langerhans (18). The initial clinical presentation, the family history of T1D and the age at diagnosis help in the diagnosis even if T1D can be diagnosed at a later age (Latent Autoimmune Diabetes in Adults, LADA). The initial clinical presentation is classically in the form of diabetic ketoacidosis with an acidic pH (<7.35), increased blood glucose and presence of plasmatic ketone bodies. However, it should be considered that the number of patients affected by T2D is increasing in the young population (19) making the diagnosis of T1D less obvious in young patients. Usually, the disease presents in 3 stages: The first stage consists in the destruction of beta cells with normal blood glucose levels and no symptoms. The second stage is characterized by the presence of hyperglycemia, but the patient usually remains asymptomatic. Finally, the third stage is the time of diagnosis characterized by the presence of symptoms such as polydipsia, polyuria, weight loss, dehydration, etc. (16). It is common to find, in addition to T1D, other autoimmune diseases such as Hashimoto's thyroiditis, celiac disease, pernicious anemia, etc. Therefore, screening for other autoimmune diseases is recommended in patients with T1D (16). Apart from the fact that type 1 diabetics are usually thinner and younger than type 2 diabetics and that they have positive antibodies, unlike type 2 diabetics, another way to distinguish them is the measurement of C-peptide in the blood. The latter determines the insulin reserve that the pancreas produces and is very low in T1D (20). The only treatment for T1D remains the subcutaneous injection of long-acting and short-acting insulins. With the technological advances of recent years, patients with T1D can benefit from insulin pump systems that will, when coupled to a blood glucose sensor and a correction algorithm, adjust the dose of insulin

given continuously to keep blood glucose within the targets. Recent studies are looking at possible treatments that could prevent or slow the progression of T1D in people at risk. Teplizumab is a monoclonal antibody that appears to slow the progression of T1D in newly diagnosed patients. Its action seems to protect the remaining beta cells against autoantibodies (16). This area is still unexplored and there is a lot of research to do in immunotherapy for T1D.

Diagnosis of NAFLD

Simple hepatic steatosis in NAFLD can be assessed by histology following a biopsy or by imaging like ultrasound imaging or magnetic resonance imaging (MRI). Liver biopsy is the gold standard for diagnosing and characterizing liver histologic alterations in NAFLD (3, 21). Histologically, NAFLD is defined as the presence of at least 5% hepatic steatosis without evidence of hepatocellular injury such as hepatocyte ballooning, whereas NASH is characterized by the presence of hepatocellular injury with lobular inflammation and hepatocellular ballooning (22, 23).

Liver biopsy is an invasive procedure, and imaging is therefore more frequently used to diagnose NAFLD. Hepatic fat content can be evaluated using conventional imaging such as ultrasonography, computed tomography (CT) and MRI. Nonetheless, these conventional imaging are limited for different reasons, such as lack of sensitivity and specificity (for ultrasonography and CT), lack of objectivity (for ultrasonography and MRI), radiation safety issues (CT) and different confounding factors (for all conventional imaging) (24). One of the main confounding factors is differential diagnosis, particularly hepatic glycogenesis and glycogenic hepatopathy (25). Nevertheless, recent advances in imaging such as multi-parametric MRI can help detect hepatic fat more efficiently. The multi-parametric MRI with, notably, the proton density fat fraction allows to overcome these limitations and has become a virtual liver biopsy method which can help avoid unnecessary biopsies and can also be used for the follow-up during therapy (24). Given the high and growing prevalence in NAFLD, this new imaging method can turn out to be crucial.

NAFLD prevalence in type 1 diabetes

As discussed above, there is a clear and known link between NAFLD and T2D. However, in recent years, there has been a significant increase in type 1 diabetic patients affected by NAFLD, although studies on this subject are scarce (25). It is known that the prevalence of NAFLD in type 2 diabetics (55.5%) is more than two times higher than in the general population (25%) (26). The association between T2D and NAFLD has been known and studied for several decades and the interest in the subject is significant, while the association with T1D has only been recently explored.

A recent systematic review and meta-analysis included twenty studies about the prevalence of NAFLD in T1D. In total, 3'901 subjects were included in this study. Overall, 19.3% of subjects with T1D had NAFLD, whereas NAFLD prevalence was 22% in type 1 diabetic adults only, which is less than in the general population (3). However, there were significant differences between the 20 studies included in this meta-analysis, depending on the way NAFLD was diagnosed. Three ways were used to diagnose NAFLD: Ultrasound, MRI and liver biopsy. When looked separately, NAFLD was found in 27.1% of subjects using ultrasound, in 8.6% using MRI and 19.3% using liver biopsy, the latter being the gold standard (3). To interpret these results in the context of the general population, other parameters must be taken in consideration. Indeed, patients with T1D are younger and mostly non obese.

Another study compared NAFLD prevalence in type 1 diabetics, type 2 diabetics and healthy individuals who were matched for age and BMI. This study showed that only 4.7% (6 out of 128) of type 1 diabetics had NAFLD, versus 13.4% (9 out 67) of healthy individuals, versus 62.8% (166 out of 264) of type 2 diabetics (4). In this study, the diagnostic modality used to evaluate liver fat content was MRI and hepatic steatosis was defined as liver fat content > 5.5%. In a more recent meta-analysis, the prevalence of NAFLD in lean/nonobese healthy individuals was reported to range from 10.2% to 15.7% (5).

When compared to the above discussed meta-analysis, which showed that 19.3% of subjects (including children, adolescents and adults) with T1D had NAFLD, this is in contrast with the second one which showed that 4.7% of people with T1D had NAFLD. Considering the sample sizes, 3901 individuals in the first study versus 128 in the second study, we can presume that the statistical power of the first study is much higher and therefore potentially more representative of NAFLD prevalence in T1D.

Another study looked into etiologic factors of NAFLD development in patients with T1D and T2D using transient elastography to diagnose NAFLD and to assess the presence or absence of advanced liver fibrosis. This study reported that NAFLD prevalence in T1D patients (N=150) was 20% (N=30) and 76% (N=76) in T2D patients (N=100) (27). Advanced liver fibrosis was found in 2% (N=3) of T1D patients and in 22% (N= 22) of T2D patients. Hepatic steatosis was estimated by controlled attenuation parameter and hepatic fibrosis by liver stiffness measurement using transient elastography (27). Interestingly, larger waist circumference, higher BMI and presence of metabolic syndrome were all positively associated with the presence of NAFLD in both groups, whereas insulin sensitivity, calculated with estimated glucose disposal rate and SEARCH estimated insulin sensitivity, were negatively associated with the presence of NAFLD (27).

In conclusion, we can say that most studies report a higher prevalence of NAFLD in T1D, but further analyses must be done to support this statement. Therefore, it remains difficult to establish whether individuals with T1D are more likely to develop NAFLD and a lot of limitations can explain the difficulty to prove a clear link between those two diseases, one of them being the diagnostic modality used.

NAFLD pathophysiology in T1D and T2D

NAFLD pathophysiology in T2D might differ in some points from NAFLD pathophysiology in T1D but it can help understand how T1D may contribute to the development of NAFLD (Table 1). In T2D, insulin resistance plays a key role in the development of NAFLD (2). Additionally, some lipid intermediates found in the development of NAFLD, such as diacylglycerols and ceramides, are more likely to cause hepatic insulin resistance than others, thus alimenting a vicious cycle leading to the increase of NAFLD (28). Insulin resistance is in fact associated with increased circulating free fatty acids and ectopic lipid accumulation in the liver, which can further promote inflammation and endoplasmic reticulum stress, participating also in this vicious cycle of the insulin resistance state (29). Inflammation seems to play an important role in both insulin resistance and NAFLD, with inflammatory mediators such as cytokines and adipokines playing a primordial role not only in inflammation but also in metabolic energy balance and immune response (30). Oxidative stress, which is caused by the excessive presence of intracellular reactive oxygen species (ROS), also plays a key role in the development of NAFLD. NADPH Oxidase (NOX) enzymes are the main producers of ROS, and it has been shown that their increased activity is linked to NAFLD and insulin resistance due to hepatic lipid overload (31, 32). Also, it is known that obesity and unhealthy food habits lead to excessive production of ROS by creating an imbalance between ROS production and elimination, and therefore participate even more in the development of insulin resistance and liver tissue damage participating in the vicious cycle (31). Subjects with NAFLD have in general lower plasma adiponectin concentrations than individuals without NAFLD and it is known that adiponectin plays an anti-inflammatory role and improves hepatic insulin sensitivity (33).

In T1D, it has been shown that insulin resistance and obesity are increasing with time and these described mechanisms in T2D may be likely to occur in T1D (6). Since T1D only relies on exogenous insulin subcutaneous administration, one of the factors influencing the pathophysiology of NAFLD development in these individuals is the altered dynamic of insulin delivery and of insulin clearance. Hyperinsulinemia in patients with NAFLD appears to be much more correlated with impaired insulin clearance than with increased insulin secretion (34). A recent study assessed the role of metabolic determinants of NAFLD in T1D individuals. Poor glycemic control (HbA1c > 7%) doubled the risk of NAFLD, and the prevalence in patients with BMI > 25 kg/m^2 was higher (66%) than the overall NAFLD prevalence (47%). Interestingly, 37% of the lean individuals (BMI < 25 kg/m^2) had NAFLD and this was correlated with total insulin dose. This study shows in patients with T1D the potential importance of exogenous injected insulin and the crucial impact of obesity in the development of NAFLD (35).

CEACAM1 (Carcinoembryonic antigen-related cell adhesion molecule 1) is a cell transmembrane protein playing a key role in insulin degradation and thus its clearance and is abundantly found in hepatocytes to help regulation of insulin homeostasis. CEACAM1 mediates excess insulin removal through its phosphorylation induced by the ligand activated insulin receptor to maintain normal insulinemia (36). There are two main mechanisms that can compromise CEACAM1 phosphorylation and action: hyperinsulinemia and impaired pulsatility of insulin secretion. As a reminder, it has been known for a long time now that beta cells release insulin in two phases: following blood glucose increase with a peak secretion, then followed by a slower release to maximal secretion levels until glycemia is back to normal (37). Considering the importance of insulin secretion pulsatility for CEACAM1's efficiency to clear insulin, continuous high insulinemia exposure not only downregulates insulin receptor

TABLE 1 Comparison of NAFLD Pathophysiology mechanisms between in T1D and T2D.

NAFLD pathophysiological mechanisms	T1D	T2D
Insulin resistance	+	+++
Altered dynamic of insulin delivery	++	–
Altered insulin clearance	+++	+
Relative insulin resistance in hepatocytes	++	+++
SREBP and ChREBP activation by hyperglycemic state and high fructose intake	+	++
Hyperglucagonemia and hepatic glucagon resistance (worsened by amylin deficiency)	+++	–
Low GLP-1 blood concentration	+	++

(-: unlikely; +: not unlikely; ++: likely; +++: very likely). T1D, type 1 diabetes; T2D, type 2 diabetes; SREBP, sterol regulatory element-binding proteins; ChREBP, carbohydrate response element-binding protein; GLP-1, glucagon-like peptide-1.

density, but also downregulates insulin clearance, therefore increasing insulinemia and insulin resistance. The less variation in insulin concentration, the more insulin is needed to be effective.

Poor glucose control leads to hyperglycemia which then increases expression of GLUT-2, a glucose transporter in hepatocytes. In this state of insulin resistance and hyperinsulinemia with hyperglycemia, hepatic lipogenesis is upregulated because of the increase of lipogenic substrate (glucose) availability through GLUT-2 increase and because of the lipogenic effect of insulin (*de novo* lipogenesis) (25). Because of the high blood glucose level and insulin action, glycogen synthesis is enhanced but when glycogen synthesis pathways are saturated due to long-lasting hyperglycemia exposure, glucose is shunted to lipogenic pathways thus favoring NAFLD development (23, 38).

In T1D, subcutaneous insulin injections are required to maintain normal blood glucose levels and it is unlikely that all injected insulin reaches the liver through the portal vein as in endogenous insulin production, then implying a relative state of insulin resistance and increased insulin requirement (25, 39) (23, 40).

Intrahepatic lipogenesis is enhanced by insulin notably by increasing sterol regulatory element-binding proteins (SREBPs) in hepatocytes and stimulating them (41). These proteins not only help for cholesterol, free fatty acids, triglycerides and phospholipids synthesis and uptake, but are also essential for enzymes expression that are required for lipogenesis (42). SREBP-1c protein, which is upregulated by hyperglycemia, is crucial for glucokinase, liver-type pyruvate kinase (LPK), fatty acid synthase (FAS), and acetyl-CoA-carboxylase (ACC) expression, which all participate in the increase of lipogenesis (43). LPK gene transcription is also stimulated by another transcription factor called ChREBP (carbohydrate response element-binding protein) but is only highly activated in hyperglycemic state without the influence of insulinemia (44). We can then hypothesize that SREBP and ChREBP are important factors and contributors for the development of NAFLD in T1D.

These factors are also activated by chronic fructose consumption usually found in individuals with metabolic syndrome and T2D (45). However, fructose consumption by T1D individuals is very common given the potentially frequent hypoglycemias experienced by these individuals. To correct their low blood sugar level, they use sugar-rich beverages which are often fructose-rich nutrients such as sodas/soft drinks, fruit juices or processed food. This behavior can occur every day for a lot of T1D and contribute not only to weight gain or obesity, but also to the activation of lipogenesis leading therefore to NAFLD susceptibility (40, 46).

T1D is also associated with other pancreatic hormones abnormalities such as hyperglucagonemia. Glucagon is a hormone secreted by alpha cells to counteract the effects of insulin to stabilize blood glucose level. It is usually suppressed by hyperglycemia and by paracrine insulin production but not by exogenous insulin administration, explaining in part hyperglucagonemia seen in T1D (47). Another cause of hyperglucagonemia in T1D is the lack of amylin secretion usually produced by beta cells simultaneously with insulin in response to nutrient stimuli. Amylin suppresses glucagon production in response to postprandial glucose increase, avoiding hepatic glucose production, and slows gastric emptying, avoiding glucose excursions (48). In normal individuals, glucagon increases hepatic lipolysis with free fatty acids oxidation, and suppresses lipogenesis, thus having likely a protective effect against fat accumulation in the liver (49). Nonetheless, hepatic glucagon resistance has been found in patients with NAFLD, thereby promoting fat accumulation in the liver and hyperglycemia through lack of neoglucogenesis inhibition (50). Therefore, hyperglucagonemia found in T1D could contribute to the development and worsening of NAFLD, although there is not enough evidence yet.

Another hormone that rose a lot of interest these recent years is glucagon-like peptide-1 (GLP-1). GLP-1 is an incretin hormone secreted by intestinal L cells upon food intake with effects on satiety, glycemia and gastric emptying. It has been shown that GLP-1 agonists reduce liver fat accumulation and reduce NASH activity (51). GLP-1 agonists have also shown to upregulate CEACAM1 transcription, thus increasing insulin clearance, which helps protecting the liver from insulin resistance and from fat deposition (36). In some studies, GLP-1 blood concentrations have been shown to be lower in patients with T1D and as such could also be one of the factors contributing to NAFLD development (40, 52–54). This hypothesis should be further studied since other work seems to support the fact that there is no significant difference in GLP-1 blood concentrations between T1D patients and the general population (55, 56).

Glycogenic hepatopathy: A differential diagnosis

One of the main differential diagnoses of NAFLD that can be seen on imaging, especially ultrasonography, is glycogenic hepatopathy. This is a rare condition characterized by the accumulation of glycogen in the hepatocytes, mostly affecting children and adolescents with poorly controlled T1D (25). Initially, glycogenic hepatopathy was considered to be part of Mauriac syndrome, which is a complication of badly controlled T1D with delayed puberty, dwarfism, cushingoid features and liver enlargement due to glycogen deposition (57). However, glycogenic hepatopathy was later dissociated from Mauriac syndrome and characterized by glycogen accumulation in hepatocytes due to poor glycemic control without any other features of Mauriac syndrome (58). To diagnose glycogenic

hepatopathy, a liver biopsy is required (59). Imaging is also used to help diagnose glycogenic hepatopathy, for example using ultrasonography. The main difficulty with ultrasonography remains its poor specificity due to similarities found in both NAFLD and glycogenic hepatopathy even though both diseases can coexist at the same time considering that their cause is identical: poor glucose control (25). Since MRI can distinguish fat from glycogen, it can be used to distinguish these two pathologies much more efficiently than ultrasonography or CT. To distinguish one from the other, there are some biological and clinical characteristics that can help, such as abdominal discomfort and elevation of liver enzymes, both found more often in glycogenic hepatopathy (Table 2) (60).

Discussion

NAFLD in T1D has become a subject of interest these recent years with more studies assessing a potential link between these diseases, since NAFLD is a rising disease that we still know little about despite more studies now being published in this field. Nonetheless, it seems very likely that there is a causative link between T1D and NAFLD, and exploring this association with further studies will help understand and treat NAFLD in T1D. It is not totally clear if patients with T1D are more susceptible to develop NAFLD as studies seem to be contradictory about whether NAFLD prevalence in T1D is higher than in the general population or not (3–5). Although most studies seem to show a higher prevalence of NAFLD in T1D, further work must be done to support this statement.

The main limitation of these studies assessing NAFLD prevalence in T1D remains the diagnostic modality used. Since the gold standard to diagnose NAFLD remains liver biopsy, but is expensive and risky to perform in a large population and since there is no blood biomarkers specific enough for NAFLD, imaging diagnosis remains the best way to diagnose NAFLD for now with multi parametric MRI being considered as a virtual biopsy with great specificity and sensitivity (24). However, given the cost of MRI, applying it to a large number of individuals will be a limitation for further studies.

NAFLD encompasses a whole spectrum of liver injuries including NASH. However, there is currently very little data on NASH prevalence in patients with T1D. It has been shown in a study that NASH has been histologically diagnosed in 20.4% of T1D individuals (10 out of 49 individuals) whereas it has been diagnosed in 44.4% of the T2D individuals (20 out of 45 individuals) (61). The T1D cohort in this study was younger but diabetes duration before liver biopsy was longer in the T2D cohort. A recent study in 2021 compared 30 T1D patients with 37 T2D patients in order to assess the relationship between hepatic energy metabolism and diabetes-related NAFLD. This study showed that, as expected, T2D individuals had higher hepatocellular lipid content (38% in T2D *vs.* 7% in T1D) and higher insulin resistance despite similar glycemic control. The follow-up after 5 years showed that hepatocellular lipid content doubled in T2D individuals with an increase of visceral adipose tissue, increasing the prevalence of NAFLD up to 70%. This was correlated with insulin resistance, and hepatic energy metabolism, estimated with γATP and inorganic phosphate (Pi) concentrations, was impaired in both individuals but significantly more in T2D individuals (17% *vs.* 10% in T1D). Altogether, this study suggests that fat tissue mass and liver mitochondria have an important role in the development of NAFLD in patients with diabetes (62). This can suggest the important role of excessive visceral adipose tissue in NAFLD and NASH emergence. Since there is only little data regarding NASH prevalence in T1D, further work is therefore required to specifically address this question.

Another area yet to be explored is searching for biological blood biomarkers that would be highly specific for NAFLD in T1D. A potential candidate is CEACAM1, which is known to be downregulated in NAFLD and upregulated with GLP-1 analogs (36). CEACAM1, a transmembrane protein acting in hepatocytes to get rid of insulin excess hence limiting insulin resistance, has been shown to be lower in T1D and could be the link between NAFLD and T1D (36). Another interesting biomarker that can help diagnose NAFLD is an elevated alanine transaminase (ALT) blood concentration. Indeed, elevated ALT concentration is frequently encountered in T1D-associated NAFLD (63).

TABLE 2 Comparison between Glycogenic Hepatopathy and Non-Alcoholic Fatty Liver Disease (NAFLD).

	Glycogenic Hepatopathy	Non-Alcoholic Fatty Liver Disease
Age at onset	Mostly children and adolescents	Mostly Adults
Uncontrolled T1D with extremely poor glucose control	Yes	Not necessarily
Symptoms	Present (abdominal discomfort)	Uncommon
Signs	Tender hepatomegaly	Ascites in advanced NAFLD
Liver Enzymes	Mild to severe elevation	No or mild elevation (mostly alanine transaminase)
Ultrasonography findings	Hyperechogenic: due to glycogen deposition	Hyperechogenic: due to fat deposition
Magnetic Resonance Imaging findings	Absence of steatosis (no difference in intensities)	Presence of steatosis (difference in intensities)
Diagnosis: Gold Standard	Histology (liver biopsy)	Histology (liver biopsy)

T1D, type 1 diabetes.

Nevertheless, elevated liver enzymes can have many causes and raised liver enzymes are not necessarily present in NAFLD (25).

Since CEACAM1 plays a crucial role in insulin resistance and in NAFLD development, we can hypothesize that pharmacologically upregulating CEACAM1 could be a promising therapeutic approach for the treatment of NAFLD in T1D. As described above, GLP-1 analogs along with PPARγ (peroxisome proliferator-activated receptor γ) agonists have both shown good potential since they both increase CEACAM1 transcription. Other potential therapeutic targets include molecules such as GIP (gastric inhibitory polypeptide) analogs, which are also part of the incretin hormones family like GLP-1 analogs, or a combination of both GLP-1 analog and GIP analog such as the dual agonist tirzepatide. Nevertheless, studies in this area are still needed to evaluate the potential of this group of molecules on NAFLD, not only in T2D, but also in T1D. Another potential therapy could be amylin analogs since amylin in T1D is lacking and it was demonstrated that pramlintide, a synthetic amylin analog, showed improvement in metabolic control (25, 64). A retrospective analysis showed that short-chain fatty acids can influence gut barrier health and have positive effects not only on NAFLD, but also on T1D. Short-chain fatty acids, especially butyrate, seem to prevent the destruction of gut barrier by maintaining it and strengthening it. They also participate in the regulation of gut microbiota and immune cells, and for all these reasons short-chain fatty acids represent another promising potential therapy for NAFLD and T1D (65).

Conclusion

There are several important points to keep in mind when it comes to NAFLD and T1D: the diagnostic modality used for NAFLD diagnosis is very important since NAFLD is difficult to diagnose without histological analysis and conventional imaging is often insufficient (24). Glycogenic hepatopathy is radiologically similar to NAFLD mostly in ultrasonography and it is important to remember the other differences that help distinguish them, such as elevated liver enzymes and abdominal discomfort, usually not found in NAFLD (28). New imaging techniques such as multi parametric MRI show promising results but remain costly and therefore represents a major limitation (24). Even though NAFLD in T1D can be partly explained by the increase in obesity and metabolic syndrome in T1D subjects, some other pathways different from the ones found in metabolic syndrome and T2D may be the key to understand the relation between T1D and NAFLD development (25, 44, 46). Relative hepatic insulin resistance caused by impaired insulin pulsatility and impaired insulin clearance, as well as hyperglucagonemia, both play a crucial role in NAFLD development and are both present in T1D (36, 47). GLP-1 agonists, amylin agonists and short-chain fatty acids have shown promising results in the treatment of NAFLD but must be further investigated, notably in T1D (51, 64, 65).

Author contributions

Both authors contributed equally to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sex difference in the associations among liver function parameters with incident diabetes mellitus in a large Taiwanese population follow-up study

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Background: The prevalence of diabetes mellitus (DM) in Taiwan between 2017 and 2020 was 11.05%, which is higher than the global prevalence (10.5%). Previous studies have shown that patients with DM have higher liver enzyme levels than those without DM. However, it is unclear whether there are sex differences in the association between incident DM and liver function. Therefore, the aim of this longitudinal study was to investigate this issue in a large Taiwanese cohort.

Methods: We identified 27,026 participants from the Taiwan Biobank, and excluded those with baseline DM (n = 2,637), and those without follow-up data on DM, serum fasting glucose or glycosylated hemoglobin A1c (n = 43). The remaining 24,346 participants (male: 8,334; female: 16,012; mean age 50.5 ± 10.4 years) were enrolled and followed for a median of 4 years.

Results: Of the enrolled participants, 1,109 (4.6%) had incident DM and 23,237 (95.4%) did not. Multivariable analysis showed that high levels of glutamic-oxaloacetic transaminase (AST) ($p < 0.001$), glutamic-pyruvic transaminase (ALT) ($p < 0.001$), albumin ($p = 0.003$), α-fetoprotein ($p = 0.019$), and gamma-glutamyl transpeptidase (GGT) ($p = 0.001$) were significantly associated with incident DM in the male participants. In comparison, high levels of AST ($p = 0.010$), ALT ($p < 0.001$), albumin ($p = 0.001$) and GGT ($p < 0.001$), and low total bilirubin ($p = 0.001$) were significantly associated with incident DM in the female participants. There were significant interactions between total bilirubin and sex ($p = 0.031$), and GGT and sex ($p = 0.011$) on incident DM.

Conclusion: In conclusion, liver function parameters were significantly associated with incident DM. Further, there were differences in the associations between the male and female participants.

KEYWORDS

liver function parameters, incident diabetes mellitus, sex difference, follow-up, Taiwan Biobank

Introduction

Diabetes mellitus (DM) is a heterogeneous group of disorders characterized by hyperglycemia (1). Type 2 is the most common form of DM, and is cause by multiple pathophysiologic abnormalities. While insulin resistance in muscle/liver and β-cell failure remain the core defects, dysfunction of adipocytes, gastrointestinal tract, α-cells, kidney, and brain had also been found to be important in development of glucose intolerance in Type 2 DM population which form the concept of ominous octet (2). The International Diabetes Federation Diabetes Atlas estimated that the global prevalence of DM in people aged 20–79 years in 2021 was 10.5% (536.6 million people) (3). According to the Taiwan Health Promotion Administration, the prevalence of DM in Taiwan between 2017 and 2020 was 11.05%, which is higher than the global prevalence (10.5%) (4). Common risk factors for DM include overweight or obesity, high-risk race/ethnicity, history of cardiovascular disease, hypertension, physical inactivity, smoking and aging (5). The complications associated with DM include microvascular (diabetic nephropathy, neuropathy, and retinopathy), macrovascular (coronary artery disease, cerebrovascular disease), and miscellaneous types (6). The global diabetes-related health expenditure was estimated to be USD 966 billion in 2021 (3), highlighting the importance of detecting the potential risk factors for DM.

Liver function parameters could be classified to 3 main categories according to their functions: (1) Detection of hepatocellular injury such as glutamic-oxaloacetic transaminase (AST), glutamic-pyruvic transaminase (ALT) and gamma-glutamyl transpeptidase (GGT); (2) Liver's biosynthetic capacity such as albumin and α-fetoprotein (AFP); (3) Liver's capacity of transportation of the organic anions and to metabolize drugs such as total serum bilirubin (7). In the first category, detection of hepatocellular injury, marked elevations in ALT levels suggest hepatocellular injury such as viral hepatitis, ischemic liver injury and toxin-induced liver damage (8). AST is present in a wide variety of tissues including the heart, skeletal muscle, kidney, brain and liver, however it is not as sensitive as ALT to detect hepatocellular injury (9). In addition, an elevated GGT level may indicate liver diseases such as acute viral hepatitis, chronic hepatitis C and non-alcoholic fatty liver disease (NAFLD) (10), however it may also indicate the presence of non-liver diseases such as uncomplicated DM, acute pancreatitis and myocardial infarction (11). In the second category, liver's biosynthetic capacity, albumin is synthesized in the liver and it is one of the most important proteins in plasma. Since albumin is only synthesized in the liver, it is a useful indicator of hepatic function, and a decrease in albumin may indicate chronic liver disease or liver cirrhosis (12). An elevated level of AFP may also indicate liver injury and the early stages of chemical hepatocarcinogenesis (13), so it can be an indicator of hepatocellular carcinoma (HCC). In the last category, liver's capacity of transportation of the organic anions and to metabolize drugs, bilirubin is derived from the breakdown of hemoglobin, and an elevated level of the unconjugated form in the liver may suggest underlying liver disease or hemolysis (8). Since the liver performs a variety of functions, no single test is sufficient to completely evaluate its function (7).

Previous studies showed that patients with abnormal liver functions test were related to incident DM (14–17). However, it is unclear whether there are sex differences in the association between incident DM and liver function. Therefore, the aim of this longitudinal study was to investigate sex differences in the association between incident DM and liver function parameters (AST, ALT, albumin, AFP, total bilirubin, and GGT) in a large cohort derived from the Taiwan Biobank (TWB).

Materials and methods

TWB

The TWB is the largest biobank in Taiwan. It was established by The Ministry of Health and Welfare with the goals of promoting healthcare and preventing diseases, with a focus on the aging population in Taiwan. The TWB collects health-related data on ∼200,000 healthy volunteers around Taiwan, as detailed below (18, 19). Ethical approval for the TWB was granted by the Ethics and Governance Council of the TWB and Institutional Review Board (IRB) on Biomedical Science Research, Academia Sinica, Taiwan.

The data collected by the TWB include body mass index (BMI), age, sex, and the presence of hypertension and DM. Fasting blood samples were obtained from all of the patients, and laboratory tests were conducted using an autoanalyzer (Roche Diagnostics GmbH, D-68298 Mannheim COBAS Integra 400). Overnight fasting blood and urine tests are also performed to collect data on uric acid, glucose, glycosylated hemoglobin A1c (HbA1c), triglycerides, total cholesterol, high-/low-density lipoprotein (HDL/LDL) cholesterol, estimated glomerular filtration rate (eGFR) [using the MDRD equation (20)], and liver function parameters (AST, ALT, albumin, AFP, total bilirubin, and GGT).

Data on blood pressure (BP) are also obtained, with the measurements made digitally by a TWB researcher three times with a 1–2-min gap between measurements. The participants are requested to avoid caffeine, exercise, and smoking for a minimum of 30 min prior to the measurements. Average systolic and diastolic BP measurements were analyzed in this study. Data on regular exercise, defined as ≥30 min of physical activity ≥3 times a week, were also recorded. This study was conducted according to the Declaration of Helsinki, and approved by the IRB of Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20210058).

Assessment of alcohol drinking and cigarette smoking history

All the participants also underwent a face-to-face interview with a researcher, during which they completed a questionnaire asking about alcohol drinking and cigarette smoking history. Subjects who had had smoked one cigarette or more per day for at least 1 year were defined as ever-smokers. Subjects who had drunk an alcoholic beverage, including beer, liquor, wine or Chinese herd wine, more than four times a week for at least 1 year were defined as ever drinkers.

Participants

A total of 27,026 participants (male: 9,552; female: 17,474) were identified in the TWB. The participants who enroll in the TWB follow up after 2–4 years. Information, including a questionnaire, physical examination and blood examination, is collected upon first enrollment and second follow-up. Of whom those with no follow-up data on DM, serum fasting glucose or HbA1c ($n = 43$), and those with baseline DM ($n = 2,637$) were excluded. The remaining 24,346 participants were enrolled and followed for a median of 4 years (Figure 1). All of the enrolled participants gave written informed consent.

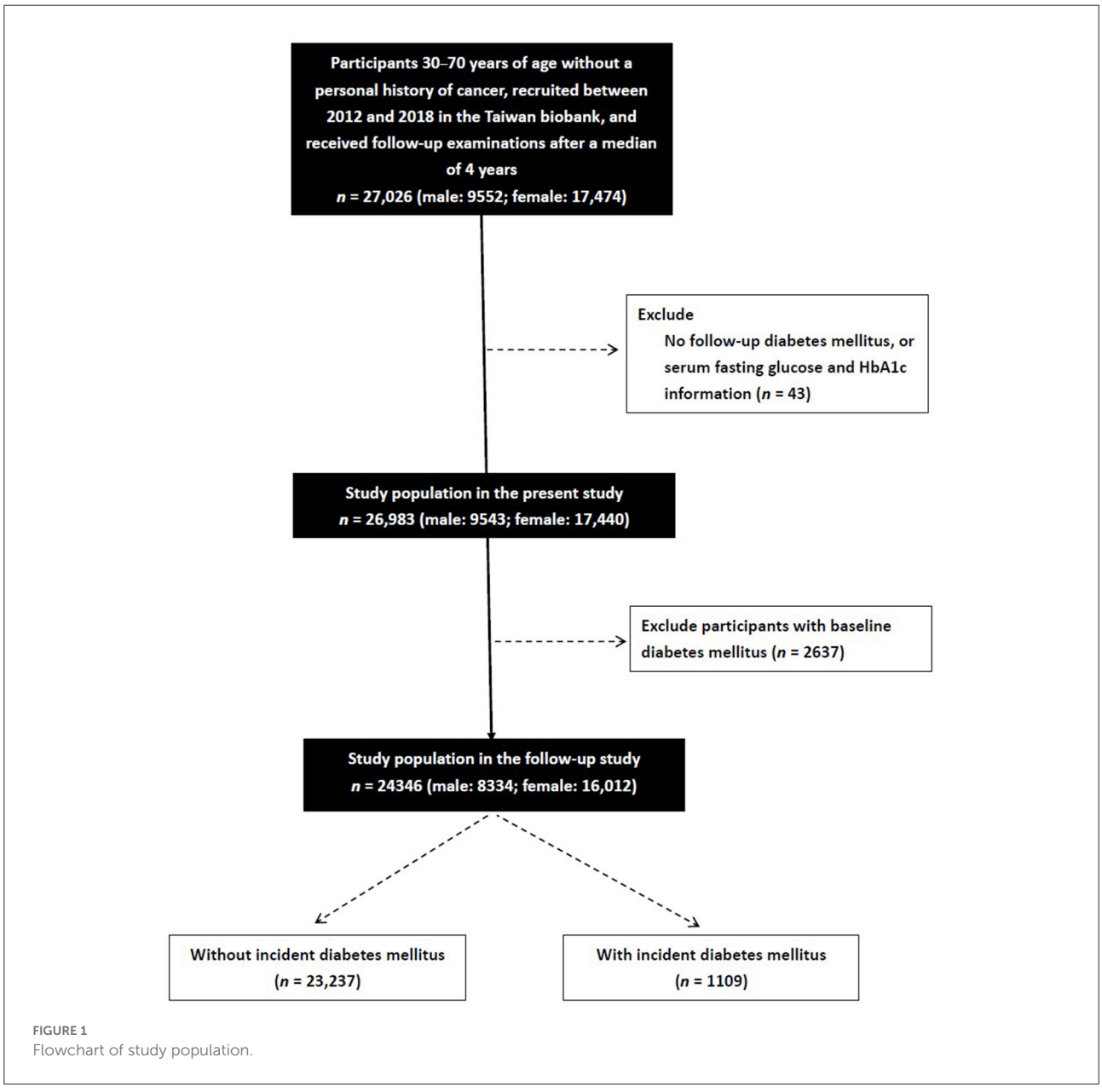

FIGURE 1
Flowchart of study population.

Definition of incident DM

Participants with no past history of DM (self-reported) with a fasting glucose level <126 mg/dL and HbA1c <6.5% were defined as not having DM. Incident DM was defined as developing DM (self-reported, fasting glucose level ≥126 mg/dL or HbA1c ≥6.5%) during the follow-up period.

Statistical analysis

Statistical analysis was done using SPSS version 19 (IBM Inc., Armonk, NY). Variables are shown as percentage or mean (±SD). Continuous variables were compared using the independent *t*-test, and categorical variables were compared using the chi-square test. Multivariable logistic regression analysis was used to examine associations between the development of incident DM and the studied liver function parameters (AST, ALT, albumin, AFP, total bilirubin, and GGT) in the male and female participants. An interaction *p* in logistic analysis was defined as: model disease $(y) = x1 + x2 + x1 \times x2$ + covariates; where $x1 \times x2$ is the interaction term, $y =$ incident DM, $x1 =$ sex, and $x2 =$ the studied liver function parameters. The covariates were significant variables in univariable analysis. Receiver operating characteristic (ROC) curves were assessed the performance of the liver function parameters to identify incident DM, and areas under the ROC curves (AUCs) were used to assess their predictive ability. A two-tailed p-value < 0.05 was considered statistically significant.

Results

Of the 24,346 enrolled participants (male: 8,334; female: 16,012; mean age, 50.5±10.4 years), 1,109 (4.6%) had incident DM and 23,237 (95.4%) did not. The incidence rates of DM were 5.7 and 4.0% in the males and females ($p < 0.001$), respectively.

Characteristics of the with and without incident DM groups

The characteristics of the with and without incident DM groups are shown in Table 1. The incident DM group had a higher percentage of males, were older, had higher rates of hypertension, smoking, alcohol drinking, menstruation (in females), and higher systolic and diastolic BP, BMI, fasting glucose, HbA1c, triglycerides, total cholesterol, uric acid and LDL-cholesterol, and lower HDL-cholesterol and eGFR than the without incident DM group. With regards to the liver function parameters, the incident DM group had higher AST, higher ALT, higher albumin, lower total bilirubin and higher GGT. However, there was no significant difference in AFP.

Comparisons of liver function parameters between the with and without incident DM groups in the male and female participants

The male participants with incident DM had higher AST, ALT, AFP, and GGT, but lower total bilirubin than the male participants without incident DM (Table 2). However, there was no significant difference in albumin. In addition, the female participants with incident DM had higher AST, ALT, albumin, and GGT, but lower total bilirubin than the female participants without incident DM. However, there was no significant difference in AFP.

Associations among liver function parameters with incident DM in the male and female participants

Multivariable logistic regression analysis was performed to examine associations among the liver function parameters with incident DM by sex (Table 3). In the male participants, after adjusting for age, hypertension, systolic and diastolic BPs, smoking and alcohol history, BMI, triglycerides, total cholesterol, LDL/HDL-cholesterol, eGFR and uric acid (significant variables in Table 1), high AST (per 1 U/L; odds ratio [OR], 1.013; 95% confidence interval [CI], 1.008–1.019; $p < 0.001$), high ALT (per 1 U/L; OR, 1.009; 95% CI, 1.006–1.012; $p < 0.001$), high albumin (per 1 g/dL; OR, 1.975; 95% CI, 1.254–3.110; $p = 0.003$), high AFP (per 1 g/mL; OR, 1.021; 95% CI, 1.003–1.039; $p = 0.019$), and high GGT (per 1 U/L; OR, 1.003; 95% CI, 1.001–1.005; $p = 0.001$) were significantly associated with incident DM. However, total bilirubin was not associated with incident DM in the male participants. In the female participants, after adjusting for the variables listed above for the male participants plus menstruation status, high AST (per 1 U/L; OR, 1.007; 95% CI, 1.002–1.012; $p = 0.010$), high ALT (per 1 U/L; OR, 1.007; 95% CI, 1.003–1.010; $p < 0.001$), high albumin (per 1 g/dL; OR, 2.018; 95% CI, 1.356–3.003; $p = 0.001$), low total bilirubin (per 1 mg/dL; OR, 0.515; 95% CI, 0.348–0.762; $p = 0.001$), and high GGT (per 1 U/L; OR, 1.006; 95% CI, 1.004–1.009; $p < 0.001$) were significantly associated with incident DM. However, AFP was not associated with incident DM in the female participants.

Interactions among liver function parameters and sex on incident DM

Significant interactions were found between total bilirubin and sex ($p = 0.031$), and GGT and sex ($p = 0.011$) on incident DM (Table 3).

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TABLE 1 Comparison of clinical characteristics among participants without or with incident DM.

Characteristics	Incident DM (−) (n = 23,237)	Incident DM (+) (n = 1,109)	p
Age (year)	50.3 ± 10.4	54.7 ± 9.0	<0.001
Male gender (%)	33.8	42.9	<0.001
Hypertension (%)	10.2	25.5	<0.001
Systolic BP (mmHg)	116.1 ± 17.2	125.3 ± 17.7	< 0.001
Diastolic BP (mmHg)	72.0 ± 10.8	76.3 ± 10.6	<0.001
Smoking history (%)	24.5	31.1	<0.001
Alcohol history (%)	2.7	4.7	<0.001
Regular exercise habits (%)	47.6	48.7	0.493
BMI (kg/m^2)	23.7 ± 3.4	26.1 ± 3.7	<0.001
Menstruation in female (%)	47.9	27.8	<0.001
Laboratory parameters			
Fasting glucose (mg/dL)	91.7 ± 7.3	101.5 ± 10.0	<0.001
HbA1c (%)	5.6 ± 0.3	6.0 ± 0.3	<0.001
Triglyceride (mg/dL)	107.2 ± 72.6	159.3 ± 134.5	<0.001
Total cholesterol (mg/dL)	195.5 ± 34.8	203.1 ± 37.2	<0.001
HDL-cholesterol (mg/dL)	55.2 ± 13.2	48.7 ± 11.3	<0.001
LDL-cholesterol (mg/dL)	121.7 ± 31.1	128.9 ± 33.7	<0.001
eGFR (mL/min/1.73 m^2)	109.6 ± 25.0	106.0 ± 24.0	<0.001
Uric acid (mg/dL)	5.4 ± 1.4	6.1 ± 1.5	<0.001
Liver function parameters			
AST (U/L)	24.1 ± 10.9	28.3 ± 16.2	<0.001
ALT (U/L)	22.4 ± 17.9	31.6 ± 26.5	<0.001
Albumin (g/dL)	4.55 ± 0.23	4.58 ± 0.24	<0.001
AFP (ng/mL)	3.32 ± 6.50	3.41 ± 5.75	0.636
Total bilirubin (mg/dL)	0.67 ± 0.28	0.64 ± 0.27	0.003
GGT (U/L)	22.6 ± 26.5	33.9 ± 40.8	<0.001

DM, diabetes mellitus; BP, blood pressure; BMI, body mass index; HbA1c, glycosylated hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; eGFR, estimated glomerular filtration rate; AST, glutamic-oxaloacetic transaminase; ALT, glutamic-pyruvic transaminase; AFP, α-fetoprotein; GGT, gamma-glutamyl transpeptidase.

Performance and predictive ability of the liver function parameters to identify incident DM

The AUCs of the liver function parameters to incident DM in the male and female participants are shown in Table 4. In the male participants, GGT had the highest AUC (0.631), followed by ALT (0.617), AST (0.570) and total bilirubin (0.472). Albumin and AFP were not significantly associated with incident DM. In the female participants, GGT also had the highest AUC (0.701), followed by ALT (0.679), AST (0.614), total bilirubin (0.441), AFP (0.558) and albumin (0.540).

Discussion

In this study, we investigated sex differences in the associations between incident DM and liver function parameters after a median 4-year follow-up period. We found that high AST, ALT, albumin and GGT were associated with incident DM in both sexes. However, high total bilirubin was only associated with incident DM in the females, and high AFP was only associated with incident DM in the males. Further, we found significant interactions between total bilirubin and GGT and sex on incident DM.

The first important finding of this study is that high AST, ALT, albumin and GGT were associated with incident DM in

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TABLE 2 Comparison of clinical characteristics of the study participants classified by the presence of different sex and incident DM.

Characteristics	Male (n = 8,334)			Female (n = 16,012)		
	Incidence of DM (−) (n = 7,858)	Incidence of DM (+) (n = 476)	p	Incidence of DM (−) (n = 15,379)	Incidence of DM (+) (n = 633)	p
Age (year)	50.4 ± 11.0	53.9 ± 9.9	<0.001	50.3 ± 10.1	55.3 ± 8.3	<0.001
Hypertension (%)	14.3	29.0	<0.001	8.2	22.9	<0.001
Systolic BP (mmHg)	121.1 ± 16.0	126.4 ± 16.6	<0.001	113.5 ± 17.2	124.4 ± 18.5	<0.001
Diastolic BP (mmHg)	76.8 ± 10.4	79.2 ± 10.3	<0.001	69.6 ± 10.2	74.1 ± 10.3	<0.001
Smoking history (%)	57.9	64.3	0.006	7.5	6.2	0.201
Alcohol history (%)	6.8	10.5	0.002	0.7	0.3	0.446
Regular exercise habits (%)	48.4	45.6	0.228	47.2	51.0	0.061
Menstruation in female (%)	–	–	–	47.9	27.8	<0.001
BMI (kg/m^2)	24.8 ± 3.1	26.7 ± 3.6	<0.001	23.2 ± 3.4	25.7 ± 3.7	<0.001
Laboratory parameters						
Fasting glucose (mg/dL)	93.9 ± 7.2	102.8 ± 9.8	<0.001	90.5 ± 7.0	100.5 ± 10.0	<0.001
HbA1c (%)	5.57 ± 0.33	6.01 ± 0.29	<0.001	5.54 ± 0.33	6.04 ± 0.28	<0.001
Triglyceride (mg/dL)	127.3 ± 90.8	181.9 ± 168.5	<0.001	96.9 ± 58.6	142.3 ± 95.4	<0.001
Total cholesterol (mg/dL)	192.4 ± 33.8	197.6 ± 38.1	0.001	197.1 ± 35.2	207.3 ± 36.0	<0.001
HDL-C (mg/dL)	48.8 ± 11.1	43.8 ± 8.9	<0.001	58.5 ± 13.0	52.4 ± 11.6	<0.001
LDL-C (mg/dL)	123.0 ± 30.8	126.3 ± 34.2	0.023	121.0 ± 31.3	130.9 ± 33.1	<0.001
eGFR (mL/min/1.73 m^2)	99.2 ± 19.8	96.9 ± 20.6	0.018	114.9 ± 25.7	112.9 ± 24.1	0.041
Uric acid (mg/dL)	6.5 ± 1.3	6.9 ± 1.5	<0.001	4.9 ± 1.1	5.5 ± 1.1	<0.001
Liver function parameters						
AST (U/L)	25.9 ± 11.3	30.1 ± 19.2	<0.001	23.2 ± 10.6	26.9 ± 13.4	<0.001
ALT (U/L)	27.4 ± 20.3	36.5 ± 31.5	<0.001	19.9 ± 16.0	28.0 ± 21.3	<0.001
Albumin (g/dL)	4.62 ± 0.23	4.63 ± 0.25	0.500	4.51 ± 0.22	4.54 ± 0.23	0.005
AFP (ng/mL)	3.14 ± 2.29	3.53 ± 8.60	0.007	3.41 ± 7.82	3.32 ± 1.52	0.255
Total bilirubin (mg/dL)	0.76 ± 0.32	0.73 ± 0.29	0.028	0.62 ± 0.24	0.58 ± 0.22	<0.001
GGT (U/L)	29.9 ± 36.3	41.5 ± 51.5	<0.001	18.9 ± 18.6	28.1 ± 29.1	<0.001

Abbreviations are the same as in Table 1.

both sexes. ALT is an enzyme primarily found in the liver, and it is more closely related to hepatocellular injury or fat deposition (21). Although AST is present in the liver, it is also present in other organs including cardiac and skeletal muscles, kidneys and brain, and it is less specific for hepatic damage then ALT (22). The AST to ALT ratio has been use to discern the different etiologies of hepatic injury (23). Previous studies have reported associations between ALT and type 2 DM (24, 25). Ohlson et al. (24) reported that an increase in ALT was associated with a higher relative risk of incident DM in middle-aged Swedish men. Vozarova et al. (25) analyzed 451 Pima Indians, and found that high ALT was an independent predictor of incident type 2 DM after adjusting for age, sex, body fat, insulin sensitivity and acute insulin response. In addition, Goessling et al. (26) reported that both ALT and AST were associated with a greater risk of incident DM after adjusting for baseline blood glucose and changes in weight. Moreover, they also found that only ALT was associated with incident DM when using normal values in the analysis (26). Previous studies have also shown a close relationship between NAFLD with type 2 DM (27). In patients with NAFLD, increases in ALT and AST by more than 2–5 times the normal limit and an AST/ALT ratio <1 have consistently been reported, possibly due to the effect of hepatocyte damage (23, 28). Another possible link between DM and NAFLD may be due to insulin resistance and visceral fat deposition, both of which can affect the regulation of lipoprotein and glucose. Under conditions of increasing insulin resistance, the downregulation of lipolysis by insulin can lead to further adipose deposition on hepatocytes, thereby further

TABLE 3 Association of liver function parameters with incident DM using multivariable logistic regression analysis in different sex.

Liver function parameters	Male (n = 8,334)			Female (n = 16,012)			Interaction p
	Multivariable*			Multivariable#			
	OR	95% CI	p	OR	95% CI	p	
AST (per 1 U/L)	1.013	1.008–1.019	<0.001	1.007	1.002–1.012	0.010	0.126
ALT (per 1 U/L)	1.009	1.006–1.012	<0.001	1.007	1.003–1.010	<0.001	0.315
Albumin (per 1 g/dL)	1.975	1.254–3.110	0.003	2.018	1.356–3.003	0.001	0.586
AFP (per 1 g/mL)	1.021	1.003–1.039	0.019	0.992	0.967–1.016	0.503	0.069
Total bilirubin (per 1 mg/dL)	0.941	0.688–1.288	0.706	0.515	0.348–0.762	0.001	0.031
GGT (per 1 U/L)	1.003	1.001–1.005	0.001	1.006	1.004–1.009	<0.001	0.011

Values expressed as odds ratio (OR) and 95% confidence interval (CI). Abbreviations are the same as in Table 1.
*Adjusted for age, hypertension, systolic and diastolic blood pressures, smoking and alcohol history, body mass index, triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol, eGFR and uric acid (significant variables in Table 1).
#Adjusted for age, hypertension, systolic and diastolic blood pressures, smoking and alcohol history, body mass index, triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol, eGFR, uric acid and menstruation status (significant variables in Table 1).

TABLE 4 Area under curve of liver function parameters for incident DM of different sex.

Liver function parameters	Male (n = 8,334)			Female (n = 16,012)		
	AUC	95% CI	p	AUC	95% CI	p
AST	0.570	0.542–0.598	<0.001	0.614	0.591–0.636	<0.001
ALT	0.617	0.591–0.644	<0.001	0.679	0.658–0.699	<0.001
Albumin	0.515	0.488–0.542	0.273	0.540	0.517–0.563	0.001
AFP	0.516	0.490–0.542	0.230	0.558	0.536–0.579	<0.001
Total bilirubin	0.472	0.445–0.499	0.039	0.441	0.419–0.464	<0.001
GGT	0.631	0.605–0.656	<0.001	0.701	0.682–0.719	<0.001

Values expressed as area under curve (AUC) and 95% confidence interval (CI). Abbreviations are the same as in Table 1.

inducing steatosis (21). Taken together, these explanations may partially explain our findings of associations between high AST and ALT with incident DM.

Another key finding is that we found an association between high albumin and incident DM in both sexes. Serum albumin is mostly produced by the liver, and it accounts for around half of all human plasma proteins. Albumin regulates the oncotic pressure of blood and transports many small molecules (29). A decrease in liver function may result in hypoalbuminemia, which can lead to general edema, fluid loss to the third space, and hyperlipidemia (30). Kunutsor et al. (16) found a nearly linear independent positive association between type 2 DM and serum albumin. In addition, Bae et al. (31) reported that increased serum albumin was positively associated with insulin resistance, but that it was not an independent factor for incident DM. In contrast, Schmidt et al. (32) found that a low serum albumin level was associated with an increased risk of type 2 DM among 12,330 men and women aged from 45 to 64 years. Chang et al. (33) also found that a decrease in albumin level was associated with an increased risk of type 2 DM, and the authors attributed this result to a decrease in hepatic albumin synthesis and increase in glycated albumin, which may increase oxidative stress and inflammation. Although we found that high albumin was associated with incident DM, further studies are needed to clarify the underlying mechanisms.

High GGT was associated with incident DM in both sexes in this study. GGT is an enzyme which catabolizes extracellular glutathione, and it has been widely used as a parameter of liver function. GGT is metabolized in the epithelial cells of the intrahepatic duct, which play an important role in glutathione equilibrium (34). An elevation in GGT has been linked to greater oxidative stress due to increasing glutathione catabolism (antioxidant agent), which may lead to ß-cell dysfunction and a decrease in insulin activity (35). In a cross-sectional study of 7,976 participants from the National Health and Nutrition Examination Survey from 1999 to 2002, Sabanayagam et al. (36) found that higher serum GGT levels were positively associated with DM, independent of alcohol consumption, BMI, hypertension and other confounders. Fraser et al. (14) conducted a meta-analysis of 18 prospective population-based studies, and also found a positive association between GGT and incident DM. Kunutsor et al. (15) conducted another meta-analysis of 24 cohort studies with 177,307 participants focusing on the nature of the dose-response relationship between GGT

and incident DM, and found a non-linear association between GGT and the risk of type 2 DM in both sexes. Moreover, the interactions between GGT and sex on incident DM were also statistically significant. We found that high GGT was more strongly associated with incident DM in the females than in the males in our study. This could be explained from several aspects. Previous studies have shown that GGT level may be affected by estrogen, menopausal stage, and even the use of oral contraceptives. Nilssen and Førde (37) found that starting to use oral contraceptives and menopause were associated with an increase in GGT level, and Serviddio et al. (38) found that estrogen was negatively associated with glutathione. As a catalyzer of glutathione, GGT may also be positively related to the level of estrogen. Moreover, Wang et al. (39) found that the association between elevated GGT and cardiovascular mortality was stronger in females than in males. Hozawa et al. (40) also found a strong positive association between elevated GGT and cardiovascular mortality among Japanese women, but not in men. Both studies concluded that their findings were due to the high percentage of alcohol consumption in men, which may also affect circulating oxidative stress. Female hormones and excessive alcohol consumption in men may play an important role, however the mechanisms underlying sex differences in the association between GGT and incident DM are still not fully understand, and further research is needed.

Another important finding of this study is the association between high AFP with incident DM in males but not in females. AFP belongs to the family of serum albumins produced by the yolk sac and fetal liver during fetal development (41). It is usually at the highest level in infants, and then decreases to normal range before 1 year of age (42). It is used to screen for specific malignancies such as HCC (43) and developmental abnormalities from maternal blood or amniotic fluid (44) in current clinical practice. Moreover, the incidence and mortality rates of HCC are higher in people with DM (45). Obesity is an important risk factor shared between DM and HCC. The pathogenesis is associated with lipid peroxidation, which can lead to an increase in free radical oxidative stress (46) and mutations of p53 tumor suppressor (47), which can both lead to hepatic carcinogenesis (48). Moreover, obesity may cause insulin resistance with hyperinsulinemia, further leading to an increase in insulin-like growth factor-1 which then promotes proliferation and inhibits apoptosis through receptor-mediated pathways, resulting in hepatic carcinogenesis (49). Since the prevalence of HCC is about 2–3 times higher in males compared with females (43), this may explain why a higher AFP level was only associated with incident DM in the males and not females. Another possible explanation for the relationship between AFP and incident DM may be related to metabolic syndrome, which is related to the development of DM, cardiovascular disease, and NAFLD. A possible mechanism for the association between metabolic syndrome and elevated AFP may be due to insulin resistance and fatty liver disease. Both are usually accompanied with each other and they may influence the hemostasis of hepatic glucose, further leading to a chronic inflammatory status of the liver (50).

The last important finding of this study is that low total bilirubin was associated with incident DM in the females but not in the males. Bilirubin is traditionally considered to be derived from the breakdown of hemoglobin *via* normal catabolic pathways, and it is clinically related to jaundice (51). However, recent studies have shown that it is also a potential antioxidant which is inversely related to a lower prevalence of oxidative stress-mediated diseases (52). In a meta-analysis of cross-sectional studies, Nano et al. (53) found an inverse association between bilirubin level and type 2 DM. Several studies have also revealed similar results of an inverse relationship between serum bilirubin level and incident type 2 DM (17). In our study, we found that bilirubin level was only significantly inversely related to incident DM in the females but not in the males. A possible explanation for this finding may be related to different interactions between hormones and bilirubin metabolism in males and females. Kao et al. (54) found that estrogen may facilitate bilirubin metabolism in a regenerating liver by enhancing the expression of cytochrome (CYP2A6). Moreover, Muraca et al. found that hepatic bilirubin UDP-glucuronosyltransferase activity, an enzyme that catalyzes the conjugation of bilirubin and plays an important role in bilirubin excretion, was higher in female than in male rats, but that decreased enzyme activity in female rats and increased activity in male rats were noted after gonadectomy. Therefore, the excretion of bilirubin decreased in the female rats but increased in the males rats after gonadectomy (55), which may partially explain our findings. Another possible mechanism of differences in the association between bilirubin and incident DM between sex maybe related to difference of heme oxygenase (HO) expression between male and female. HO is an enzyme play an important role of heme catabolism to produce biliverdin, and carbon monoxide and eventually increase bilirubin which is the end product of heme catabolism (56). The HO system is related to antioxidant and anti-apoptotic because of its byproducts, bilirubin/biliverdin and carbon monoxide (57). HO-1 is induced by oxidant stress and plays a crucial role of antioxidant in diabetes by improving insulin sensitivity, reduces adipose tissue volume, and causes adipose tissue remodeling (58). An animal study of rats found that trauma and hemorrhage induced a twofold increase in hepatic HO-1 expression in proestrus females compared with males (59). This may explain the mechanism of differences in the association between bilirubin and incident DM between sex.

The strengths of this study include that the analysis involved a large cohort, and the comprehensive follow-up data to analyze sex differences in the association between liver function and incident DM. Despite these strengths, several limitations should be noted. First, information on the presence/absence of fatty liver, dietary issues, and certain medications (ex.

renin-angiotensin-aldosterone system blockers, and statins) which could affect the development or prevention of incident DM is not available in the TWB, which may have resulted in underestimation of the association between liver function and incident DM. In addition, information on factors which could lead to incident DM such as proteinuria is also not available in the TWB. Another limitation is that we only enrolled participants of Han ethnicity residing in Taiwan, and thus our findings may not be generalizable to other ethnicities/areas. Finally, sample bias may have been introduced, as only around 25% of participants in the TWB return for follow-up evaluations.

In conclusion, liver function parameters were significantly associated with incident DM. Further, there were differences in the associations between the male and female participants.

Data availability statement

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

Ethics statement

This study was conducted according to the Declaration of Helsinki, and approved by the IRB of Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20210058). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, methodology, validation, formal analysis, writing—review and editing, supervision, and data curation: Y-KC, P-YW, J-CH, S-CC, and J-MC. Software, investigation, resources, project administration, and funding acquisition: S-CC. Writing—original draft preparation: Y-KC and S-CC. Visualization: S-CC and J-MC. All authors have read and agreed to the published version of the manuscript.

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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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Diet was less significant than physical activity in the prognosis of people with sarcopenia and metabolic dysfunction-associated fatty liver diseases: Analysis of the National Health and Nutrition Examination Survey III

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Background: Sarcopenia is prevalent in metabolic dysfunction-associated fatty liver diseases (MAFLD), and the primary treatment for both diseases is lifestyle modification. We studied how dietary components and physical activity affect individuals with sarcopenia and MAFLD.

Materials and methods: We conducted a study utilizing National Health and Nutrition Examination Survey (NHANES) III (1988–1994) data with Linked Mortality file (through 2019). The diagnosis of fatty liver disease (FLD) was based on ultrasound images revealing moderate and severe steatosis. Using bioelectrical measures, sarcopenia was assessed. Using self-report data, dietary intake and physical activity levels were evaluated.

Results: Among 12,259 participants, 2,473 presented with MAFLD, and 290 of whom had sarcopenia. Higher levels of physical activity (odds ratio [OR] = 0.51 [0.36–0.95]) and calorie (OR = 0.58 [0.41–0.83]) intake reduced the likelihood of sarcopenia in MAFLD patients. During a median follow-up period of 15.3 years, 1,164 MAFLD and 181 MAFLD patients with sarcopenia perished. Increased activity levels improved the prognosis of patients with sarcopenia (Insufficiently active, HR = 0.75 [0.58–0.97]; Active, HR = 0.64 [0.48–0.86]), which was particularly pronounced in older patients.

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Conclusion: In the general population, hyperglycemia was highly related to MAFLD prognosis. Physical inactivity and a protein-restricted diet corresponded to sarcopenia, with physical inactivity being connected to poor outcomes. Adding protein supplements would be beneficial for older people with sarcopenia who are unable to exercise due to frailty, while the survival benefits were negligible.

KEYWORDS

sarcopenia, MAFLD, mortality, physical activity, nutrition, NHANES

1 Introduction

Non-alcoholic fatty liver disease (NAFLD), initially defined as fatty liver disease in the absence of significant alcohol intake and other causes of steatosis, is a common liver disorder that is strongly associated with features of the metabolic syndrome (1). With a prevalence of approximately 25% in the general population, NAFLD has emerged as a leading cause of advanced liver disorders, posing an underestimated global healthcare burden (2). However, the term "non-alcoholic" overemphasized the absence of alcohol consumption while underemphasizing the significance of metabolic factors, which are the primary drivers of the course of the disease (3). It has been suggested that metabolic (dysfunction)-associated fatty liver disease (MAFLD), which endorsed a list of positive diagnostic criteria and offered a more comprehensive description of its metabolic-related natural courses, may represent the importance of metabolic risk factors and improve the detection of the disease (4, 5). Despite the rising prevalence and increasing impact of MAFLD (5, 6), there is an absence of approved pharmacotherapy for this significant condition, whose treatment remains limited to lifestyle modification (7, 8).

Sarcopenia is a geriatric syndrome characterized by generalized loss of muscle mass and its function, and is associated with adverse outcomes (9, 10). Since age-related sarcopenia is inevitable, inactivity and poor diet can accelerate the process. Physical inactivity may contribute to the development of sarcopenia (11, 12), and an increase in moderate-to-vigorous physical activity levels could potentially prevent sarcopenia from developing (13). A cohort study demonstrated that malnutrition is related to a fourfold increased risk of developing sarcopenia over a four-year follow-up period (14).Moreover, lean muscle mass in older individuals is positively associated with protein consumption (15), where insufficient protein intake and a lack of amino acid availability contribute to deficits in muscle protein synthesis (16). Physical exercise has a protective effect on muscle mass and function maintenance, in comparison, the effect of supplemental nutrition on muscle function is uncertain (17–19). A number of studies have revealed that dietary supplements may enhance the benefit of exercise training despite the relatively low quality of the evidence (20); however, the existing evidence for nutrition interventions is based on groups with varying ages, frailties, and nutritional conditions, and the findings are inconsistent (21, 22). Currently, large scale clinical trials are addressing the role of exercise and nutritional interventions in the treatment of sarcopenia, such as the European SPRINTT trial (NCT02582138) (23). In addition to aforementioned variables, sarcopenia is secondary with chronic illness, such as liver diseases, renal diseases, inflammatory diseases, and malignancies (24). Recent studies have observed a significantly higher prevalence of sarcopenia among obese and NAFLD patients (25–27). Multiple potential mechanisms evolved in the link between sarcopenia and NAFLD, including insulin resistance, elevated inflammation, myokines secreted by skeletal muscle, vitamin D deficiency and physical inactivity, but the specific mechanism is yet unclear (28).

Lifestyle modification remains the first-line intervention for fatty liver diseases (FLD), and a standard approach consists of a 7%–10% weight loss from baseline. Similarly, there are no approved pharmacological treatment for sarcopenia. In liver cirrhosis, the severity of sarcopenia increased as the liver disease progress (29), which was primarily regarded as a sign of malnutrition and required nutritional supplementation. However, these treatments had minimal benefits for survival improvement (30).

The Third National Health and Nutrition Examination Survey (NHANES III) was a well-designed population-based program, collecting data from US adults from 1988 to 1994. In this context, we aim to analyze the associations between diet, physical activity and sarcopenic MAFLD using the population-based survey data.

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; BIA, bioelectrical impedance analysis; BMI, body mass index; CRP, C-reactive protein; FIB-4, Fibrosis-4 index; FLD, fatty liver disease; FPG, fasting plasma glucose; GGT, gamma-glutamyl transferase; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HL, hyperlipidemia; HTN, hypertension; IQR, interquartile range; HOMA-IR, homeostasis model assessment of insulin resistance score; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; LSM, liver stiffness measurement; MAFLD, metabolic fatty liver disease; MET, metabolic equivalent; NCHS, National Center for Health Statistics; NFS, Non-Alcoholic Fatty Liver Diseases Fibrosis Score; NHANES, Nutritional Health and Nutritional Examination Survey; SMM, skeletal muscle mass; SMI, skeletal muscle index; TC, total cholesterol; TG, triglyceride; T2DM, type 2 diabetes mellitus.

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2 Material and methods

2.1 Data source and population

National Health and Nutrition Examination Survey (NHANES) is a population-based survey program carried out by the National Center for Health Statistics (NCHS), which aims to evaluate the health and nutritional status of civilian, non-institutionalized members in the US population (31). Our work is predicated on the database of NHANES III (1988–1994) (32), which is the only survey that recorded liver ultrasonography data using a Toshiba Sonolayer SSA-90A and Toshiba video recorders (33). The steatosis severity of participants was reevaluated and graded by experts between 2009 and 2010, and FLD was defined as moderate or severe hepatic steatosis based on hepatic ultrasound imaging. Household interviews were conducted by qualified health technicians utilizing a computer-assisted personal interview system to collect data on demographic variables and health history. Body mass index (BMI) was computed by dividing weight in kilograms by height in meters squared, rounding to the nearest decimal. The Linked Mortality Files (LMF) have been updated with mortality follow-up data through December 31, 2019 (34). During the follow-up phase, respondents without matched death records were presumed alive. Survival time was counted from a subject who participated in the survey to death or December 31, 2019. Informed consent was obtained from all participants, and ethical approval was obtained from the NCHS Ethics Review Board.

A total of 20,050 subjects were included in the NHANES III survey. Among these subjects, 7,791 were excluded based on the following criteria (1): missing data of BIA (n=4186); (2) missing data of height or weight (n=25); (3) positive serologic markers for hepatitis B (n=73) or C (n=348) virus; (4) patients with missing data of liver ultrasounds (n=3159); After applying the above exclusion criteria, we included 12,259 subjects aged 18 to 75 years, of which 2,473 were MAFLD patients, and 9,786 were non-MAFLD patients (Figure 1).

2.2 Definition of MAFLD

MAFLD was diagnosed in individuals with FLD and any of the following three medical conditions: overweight/obesity (body mass index [BMI] ≥ 25 kg/m^2), type 2 diabetes mellitus (T2DM), or the existence of metabolic dysregulation (5). Metabolic dysregulation was defined by the presence of at least two metabolic risk abnormalities: (a) waist circumference ≥ 102 cm in men and ≥ 88 cm in women; (b) blood pressure ≥ 130/85 mmHg or specific drug treatment; (c) TG ≥ 150 mg/dL or specific drug treatment; (d) HDL-C < 40 mg/dL for men and < 50 mg/dL for women; (e) prediabetes (FPG = 100–125 mg/dL or HbA1c = 5.7%–6.4%); (f) homeostasis model assessment of insulin resistance score (HOMA-IR) ≥ 2.5; and/or (g) CRP > 2 mg/L. The classification of individuals into MAFLD and non-MAFLD categories was based on their diagnoses.

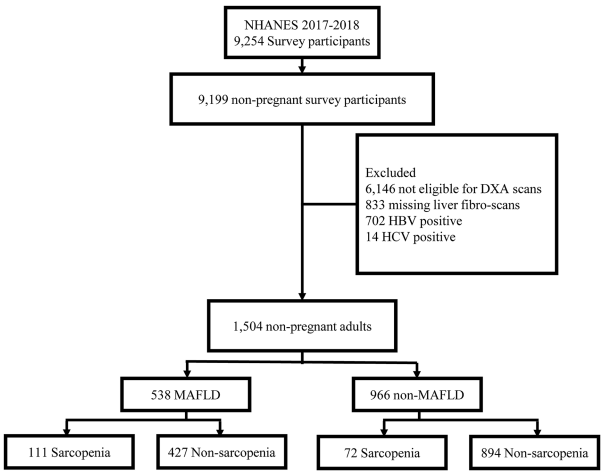

FIGURE 1
Flow Chart of Participants for the Study.

2.3 Definition of sarcopenia

Following the recommendation of 2nd edition of European Working Group on Sarcopenia in Older People (EWGSOP2), this study employs bioelectrical impedance analysis (BIA) to diagnose sarcopenia based on the existence of decreased muscle quantity or quality (9). For the NHANES III database, BIA was measured as the resistance at 50 kHz between the right wrist and ankle of a supine participant using A Valhalla 1990B Bio-Resistance Body Composition Analyzer (Valhalla Medical, San Diego, California, USA).

Here, Skeletal muscle mass (SMM) was calculated by BIA from NHANES III database using Janssen's equation: SMM (kg)= (height in cm)^2/BIA-resistance × 0.401 + (sex × 3.825) + (age in years × −0.071) + 5.102, where BIA-resistance is measured in ohms, and sex is encoded as 1 for male and 0 for female (35). Using the following formula, skeletal muscle mass index (SMI) was calculated: SMI = skeletal muscle mass in kg/body weight in kg × 100. Participants were considered to have sarcopenia if their SMI was more than two standard deviation below the sex-specific mean for young adults aged 18 to 39 (9, 35).

2.4 Physical activity level

Physical activity questionnaires were given at a home interview for all participants, inquiring about the frequency of leisure time activities (walking, running or jogging, riding, swimming, aerobics, dancing, etc.) in the previous month. The intensity of each activity was evaluated by metabolic equivalent (MET) based on the criteria from the Compendium of Physical Activities (36), which defines one MET as the energy expended at resting metabolic rate.

The NHANES III datasets collected information on the intensity rating and frequency of each individual's daily physical activity. The activities are classified into moderate (METs ranging from 3 to 6) and vigorous (METs above 6) categories based on their

intensity rates. Active group was characterized as those who engaged in moderate or vigorous activity at least five or three times per week. The inactive group was defined as those who participate in no physical activity during their leisure time. The insufficiently active group fell in the middle between active and inactive levels of physical activity (37, 38).

2.5 Ascertainment of nutrient components intake

A nutritional interview comprising a 24-hour recall of dietary intake was conducted, with participants providing information on specific foods and quantities. Following the instruction of the Nutrient Composition Data Bank, the grams of nutrient components (carbohydrate, protein, fat, cholesterol, saturated fatty acids, monounsaturated fatty acid, and polyunsaturated fatty acid) were recorded and calculated.

In our study, the absolute quantity and percentage of energy intake from each macronutrient were categorized into gender-specific quartiles (Q1, Q2, Q3, and Q4). Additionally, the contribution of carbohydrates, proteins, and fatty acids to the overall amount of energy intake (% of total energy consumed) was calculated. The quartile variables were modeled as dummy variables, comparing each quartile to the lowest one (Q1).

In accordance with the American Gastroenterological Association's (AGA) guidelines for lifestyle modification for NAFLD management, we further grouped individuals based on their calorie and protein intake (7). The definition of a hypocaloric diet was < 1200 kcal/day for women and < 1500 kcal/day for men. In addition, the relative daily protein intake of participants was graded as low (< 1.2 g/kg), adequate (1.2–1.5 g/kg), and high (> 1.5 g/kg) based upon recommendations for patients with sarcopenia.

2.6 Other definitions

Household interviews were conducted by skilled interviewers utilizing a computer-assisted personal interview system to collect data on demographic variables and health history. The data on body measurements were gathered by qualified health technicians. Body mass index (BMI) was computed by dividing weight in kilograms by height in meters squared, and then rounding to the nearest decimal. Participants were asked to fast for 9 hours before the blood sample was collected. Serum insulin and plasma glucose concentrations were measured by radioimmunoassay and a hexokinase enzymatic array from fasting blood samples. The HOMA-IR score was determined by the following formula: HOMA-IR = (Fasting insulin in μIU/mL) × (Fasting glucose in mg/dL)/405 (39). In addition, concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, gamma-glutamyl transferase (GGT), total bilirubin, albumin, glycated hemoglobin (HbA1c), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and C-reactive protein (CRP) were measured. Details of measurements are available at http://www.cdc.gov/nchs/nhanes/index.htm.

T2DM was defined by a self-reported diabetic medical history, an FPG ≥ 126 mg/dL, or an HbA1c of ≥ 6.5%. Hypertension (HTN) was defined by self-reported medical history of HTN, systolic blood pressure readings above 130 mmHg, or diastolic blood pressure measures above 80 mmHg from an average of 3 measurements. Hyperlipidemia (HL) was defined by a reported history of HL, cholesterol ≥ 200 mg/dL, LDL-C ≥ 130 mg/dL, or HDL-C ≤ 40 mg/dL for men and ≤ 50 mg/dL for women.

NAFLD Fibrosis Score (NFS) score is a non-invasive method to separate NAFLD patients with and without advanced fibrosis, calculated as: NFS = -1.675 + (0.037 × Age in years) + (0.094 × BMI in kg/m2) + (1.13 × Impaired fasting glucose or diabetes) + (0.99 × AST in U/L/ALT in U/L) – (0.013 × Platelets in $\times 10^9$/L) – (0.66 × Albumin in g/dL), where impaired fasting glucose/diabetes is encoded as 1 and 0 for participants with or without abnormal fasting glucose (40). Fibrosis-4 (FIB-4) index was designed to predict significant fibrosis in a simple equation: (Age in years × AST in U/L)/(Platelets in $\times 10^9$/L × ALT$^{0.5}$ in U/L) (41). Advanced fibrosis was determined by a NFS > 0.675 (40) or Fibrosis-4 (FIB-4) index > 2.67 (41).

2.7 Statistical analysis

We compared the baseline characteristics of MAFLD and non-MAFLD participants using data from NHANES III. Continuous variables were expressed as means ± standard deviation (SD), while categorical variables were expressed as percentages. The Student t-test was utilized for normally distributed variables, the Chi-squared test for categorical variables, and the Mann-Whitney U-test for non-normally distributed variables. Multivariate logistic regression models adjusted for confounders were used to evaluate the association between sarcopenia and other clinical covariates. In tests of interaction, age (dichotomized into < 60 years and ≥ 60 years) modified the effect of sarcopenia, whereas gender did not interact significantly with sarcopenia. Cox proportional hazards models were developed to estimate hazard ratios (HR) and 95% confidence intervals (CI) of risk factors for all-cause mortality in participants with sarcopenia or MAFLD. Model 1 was adjusted for age, sex, race, and BMI levels. Model 2 was adjusted for age, sex, race, BMI levels, and the existence of advanced fibrosis. Model 3 was adjusted for variables mentioned in model 2 with T2DM. Model 4 was adjusted for all variables in model 3 with other medical histories (HTN, HL, smoking). No evident interactions between MAFLD and sarcopenia were found ($p > 0.05$). All tests were two-tailed, and a p value less than 0.05 was considered statistically significant. R 4.2.0 (https://www.r-project.org/) was used to conduct all analyses.

3 Results

3.1 Data characteristics

A total of 12,259 participants from NHANES III data sets were included in this analysis, of whom 2,473 (20.2%) were diagnosed with MAFLD (Figure 1). The included participants contained 5,862

(47.8%) males aged 43.8 ± 15.9 years. Individuals with MAFLD had a higher prevalence of sarcopenia than those without MAFLD (11.7% vs. 3.0%), and this tendency persisted regardless of age, sex, ethnicity, levels of physical activity, calorie consumption, and liver fibrosis (Figure 2). The statistical differences between MAFLD and non-MAFLD groups were listed in Table S3.

The demographic, laboratory, and lifestyle characteristics of participants were demonstrated in Tables S1–2, categorized by the presence of MAFLD and sarcopenia. Sarcopenia, with or without MAFLD, was characterized by female gender, advanced age, and central obesity. Moreover, self-report data demonstrated that those with sarcopenia consumed fewer calories and engaged in less physical activity than those without the condition.

3.2 Identify risk factors for sarcopenia among MAFLD participants

The fully-adjusted logistic regression model showed that the presence of MAFLD was associated with an increased risk of sarcopenia (odds ratio [OR] = 1.38 [95% CI 1.11–1.73]) (Table 1). We then generated multivariate Logistic regression models (adjusted for age, sex, and race) to identify sarcopenia-related factors by calculating their ORs amongst the MAFLD population. As shown in Table 2, sarcopenia was associated with physical activity levels (active vs. inactive, OR=0.51 [95% CI 0.36–0.95]), calorie intake (Q2 vs. Q1, OR = 0.58 [95% CI 0.41–0.83]), carbohydrates (Q2 vs. Q1, OR = 0.54 [95% CI 0.37–0.76]), and fatty acids (Q2 vs. Q1, OR = 0.62 [95% CI 0.44–0.89]) intake.

Ordinal logistic regressions were performed to further reveal the relationship between sarcopenia and lifestyle factors. Sarcopenia was significantly and negatively associated with higher levels of physical activity (OR = 0.74 [95% CI 0.62–0.87]) (Table S4) and appropriate relative protein intake (OR = 0.48 [95% CI 0.35–0.65]) (Table S5). In contrast, there was no connection between sarcopenia and absolute calorie, carbohydrates, protein, or fat consumption (Tables S6–7).

3.3 All-cause mortality

Of the overall NHANES III cohort (1988–1994), 290 (2.37%) patients presented with MAFLD and sarcopenia, of whom 181 (62.41%) individuals died after a median follow-up of 15.3 years.

Analyses of the relationships between MAFLD and sarcopenia and all-cause mortality were conducted using Models 1 through 4, which included age, sex, race, health behavior, and medical history as adjustments (Table 3). The presence of sarcopenia was associated with a poorer prognosis after modifications, whereas the presence of MAFLD was unable to predict survival when a history of T2DM was added to the model (Models 3–4).

For the purpose of modifying the interaction between age and sarcopenia, adjusted HRs calculated for individuals with sarcopenia were split into two age groups (Table 4). Higher levels of activity improved the survival of sarcopenia (Insufficiently active, HR = 0.75 [95% CI 0.58–0.97]; Active, HR=0.64 [95% CI 0.48–0.86), which was more prominent in older patients. In both age categories, adequate protein intake was not significantly associated with long-term outcomes.

Diabetes had the greatest impact on the prognosis of persons with MAFLD (HR = 1.84 [95% CI 1.59–2.12]), and increasing activity levels also improved the survival (Insufficiently active, HR = 0.85 [95% CI 0.73–0.99]; Active, HR = 0.64 [95% CI 0.67–0.93]). A daily protein intake of greater than 1.5 g/kg protein was associated with a better prognosis in older MAFLD patients, but had no significant effect on younger individuals (Table S8).

4 Discussion

In this study, we used data sets from NHANES III (1988–1994) to investigate the clinical impact of dietary components and physical activity on patients with sarcopenia and MAFLD, revealing an increased incidence of sarcopenia in patients with MAFLD. Decreased physical activity levels and insufficient protein

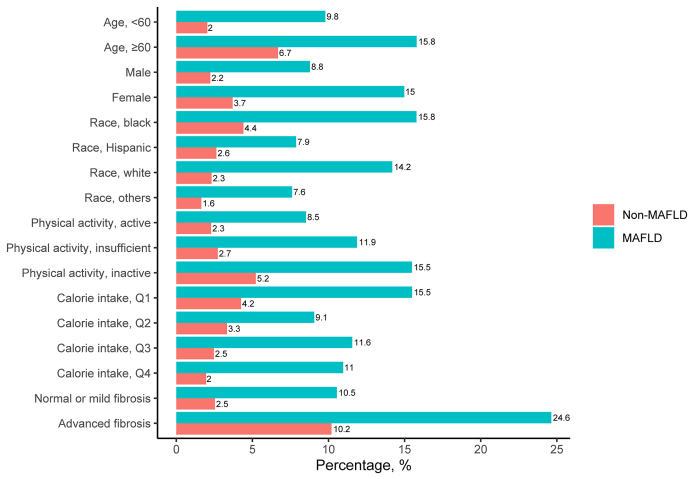

FIGURE 2
Prevalence of Sarcopenia among Participants with and without MAFLD. *MAFLD, metabolic-associated fatty liver diseases.*.

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TABLE 1 Multivariate Analysis for Sarcopenia in overall population.

	OR (95% CI)	P-value
MAFLD	1.38 (1.11–1.73)	0.004
Age	1.06 (1.05–1.07)	< 0.001
Male sex	1.37 (1.09–1.73)	0.006
Race		
Black	Reference	
Hispanic	0.81 (0.61–1.07)	0.144
White	0.83 (0.64–1.09)	0.182
Others	0.62 (0.31–1.25)	0.181
BMI	1.34 (1.31–1.36)	< 0.001
HbA1c	0.92 (0.85–0.99)	0.088
Physical activity		
Inactive	Reference	
Insufficiently active	0.79 (0.61–1.03)	0.085
Active	0.71 (0.53–0.95)	0.02
Calorie [a]		
Q1	Reference	
Q2	0.84 (0.64–1.11)	0.226
Q3	0.82 (0.61–1.10)	0.177
Q4	0.61 (0.44–0.85)	0.003

[a]Q1: 0–1763 kcal in male, 0–1230 kcal in female; Q2: 1764–2365 kcal in male, 1231–1647 kcal in female; Q3: 2366–3128 kcal in male, 1648–2148 kcal in female; Q4: >3128 kcal in male, >2148 kcal in female.
MAFLD, metabolic dysfunction-associated fatty liver diseases; BMI, body mass index; HbA1c, glycosylated hemoglobin.

consumption may contribute to sarcopenia, with reduced physical activity being related to unfavorable outcomes.

Sarcopenia is strongly age-related and primarily observed in older people, while chronic diseases may induce sarcopenia in younger individuals (42). Consistent with earlier studies that demonstrated a positive correlation between NAFLD and sarcopenia (43, 44), sarcopenia was more prevalent in MAFLD than non-MAFLD participants (11.7% vs. 3.0%) in our study and related to a higher mortality. Insulin resistance may function as the main pathologic mechanism of MAFLD and sarcopenia. Insulin could activate the mammalian target of rapamycin (mTOR) and enhance its downstream effectors, 4E-binding protein 1 and ribosomal S6 kinase 1, mediating skeletal muscle anabolism and maintaining muscle mass (45). Impaired insulin sensitivity may interrupt the glucose metabolism and result in excess glucose conversion to triacylglycerol in the liver, which also leads to hepatic insulin resistance. Other factors, such as chronic inflammation, hyperammonemia, alterations in sex hormones, and insulin-like growth factor-1 signaling may also interfere with the glucose disposal in skeletal muscles and lead to muscle loss (42, 46, 47), which helps to explain the co-existence of sarcopenia with MAFLD. The impact of sarcopenia on the long-term prognosis of MAFLD is anticipated to be substantial, since both sarcopenia and liver fibrosis caused by MAFLD are independently associated with increased risk of death from all causes (47).

Prior studies showed a strong interest in elucidating how sarcopenia contributes to adverse outcomes in patients with chronic liver diseases, particularly those with cirrhosis. Molecular studies have shown that cirrhotic patients had an increase in muscle cell autophagy (48) and a higher expression of myostatin that inhibited mTOR signaling and suppressed protein synthesis (49). Besides, hyperammonia, a common abnormality caused by liver

TABLE 2 Age, Sex and Race-adjusted Odds Ratio (OR) for Sarcopenia in patients with and without MAFLD.

Covariates	MAFLD		Non-MAFLD	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Physical activity				
Inactive	Reference		Reference	
Insufficient	0.77 (0.57–1.04)	0.089	0.65 (0.49–0.87)	0.004
Active	0.51 (0.36–0.95)	< 0.001	0.57 (0.42–0.77)	< 0.001
Calorie [a]				
Q1	Reference		Reference	
Q2	0.58 (0.41–0.83)	0.003	1.00 (0.73–1.36)	0.978
Q3	0.82 (0.58–1.14)	0.238	0.86 (0.61–1.21)	0.388
Q4	0.83 (0.58–1.20)	0.333	0.83 (0.57–1.20)	0.313
Protein [b]				
Q1	Reference		Reference	
Q2	0.98 (0.70–1.38)	0.913	0.98 (0.71–1.34)	0.898

(Continued)

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TABLE 2 Continued

Covariates	MAFLD		Non-MAFLD	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Q3	0.83 (0.57–1.20)	0.325	0.85 (0.61–1.19)	0.335
Q4	1.04 (0.73–1.48)	0.838	0.94 (0.66–1.34)	0.729
Carbohydrates [c]				
Q1	Reference		Reference	
Q2	0.53 (0.37–0.76)	< 0.001	0.85 (0.61–1.17)	0.309
Q3	0.75 (0.53–1.05)	0.09	1.12 (0.82–1.53)	0.471
Q4	0.76 (0.53–1.08)	0.128	0.64 (0.43–0.94)	0.024
Fatty acids [d]				
Q1	Reference		Reference	
Q2	0.62 (0.44–0.89)	0.009	1.01 (0.74–1.38)	0.949
Q3	0.89 (0.64–1.25)	0.503	0.86 (0.61–1.21)	0.387
Q4	0.82 (0.57–1.19)	0.297	1.02 (0.72–1.44)	0.928
Carbohydrates %				
<40%	Reference		Reference	
40%-59%	0.82 (0.60–1.13)	0.223	0.89 (0.65–1.22)	0.484
≥60%	0.85 (0.57–1.28)	0.434	1.04 (0.71–1.52)	0.832
Fatty acids %				
<40%	Reference		Reference	
≥40%	1.18 (0.89–1.57)	0.257	1.09 (0.83–1.43)	0.549
Relative protein intake				< 0.001
< 1.2 g/kg	Reference		Reference	
1.2–1.5 g/kg	0.28 (0.14–0.53)	< 0.001	0.06 (0.03–0.14)	< 0.001
>1.5 g/kg	0.39 (0.23–0.68)	< 0.001	0.27 (0.17–0.43)	< 0.001

[a]Q1: 0–1763 kcal in male, 0–1230 kcal in female; Q2: 1764–2365 kcal in male, 1231–1647 kcal in female; Q3: 2366–3128 kcal in male, 1648–2148 kcal in female; Q4: > 3128 kcal in male, > 2148 kcal in female.
[b]Q1: 0-64.0 g in male, 0-44.0 g in female; Q2: 64.1-89.2 g in male, 44.1-62.1 g in female; Q3: 89.3–121.0 g in male, 62.2-82.9 g in female; Q4: > 121.0 g in male, > 83.0 g in female.
[c]Q1: 0–203 g in male, 0–152 g in female; Q2: 204–280 g in male, 153–206 g in female; Q3: 281–371 g in male, 207–271 g in female; Q4: > 371 g in male, > 271 g in female.
[d]Q1: 0-58 g in male, 0-40 g in female; Q2: 59-87 g in male, 41-60 g in female; Q3: 88–124 g in male, 61-87 g in female; Q4: > 124 g in male, > 87 g in female.
Adjusted for age, sex, and race.
MAFLD, metabolic dysfunction-associated fatty liver diseases.

dysfunction and portosystemic shunting, may contribute to both myostatin upregulation and autophagy processes (48–50). In addition to the detrimental impact of sarcopenia on cirrhosis, additional investigation is needed to understand how sarcopenia affects the prognosis of MAFLD. Moreover, we confirmed that sarcopenia was an independent predictor of survival in individuals either with or without MAFLD. Our research further revealed a strong correlation between MAFLD and T2DM rather than severe fibrosis, indicating that metabolic dysregulation was mainly responsible for the unfavorable prognosis of MAFLD patients in the general community.

Despite compelling evidence that sarcopenia is associated with negative outcomes, no viable methods to reverse muscle mass loss have been identified (51). Previous studies supported the hypothesis that physical activity can enhance the functional capacity of skeletal muscle, but its effect on gaining muscle mass remained uncertain (52). Here, we revealed that in patients diagnosed with sarcopenia, increasing the intensity and frequency of exercises is linked to a better prognosis, especially in the older population. Exercise may boost the muscle accumulation by increasing hormone levels such as testosterone (53) and insulin-like growth factor-1 (IGF-1) (54), and it may promote mitochondrial biogenesis by inhibiting TNF-α and various other molecular mechanisms. Exercise also upregulated PGC-1α and Toll-like receptors downregulation that enhanced the anti-inflammatory and anti-atrophy effects (55, 56). Autophagy contributes to decreased synthesis and increased proteolysis of skeletal muscle in patients with chronic liver diseases. Physical exercise may rescue the impaired mTORC1 signaling by stimulating

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TABLE 3 Hazard Ratios of Risk Factors for All-cause Mortality (Multiple Imputation Analysis).

Covariate	Model 1		Model 2		Model 3		Model 4	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
MAFLD	1.11 (1.03–1.19)	0.007	1.11 (1.04–1.20)	0.003	1.05 (0.97–1.13)	0.209	1.03 (0.96–1.11)	0.427
Sarcopenia	1.18 (1.06–1.34)	0.003	1.17 (1.04–1.30)	0.009	1.16 (1.03–1.30)	0.013	1.14 (1.02–1.28)	0.025
Age	1.09 (1.09–1.09)	< 0.001	1.09 (1.09–1.09)	< 0.001	1.09 (1.08–1.09)	< 0.001	1.09 (1.08–1.09)	< 0.001
Male	1.46 (1.37–1.55)	< 0.001	1.43 (1.35–1.52)	< 0.001	1.46 (1.37–1.55)	< 0.001	1.28 (1.20–1.37)	< 0.001
Race								
Black	Reference		Reference		Reference		Reference	
Hispanic	0.69 (0.63–0.75)	< 0.001	0.73 (0.67–0.80)	< 0.001	0.70 (0.64–0.76)	< 0.001	0.74 (0.67–0.80)	< 0.001
White	0.81 (0.75–0.87)	< 0.001	0.79 (0.74–0.86)	< 0.001	0.82 (0.76–0.88)	0.002	0.83 (0.78–0.90)	< 0.001
Others	0.55 (0.45–0.66)	< 0.001	0.55 (0.46–0.67)	< 0.001	0.55 (0.46–0.66)	< 0.001	0.61 (0.50–0.73)	< 0.001
Obesity								
Normal	Reference		Reference		Reference		Reference	
Obese	0.87 (0.86–0.93)	< 0.001	0.90 (0.84–0.97)	0.005	0.87 (0.81–0.94)	< 0.001	0.86 (0.80–0.93)	<0.001
Overweight	1.03 (0.13–1.12)	0.409	1.10 (1.02–1.19)	0.014	1.03 (0.95–1.10)	0.503	0.99 (0.91–1.07)	0.767
Advanced fibrosis			1.33 (1.21–1.46)	< 0.001	1.21 (1.10–1.33)	< 0.001	1.22 (1.11–1.35)	< 0.001
T2DM					2.00 (1.83–2.18)	< 0.001	1.94 (1.77–2.11)	< 0.001
HTN							1.30 (1.22–1.39)	< 0.001
HL							0.92 (0.85–0.98)	0.017
Smoking							1.58 (1.48–1.69)	< 0.001

Model 1 was adjusted for age, sex, race, and BMI levels. Model 2 was adjusted for age, sex, race, BMI levels, and the existence of advanced fibrosis. Model 3 was adjusted for age, sex, race, BMI levels, advanced fibrosis, and T2DM. Model 4 was adjusted for age, sex, race, BMI levels, advanced fibrosis, T2DM, HTN, hypercholesterolemia, and history of smoking.
MAFLD, metabolic dysfunction-associated fatty liver diseases; BMI, body mass index; T2DM, Type 2 Diabetes; HL, hyperlipidemia; HTN, Hypertension.

phosphatidic acid (57), therefore maintaining muscle mass by activating protein synthesis and inhibiting autophagy. As resistance exercises were more effective at stimulating skeletal muscle protein synthesis (58), the effect of different types of exercise on preventing sarcopenia and improving survival required more validation.

Given that physical activity was rather compromised in older people by their frailty or diseases, a protein supplement was considered a practical choice for preserving muscle mass (59, 60). Although older and younger individuals had similar rates of protein turnover (61), elderly people have a more muted response to administered amino acids than young people (62). Lower mTOR and p70S6K concentrations (63), along with a concurrent decline in positive regulators (such as IGF-1) and an increase in negative regulators (such as AMPK) in older skeletal muscle, may explain their resistance to amino acid feedings (64). Some observational and cohort studies demonstrate that adequate protein consumption is well tolerated without major adverse events and can prevent muscle loss (65, 66), but there is insufficient evidence to support the hypothesis that protein intake can improve the long-term outcomes. Several randomized controlled trials were performed in cirrhotic individuals with sarcopenia; nevertheless, nutrition supplementation through multiple routes had little influence on sarcopenia or survival (30). Supplemental hormone therapy and mechanistic targeted treatments were produced as more precise treatments for sarcopenia, necessitating a clearer knowledge for its pathophysiological process (51, 67).

This research has a few limitations. First, the diagnosis of FLD was established by ultrasound images from NHANES III, but fibrosis data were not available with ultrasound. In the absence of liver stiffness measurement (LSM) results, advanced fibrosis was determined by NFS and FIB-4 scores. The relationship between MAFLD-related fibrosis and sarcopenia should be evaluated further. Moreover, the NHANES III database was relatively outdated in comparison to other NHANES survey cycles. Second, we calculated the skeletal muscle mass using BIA measurements, whereas dual-energy X-ray absorptiometry (DXA) is the primary method for measuring body composition. Since sarcopenia is defined as loss of both muscle mass and function, the NHANES database does not contain muscle function measurements, such as contractile strength, maintenance of contraction, and muscle fatigue in response to persistent and repetitive contraction (68). Finally, the mortality data came from a separate national database that matched the NHANES III data, where the data on liver-associated mortality was not available. Given the cross-sectional nature of the NHANES database, the progression of liver diseases cannot be determined.

TABLE 4 Hazard Ratios of Risk Factors of Patients with Sarcopenia for All-cause Mortality, Stratified by Age.

Covariate	Overall		< 60 years		≥ 60 years	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age	1.07 (1.06–1.08)	< 0.001	1.07 (1.05–1.09)	< 0.001	1.10 (1.06–1.13)	< 0.001
Male	1.47 (1.15–1.88)	0.002	1.92 (1.28–2.88)	0.002	1.21 (0.87–1.67)	0.251
Race						
Black	Reference		Reference		Reference	
Hispanic	0.87 (0.65–1.17)	0.353	0.87 (0.53–1.43)	0.581	0.85 (0.58–1.23)	0.383
White	0.98 (0.76–1.27)	0.896	0.78 (0.50–1.22)	0.276	1.14 (0.83–1.56)	0.435
Others	1.06 (0.46–2.48)	0.884	1.20 (0.35-4.08)	0.768	0.92 (0.28–3.08)	0.896
Physical activity						
Inactive	Reference		Reference		Reference	
Insufficiently active	0.75 (0.58–0.97)	0.027	0.74 (0.45–1.02)	0.176	0.78 (0.57–1.08)	0.132
Active	0.64 (0.48–0.86)	0.003	0.80 (0.51–1.30)	0.392	0.60 (0.42–0.86)	0.006
Relative protein intake						
< 1.2 g/kg	Reference		Reference		Reference	
1.2–1.5 g/kg	0.93 (0.43–1.99)	0.851	0.72 (0.26–2.00)	0.534	1.09 (0.34–3.51)	0.886
> 1.5g/kg	0.90 (0.56–1.45)	0.667	1.14 (0.57–2.29)	0.715	0.77 (0.40–1.49)	0.434
Cirrhosis	1.12 (0.86–1.45)	0.395	0.96 (0.60–1.56)	0.883	1.15 (0.84–1.59)	0.383
T2DM	1.39 (1.05–1.82)	0.02	1.76 (1.08–2.85)	0.023	1.26 (0.90–1.78)	0.182
HTN	1.12 (0.90–1.40)	0.311	1.31 (0.90–1.91)	0.165	1.00 (0.76–1.32)	0.983
HL	1.02 (0.80–1.31)	0.862	0.82 (0.52–1.31)	0.409	1.08 (0.79–1.47)	0.64
Smoking	1.40 (1.11–1.77)	0.004	1.36 (0.92–2.01)	0.123	1.54 (1.14–2.07)	0.005

Adjusted for age, sex, and race.
T2DM, Type 2 Diabetes; HL, hyperlipidemia; HTN, Hypertension.

In summary, our data demonstrate that sarcopenia is more prevalent and is associated with an increased risk of all-cause death among MAFLD participants. MAFLD patients who suffer from sarcopenia may benefit from physical activity and a proper intake of proteins. Therefore, clinicians should recognize and manage sarcopenia in patients with MAFLD in order to improve their life quality and overall survival outcome.

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

Author contributions

YY and YC: conception and design. YY, CW, YD, JH, and YL: data collection, data management, and formal statistical analysis. YY and CW: manuscript writing. YC: manuscript revising. All authors involved in writing and approved the final manuscript.

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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 effect of *G0S2* on insulin sensitivity: A proteomic analysis in a *G0S2*-overexpressed high-fat diet mouse model

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Background: Previous research has shown a tight relationship between the G0/G1 switch gene 2 (G0S2) and metabolic diseases such as non-alcoholic fatty liver disease (NAFLD) and obesity and diabetes, and insulin resistance has been shown as the major risk factor for both NAFLD and T2DM. However, the mechanisms underlying the relationship between G0S2 and insulin resistance remain incompletely understood. Our study aimed to confirm the effect of G0S2 on insulin resistance, and determine whether the insulin resistance in mice fed a high-fat diet (HFD) results from G0S2 elevation.

Methods: In this study, we extracted livers from mice that consumed HFD and received tail vein injections of AD-G0S2/Ad-LacZ, and performed a proteomics analysis.

Results: Proteomic analysis revealed that there was a total of 125 differentially expressed proteins (DEPs) (56 increased and 69 decreased proteins) among the identified 3583 proteins. Functional enrichment analysis revealed that four insulin signaling pathway-associated proteins were significantly upregulated and five insulin signaling pathway -associated proteins were significantly downregulated.

Conclusion: These findings show that the DEPs, which were associated with insulin resistance, are generally consistent with enhanced insulin resistance in G0S2 overexpression mice. Collectively, this study demonstrates that G0S2 may be a potential target gene for the treatment of obesity, NAFLD, and diabetes.

KEYWORDS

***G0S2*, insulin sensitivity, label-free proteomics, metabolic diseases, high-fat diet**

Introduction

The increasing incidence of metabolic diseases such as obesity, type 2 diabetes mellitus (T2DM), dyslipidemia, and nonalcoholic fatty liver disease (NAFLD) that are triggered by metabolic derangements has been a subject of serious concern worldwide in the past few decades. The systemic metabolic dyshomeostasis caused by impaired insulin signaling is a hallmark of metabolic disease (1, 2). The overabundance of circulating fatty acids lead to insulin resistance, and the aggravation of insulin resistance can further inhibit the antilipolytic effect of insulin and increase lipolysis. The decrease in fatty acid oxidation and increase in cytosolic levels of free fatty acids increases the overall risk of T2DM. The accumulation of lipids in the liver leads to hepatic insulin resistance and NAFLD Therefore, insulin resistance is the most important etiological factor of metabolic disorders (3, 4). Accumulating evidence has shown that simultaneous presence of obesity, NAFLD, and type 2 diabetes mellitus (T2DM) is frequently observed and acts synergistically, resulting in an increased risk of hepatic and cardiovascular clinical outcomes (5–7).

The G0/G1 switch gene 2 (*G0S2*), also known as the lipolytic inhibitor, was originally identified in lymphocytes during the phase of G0 to G1 cell cycle transition that is associated with pharmaceutical stimulation (8, 9). *G0S2* encodes a small 12-kDa protein and is abundantly expressed in the liver, adipose tissue, heart, and skeletal muscle (10, 11). In humans and mice, G0S2 is a multifaceted protein and has been shown to play various important roles in metabolism (9, 10). G0S2 mediates endoplasmic reticulum stress-induced metabolism dysfunction in mice models with metabolic disorders through the PERK-eIF2α-ATF4 pathway (12). As the rate limiting step in fat catabolism, *G0S2* knockout mice shows enhanced lipid metabolism, enhanced thermogenesis, and improved insulin sensitivity (13).

The liver is one of the primary metabolic organs involved in energy homeostasis and glycolipid metabolism and disposes off as much as one-third of the glucose and lipid load (14). Insulin resistance is a primary characteristic and underlying cause of metabolic disorders, including non-alcoholic fatty liver disease (NAFLD) (15). Liver insulin resistance in NAFLD increase the risk for metabolic diseases such as T2DM (16, 17). It has been shown that insulin resistance in adipose tissue contributes to excessive release of fatty acids into the bloodstream, which are taken up by the liver, resulting in liver insulin resistance and NAFLD through dysregulated lipolysis (18–20). It has been revealed that loss of liver glycogen synthesis, which promotes and diverts glucose toward fat synthesis, is the result of liver insulin resistance. G0S2 plays an important role in inducing hepatic steatosis through downregulation of UPR signaling, while regulating lipolysis and energy metabolism by inhibiting adipose triglyceride lipase (ATGL) (14, 21). G0S2 has been shown to exert significant influence on the metabolism of liver lipids, while it has been shown that lipid metabolism has a close relationship with insulin sensitivity (15–17). G0S2 expression was upregulated in the hepatocytes of Nagoya-Shibata-Yasuda (NSY) mice fed with high-sucrose diet (22). G0S2 can modulate the lipolysis process by interacting with ATGL, and the level of G0S2 is upregulated in the occurrence of fatty liver disease in mice (9, 14). Thus far, the precise underlying mechanisms of G0S2 in the regulation of insulin resistance-related NAFLD are still unknown. To reveal the mechanism of G0S2 in NAFLD, we performed a preliminary study of proteomic analysis of livers taken from G0S2-overexpressed mice fed high-fat diet (HFD) and control mice fed HFD by using quantitative proteomics, GO analysis, and KEGG analysis. This study shows that overexpression of the *G0S2* gene aggravates liver insulin resistance of mice through upregulating P-Foxo1, Socs3, and Ptpn1 and downregulating Gstp1 and Ppar-γ, which demonstrates that G0S2 may be a potential target gene for the treatment of NAFLD, obesity, and diabetes.

Materials and methods

Animal models

Eight-week-old male C57BL/6 mice were used in this study. The mice were housed in microisolator cages in a specific pathogen-free (SPF) animal room maintained at a controlled environment of temperature of 22 ± 2°C and humidity of 55%, under a 12-h light/dark cycle. Mice had *ad libitum* access to water and high-fat diet (HFD) (protein, 20 kcal%; fat, 45 cal%; carbohydrates, 35 kcal%, D12451, Research Diets, New Brunswick, NJ, USA) for 12 weeks. We selected the mice in *G0S2* overexpression group to receive tail vein injections of Ad-*G0S2* (2.51×10^{10} PFU/mL), and the control mice were injected with Ad-LacZ (4.5×10^{10} PFU/mL) *via* the tail vein as control. Following the operation, all mice continued on the existing diet for 4 weeks. Body weight and glucose tolerance levels were monitored routinely. At the end point, mice were euthanized to minimize suffering, and the livers were extracted, frozen, and stored in liquid nitrogen. All animal experiments in this protocol were approved by The Animal Care and Use Committee of Shandong Provincial Hospital.

Body weight was measured at the same time every week during the experiments. For the glucose tolerance test (GTT) and insulin tolerance test (ITT), mice were fasted for 6 h, and blood glucose was measured after intraperitoneal injection of glucose (2 g/kg body weight) and insulin (0.75 U/kg body weight), respectively. Blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes after the glucose or insulin injection.

Tissue sample preparation

To the lysis samples, the SDT buffer (4% SDS, 100 mM Tris-HCl, 1 mM DTT, pH 7.6) was added to the liver tissues, and an Automated Homogenizer (MP Fastprep-24, 6.0M/S, 30S) was used to homogenize the lysate twice. Boiling, centrifugation, and

filtration were used to extract the homogenate supernatant. The amount of protein was quantified as previously described (23). The protein extracts were digested with trypsin based on a filter-aided sample preparation (FASP) procedure (24). Next, 12.5% SDS-PAGE was used to separate the proteins, and Coomassie Blue R-250 staining was used to visualize the protein bands (25).

Label-free LC-MS/MS analysis

LC-MS/MS analysis was performed on a Nanoelute HPLC system (Bruker Daltonics) coupled with a timsTOF Pro mass spectrometer (Bruker) for 60, 120, and 240 min. The mass spectrometer was operated as described in previous studies (26).

Protein identification and quantification

MaxQuant software (version 1.6.14) and the Swissport_Mus_Musculus_17063_20210106 in Fasta were used to analyze the MS data (27). Trypsin/P was specified as the cleavage enzyme. The maximum number of missed cleavages were 2. Carbamidomethyl (C) was defined as fixed modification, while the oxidation (M) of methionine and the acetylation of the N-terminus of the protein was specified as variable modification. The global false discovery rate (FDR) of peptide and protein identification was <0.01. As for the experimental bias, the calculation of protein abundance was normalized by the spectral protein intensity (LFQ intensity). Proteins with a fold change >1.5 or <0.669 and p value (Student's *t*-test) <0.05 were considered differentially expressed proteins (28–30).

Protein functional classification and database search

All differentially expressed proteins' (DEPs) sequence information was aligned to the *Homo Sapiens* reference sequence (NCBIBLAST-2.2.28+-win32.exe). Blast2GO Command Line was used to complete the annotation from GO terms to proteins. The InterProScan was used to search the EBI database, and it also added functional information of motif to the proteins. The number of DEPs and total proteins correlated to GO terms was compared by Fisher's exact test to enrich the GO terms, and generate hierarchical clustering heat maps. Fold change >1.5 and the corrected p-value <0.05 is considered significant in GO (31–33).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment annotation of proteins was performed using the database (https://geneontology.org/). The enrichment of DEPs against all identified proteins were identified by Fisher's exact test, and a corrected p value <0.05 was considered to be enriched significantly. The annotation of proteins were matched into the database. Online tool KEGG mapper was used to classify these pathways into hierarchical categories.

The protein–protein interaction (PPI) network analysis of the DEPs were searched from IntAct molecular interaction database (https://www.ebi.ac.uk/intact/) or STRING software (https://www.string-db.org/) (version 11.5). The results were downloaded in the XGMML format, and Cytoscape software (https://www.cytoscape.org/, version 3.2.1) was used to visualize and further analyze functional PPI networks (34).

Real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from liver tissue with TRIzol Reagent (Invitrogen, Carlsbad, CA, United States) and PrimeScript reagent (TaKaRa, Kusatsu, Japan) was used to reverse transcribe into cDNA according to the manufacturer's instructions. To analyze the target genes' relative mRNA expression, SYBR Green PCR Master Mix Reagent Kit (Yeasen, Shanghai, China) was used to perform real time qPCR using the Roche 480 detection system. The relative mRNA expression levels were normalized by GAPDH, and 2-△△Ct method was performed to calculate the results. The primer sequences used are listed in Supplementary Table 2.

Western blot analysis

RIPA buffer containing PMSF and phosphatase inhibitor was used to lyse mice liver tissues to extract total protein. After centrifugation at 12000 ×*g* for 15 min, the supernatant was used to measure total protein concentration by BCA method. We used 10% and 12.5% SDS-PAGE gels in the experiment, respectively, based on the molecular weights of the proteins of interest, and then transferred onto a PVDF membrane. The membranes containing proteins were incubated with primary antibodies overnight at 4°C, followed by incubation at room temperature for 1 h with the secondary antibody. The Enhanced Chemiluminescene Plus imaging system was used to detect the protein–antibody immune complexes.

Antibodies

Anti-FOXO1 antibody (GB11286), Anti-Phospho-FOXO1 antibody (GB113974), Anti-PPAR gamma antibody (GB112205), Anti-SOCS3 antibody (GB113792) and β-actin antibody (GB15003) were purchased from Servicebio Technology (Wuhan, China); Anti-GSTP1(PTM-5992) antibody and Anti-PTPN1(PTM-6344) antibody were obtained from PTM BIO (Suzhou, China); Anti-G0S2 antibody (A9970), β-actin antibody (AC004), β-tubulin antibody and Hsp90α antibody were purchased from ABclonal (Wuhan, China).

Primary mouse hepatocyte isolation and culture

Primary hepatocytes were isolated from *G0S2* normal expression mice (HFD) and *G0S2* overexpression mice (HFD +G0S2 overexpress) as previously described (35). The isolated primary hepatocytes were cultured in DMEM with 10% fetal bovine serum overnight. After attachment, cells were incubated in Dulbecco's modified Eagle medium with 0.1 μM insulin or without insulin for 1 h (36).

Statistical analyses

All data were expressed as the mean ± SD values. Significant differences between the two groups were assessed using an unpaired Student's t-test, while comparisons among multiple groups were conducted using one-way ANOVA analysis, both performed with GraphPad Prism 8.0. P<0.05 was considered to indicate statistically significant differences. The experiment was repeated three times, using three independent batches of mice and three independent mice in each group.

Results

G0S2 increased HFD-induced obesity and insulin resistance

To address the effects of G0S2 on HFD-fed mice, we injected Ad-*G0S2 in vivo*, directly through the tail vein and continued the HFD for 4 weeks. However, control mice received a vehicle. Compared with control mice, the fasting body weight of *G0S2* overexpression mice was significantly increased (Figure 1A). Furthermore, the assays of the GTT and ITT indicated that G0S2 aggravated insulin resistance (Figures 1B, C). We examined the hepatic mRNA and protein levels of the *G0S2* gene in both groups

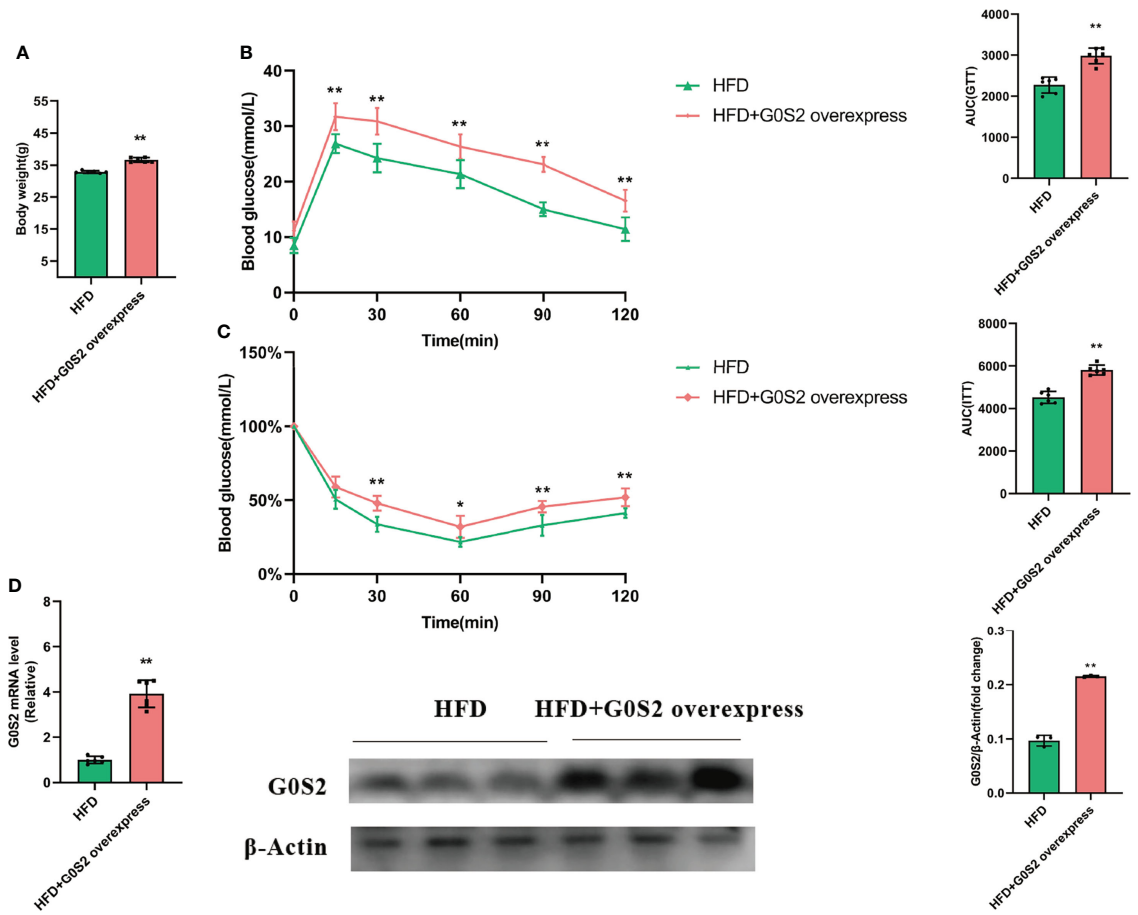

FIGURE 1
G0S2 increased HFD-induced obesity and insulin resistance. **(A)** Fasting body weight of mice in G0S2 normal expression group (HFD) and G0S2 overexpression group (HFD+G0S2 overexpress). **(B, C)** Representative GTT **(B)** and ITT **(C)** results of mice in the two given groups. **(D)** Western blot and RT-PCR were used to analyze the levels of *G0S2* gene in mice that did and did not receive tail vein injections of Ad-*G0S2* after HFD feeding for 16 weeks in all. *P<0.05; **P<0.01 compared with HFD-vehicle mice.

by RT-PCR and western blot method. The results showed *G0S2* overexpression of mice upregulation of *G0S2* genes (Figure 1D). These data indicate that the *G0S2* overexpression of the mouse model was established successfully.

G0S2 overexpression induces differential protein expression in the HFD-diet mouse liver

Insulin resistance is strongly associated with NAFLD (16). Deletion of the *G0S2* gene alleviates HFD-induced NAFLD and insulin resistance (13, 37). However, the mechanisms of G0S2 in insulin resistance-related NAFLD are still unknown. To identify the DEPs in the liver of *G0S2* overexpression mice compared to control mice, we performed label-free quantitative proteomics analysis. In all, 3583 proteins were identified by proteomics analysis; among these, 125 proteins were significantly differentially expressed, which included 56 upregulated and 69 downregulated (fold change≥1.5, P<0.05) proteins (Figure 2, Supplementary Table 1). These results show that G0S2 has an obvious impact on liver protein expression in HFD-diet mice.

GO analysis

To further identify the functions of DEPs influenced by G0S2, GO analysis was performed to analyze the proteomics data. The molecular function (MF) category was mainly enriched in "protein binding," "catalytic activity," "enzyme binding," "cell adhesion molecule binding," and "cadherin binding" (Figures 3A, B). These terms suggest a differential influence of G0S2 on NAFLD by interacting with PNPLA2, ABHD5, E-cadherin, and cell adhesions (2, 38–40).

The results of the biological process (BP) category showed that 19% of the identified DEPs were enriched in the metabolic process, while 2% of proteins were involved in fatty acid metabolic process and "response to insulin," respectively (Figures 4A, B). Nine DEPs in the liver of *G0S2* overexpression mice were possibly involved in the regulation of insulin homeostasis (four upregulated and five downregulated) (Figure 5A, Table 1). The results of PPI network analysis showed that G0S2 may interact with Forkhead box protein O1 (Foxo1), Suppressor of cytokine signaling 3 (Socs3), Tyrosine-Protein phosphatase non-receptor type 1 (Ptpn1), Acyl-CoA (8-3)-desaturase (Fads), 5-AMP-activated protein kinase catalytic subunit alpha-1 (Prkaa1), Eukaryotic translation initiation factor 6 (Eif6), Glutathione S-transferase P 1 (Gstp1), Growth factor receptor-bound protein 2 (Grb2), and Peroxisome proliferator-activated receptor gamma (PPAR-γ) (Figure 5B). To confirm the effect of DEPs on the regulation of insulin in *G0S2* overexpression mice, five DEPs were validated using WB assay. Consistent with the results of the proteomics analysis, an obvious increase of phosphatase Foxo1, Socs3, and Ptpn1, and an obvious decrease of Gstp1 and PPAR-γ was observed (Figure 6). Next, primary mouse hepatocytes isolated from mice with normal G0S2 expression (HFD) and mice overexpressing G0S2 (HFD+G0S2 overexpression) were either stimulated with insulin or left unstimulated. These DEPs were differentially regulated under basal and insulin-stimulated (0.1μM, 1h) conditions. Downregulation of phosphatase Foxo1, Socs3, and Ptpn1, and upregulation of Gstp1 and PPAR-? in the livers of HFD-G0S2 overexpression mice were determined by western blotting and RT-PCR in primary mouse hepatocytes (Figures 7, 8) and suggest that G0S2 plays an important role in the regulation of insulin sensitivity.

KEGG analysis of DEPs

KEGG enrichment analysis was used to further explore the functions of the identified DEPs. The results revealed that the enrichment of DEPs in the pathways were associated with insulin

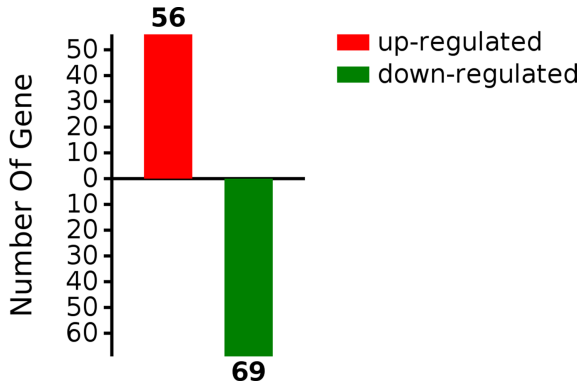

FIGURE 2
Differentially expressed proteins in the liver tissue of HFD-*G0S2* overexpression mice.

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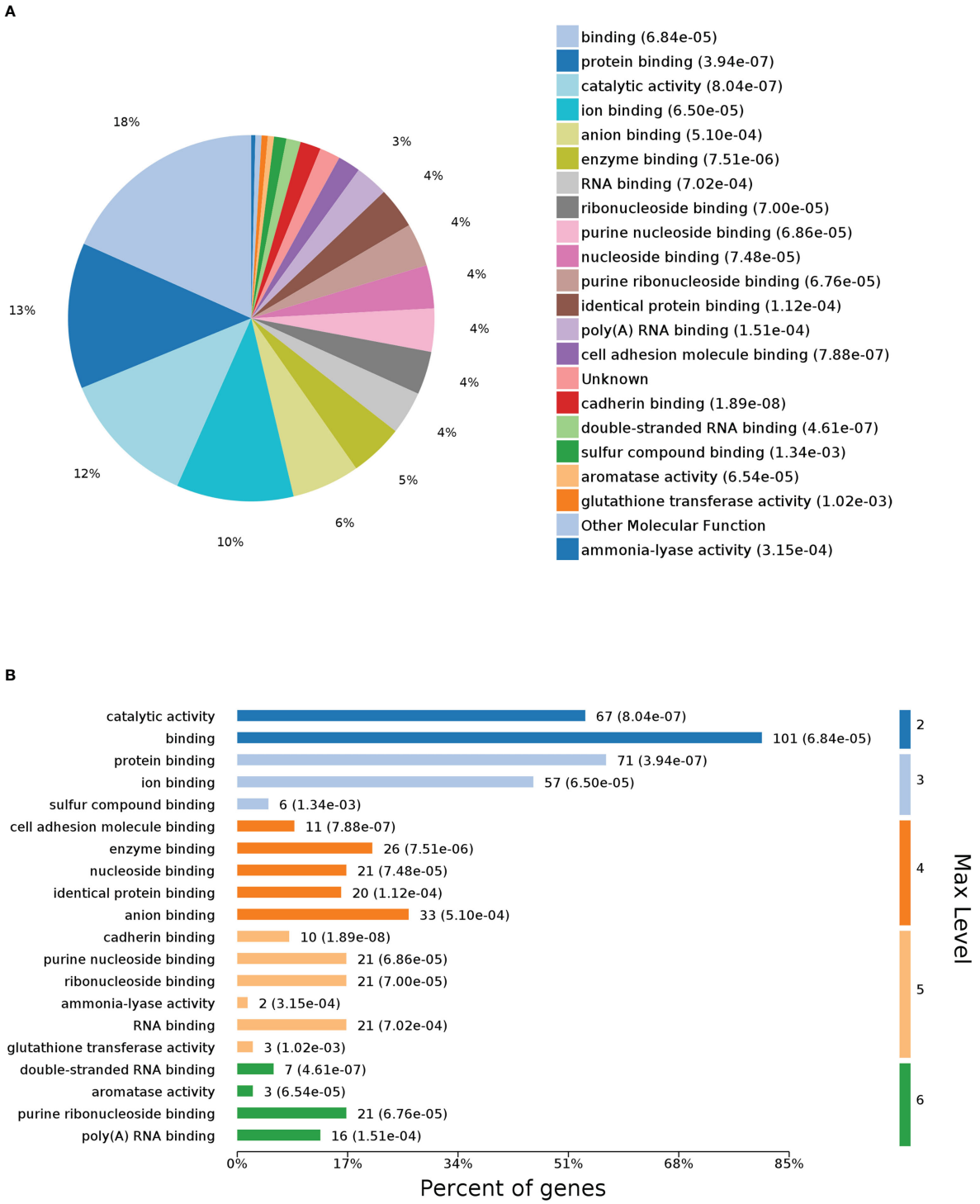

FIGURE 3
Gene Ontology (GO) enrichment analysis of molecular function for DEPs. **(A)** Pie chart of DEP-enriched GO terms for molecular function (MF). **(B)** Max level for MF.

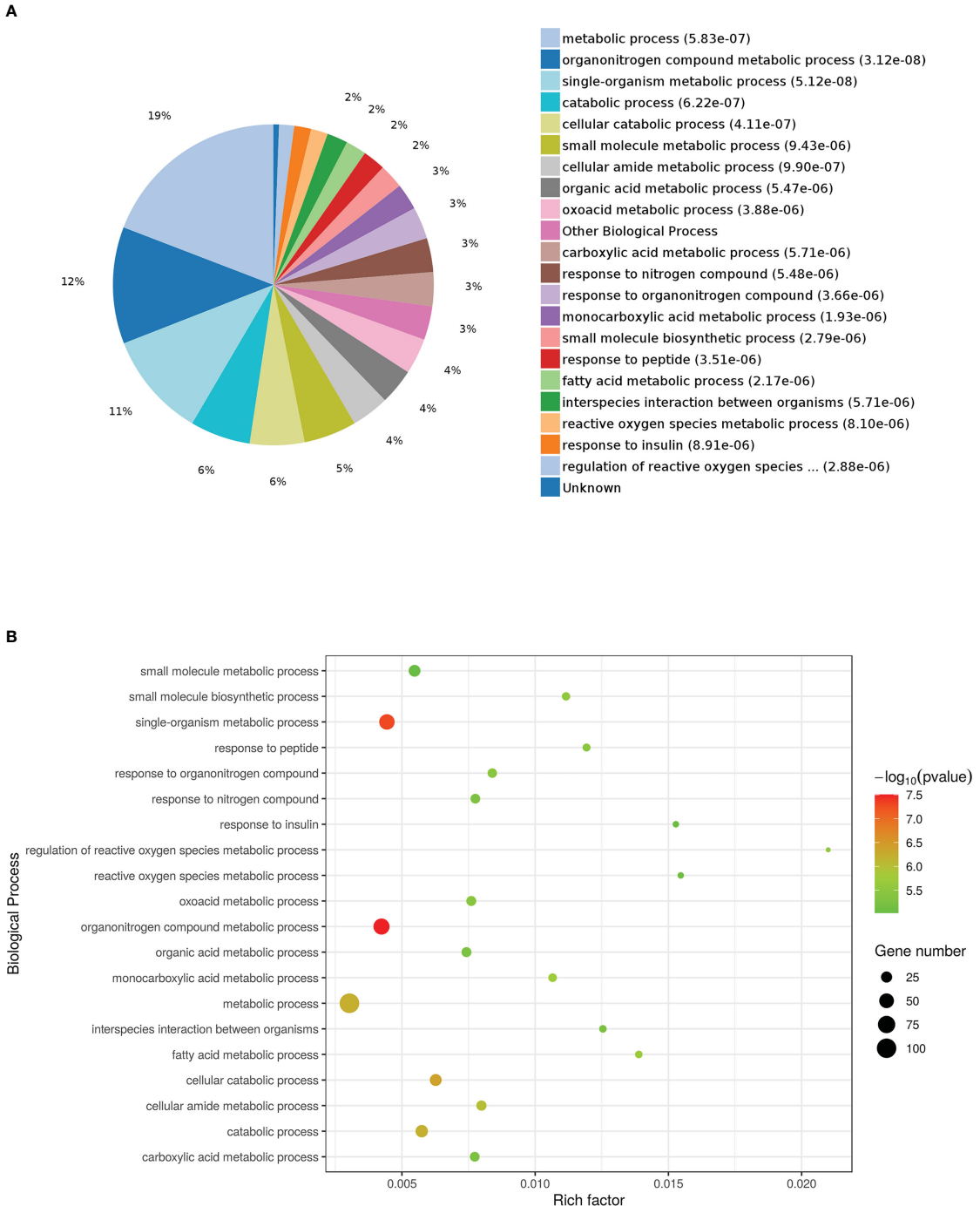

FIGURE 4
Gene Ontology (GO) enrichment analysis of biological processes for DEPs. **(A)** Pie chart of DEP-enriched GO terms for biological processes (bp). **(B)** Enriched GO terms for bp.

resistance (4%), insulin signaling pathway (4%), and AMPK signaling pathway (3%). Additionally, 3%, 3%, and 2% of DEPs were associated with "Glucagon signaling pathway," "Adipocytokine signaling pathway," and "Steroid biosynthesis" (Figures 9A, B). To better understand the relationship between the nine DEPs and insulin resistance, another network of PPI was established (Figure 10). The complicated network comprised various insulin resistance-associated proteins, which was interacted with each other, suggesting that G0S2 might be the key factor in regulating insulin sensitivity.

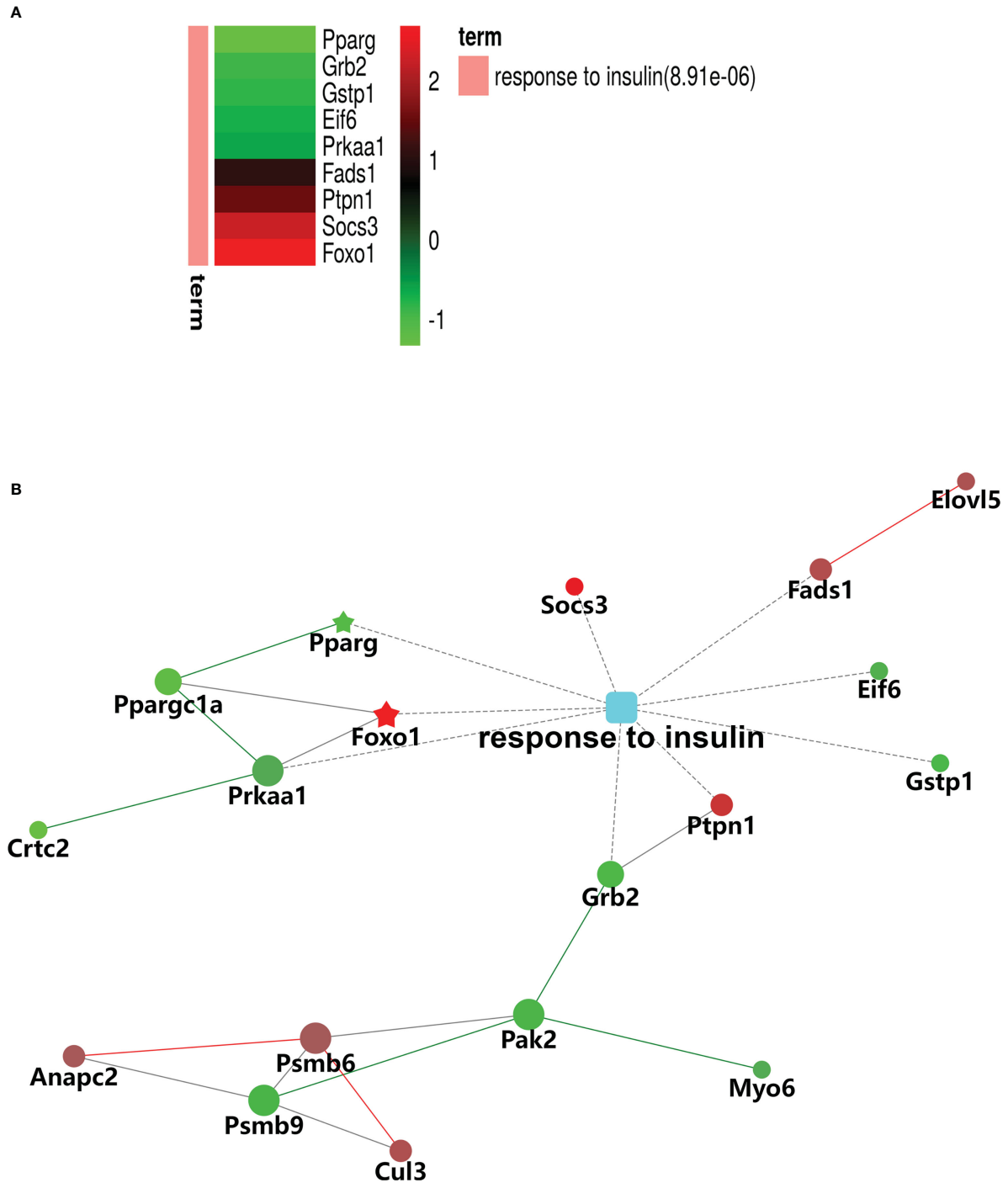

FIGURE 5
Gene Ontology (GO) enrichment analysis of biological processes for DEPs involved in insulin signaling pathways. **(A)** Heatmap of nine DEPs in response to insulin. **(B)** Protein–protein interaction (PPI) network of DEPs associated with the response to insulin. The red signal represents upregulation and green signal represents downregulation. The red pentagram represents the most pronounced upregulation and green pentagram represents the most pronounced downregulation,.

Discussion

G0S2 is primarily a cell cycle-regulated protein that was originally identified in blood mononuclear cells and has 78% homology between mouse and human isoforms (2). A further study ruled out that G0S2 is involved in various biological and pathological processes such as glycolipid metabolism, inflammation, immunization, and cancer (41–44).

Increasing research indicates that interfering hepatic G0S2 expression represents an effective change in the level of hepatic

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TABLE 1 Identification of *G0S2* overexpression-induced differentially expressed proteins associated with the response to insulin.

Change	Protein IDs	Protein Name	Gene Name	Fold Change
up	Q9R1E0	Forkhead box protein O1	Foxo1	6.420271268
up	O35718	Suppressor of cytokine signaling 3	Socs3	4.792348761
up	P35821	Tyrosine-protein phosphatase non-receptor type 1	Ptpn1	2.911627141
up	Q920L1	Acyl-CoA (8-3)-desaturase	Fads1	2.059835232
down	Q5EG47	5-AMP-activated protein kinase catalytic subunit alpha-1	Prkaa1	0.650331086
down	O55135	Eukaryotic translation initiation factor 6	Eif6	0.616771107
down	P19157	Glutathione S-transferase P 1	Gstp1	0.563400004
down	Q60631	Growth factor receptor-bound protein 2	Grb2	0.526383608
down	P37238	Peroxisome proliferator-activated receptor gamma	Pparg	0.397358829

Up, upregulated; down, downregulated.

TG and blood glucose (21, 35). G0S2 knockout mice exhibit a lower level of hepatic triglycerides and were resistant to HFD-induced liver steatosis (12). Moreover, clinical trials show that the mRNA and protein content of G0S2 are reduced in poorly controlled type 1 and type 2 diabetic subjects (41, 45). These previous studies suggested that G0S2 is critical for the regulation of physiological and pathological processes of NAFLD and diabetes.

Accumulating studies support that insulin resistance is one of the earliest manifestations of a constellation of metabolic disease, including T2DM and NAFLD (46). Some extracellular factors lead to defects in the responsiveness of cells to insulin, such as lipids and other circulating factors that perturb the intracellular concentration of ceramide (14). Insulin resistance is the main risk factor of diabetes and NAFLD (11, 47, 48). However, the mechanisms of G0S2 regulated NAFLD and diabetes is still not clearly known.

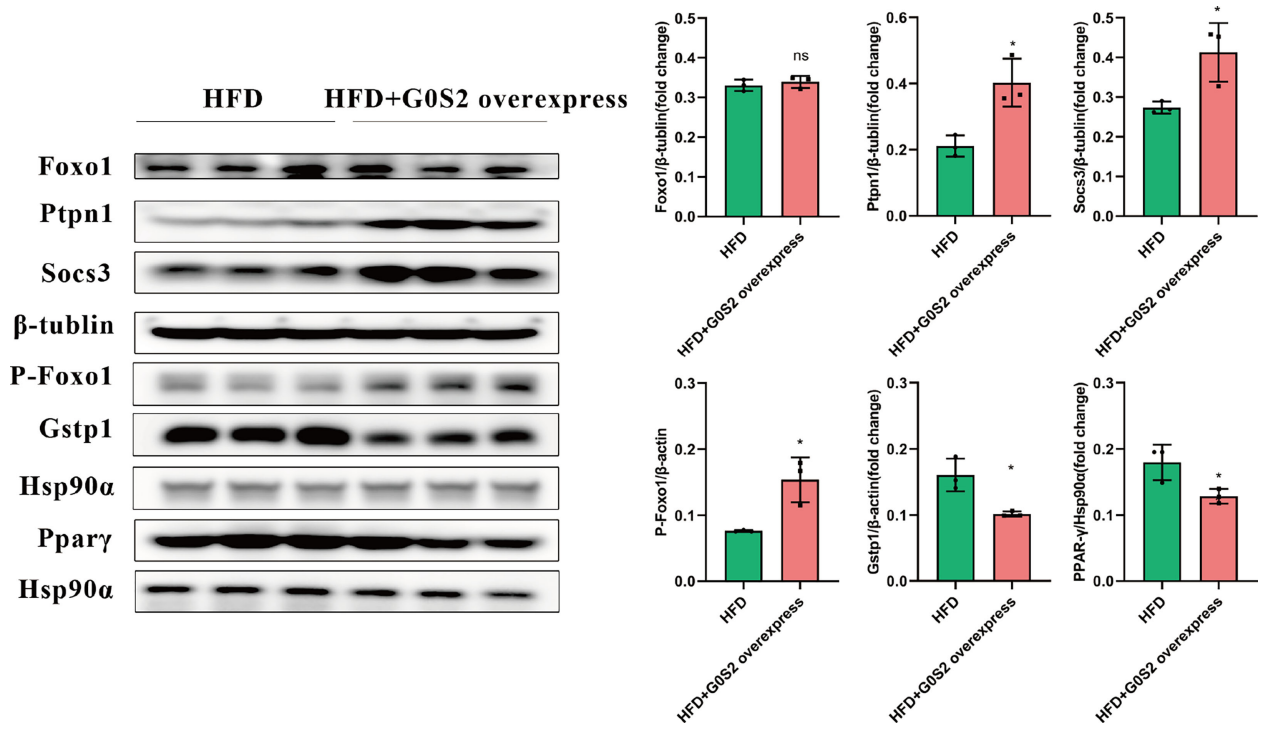

FIGURE 6
Insulin resistance was evaluated using western blotting and RT-PCR analysis. Upregulation of phosphatase Foxo1, Socs3, and Ptpn1, and downregulation of Gstp1 and PPAR-γ in the livers of HFD-*G0S2* overexpression mice were determined by western blotting and RT-PCR in liver tissue. The experiment was repeated three times, using three independent batches of mice and three independent mice in each group. *P<0.05.

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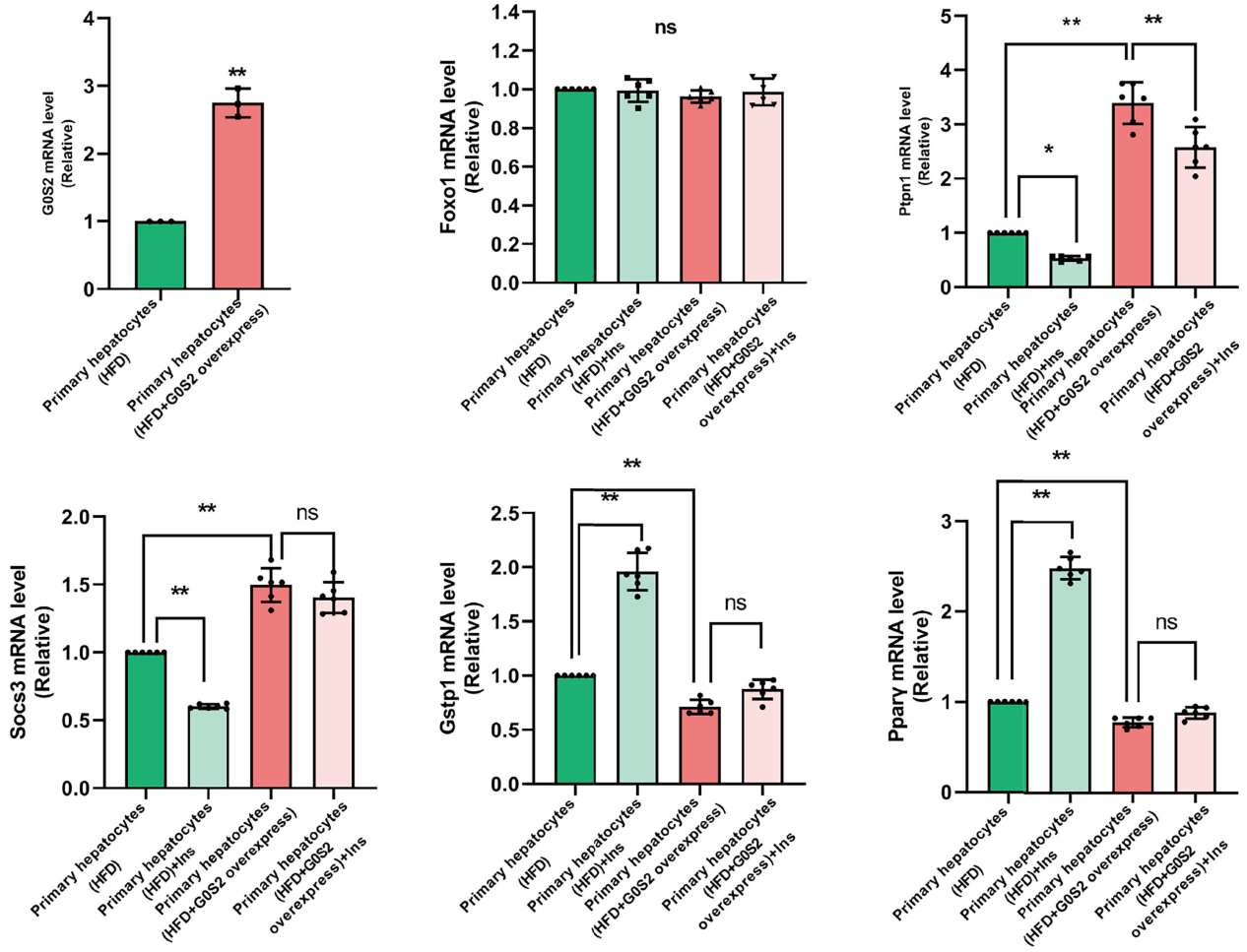

FIGURE 7
The genes involved in insulin resistance were evaluated using RT-PCR analysis in primary mouse hepatocytes. Downregulation of the phosphatases Foxo1, Socs3, and Ptpn1, as well as upregulation of Gstp1 and PPAR-γ, were determined in primary mouse hepatocytes isolated from *G0S2*-overexpressing mice (HFD+G0S2 overexpression) by RT-PCR. *P<0.05, **P<0.01. ns, P>0.05.

In our study, the protein expression in the livers of G0S2-overexpression mice was analyzed by label-free LC-MS/MS quantitative proteomics. The results of proteomics demonstrated that there were four upregulated proteins that were related to insulin signaling pathways. Foxo1 was mainly involved in insulin resistance and lipid metabolism. Previous studies have revealed that Foxo1 participates in insulin resistance and β-cell failure in T2DM patients and leads to gluconeogenesis dysfunction and cell apoptosis. However, inhibition of Foxo1 improves insulin resistance (49, 50). However, some studies show that inhibition of Foxo1 interacts with ATGL leading to hepatic steatosis (51). Our study showed that Foxo1 was upregulated by 6.4-fold and was a pro-insulin resistance protein. Hence, the above research results suggest that G0S2 exerts an important role in regulating the insulin signaling pathway in the liver.

The suppressor of cytokine signaling (SOCS) family of proteins are negative regulators of cytokine signaling. The expression of Socs3 in the liver, skeletal muscle, and adipose tissue is upregulated in obese rodents (52, 53). In obese patients with NAFLD, the abundance of Socs3 in mononuclear cells was also increased (54, 55). In an Socs3 AKO mouse model, the HFD increased the levels of Socs3 in adipose tissue of WT mice; however, Socs3 AKO mice failed to show the same results (56). Socs3 has been shown to play an important role in insulin sensitivity, because it inhibits tyrosine phosphorylation of the relevant receptor, such as insulin receptor and insulin receptor substrate-1 (IRS1) (57, 58). A recent study found that Polygoni Cuspidati ethanol extract attenuates obesity, NAFLD, and IR *via* inhibitions of Socs3 (59). The findings of our study suggest that upregulation of G0S2 induced impairment of insulin signaling. Insulin resistance is likely an important determinant of the negative effects of G0S2 targeting NAFLD and diabetes.

Ptpn1, the gene coding for Protein Tyrosine Phosphatase-1B, plays a critical role in negative regulation of insulin signaling. The upregulation of Ptpn1 in tissues and cells inactivates protein tyrosine kinase (PTK), blocks the effect of insulin on binding to

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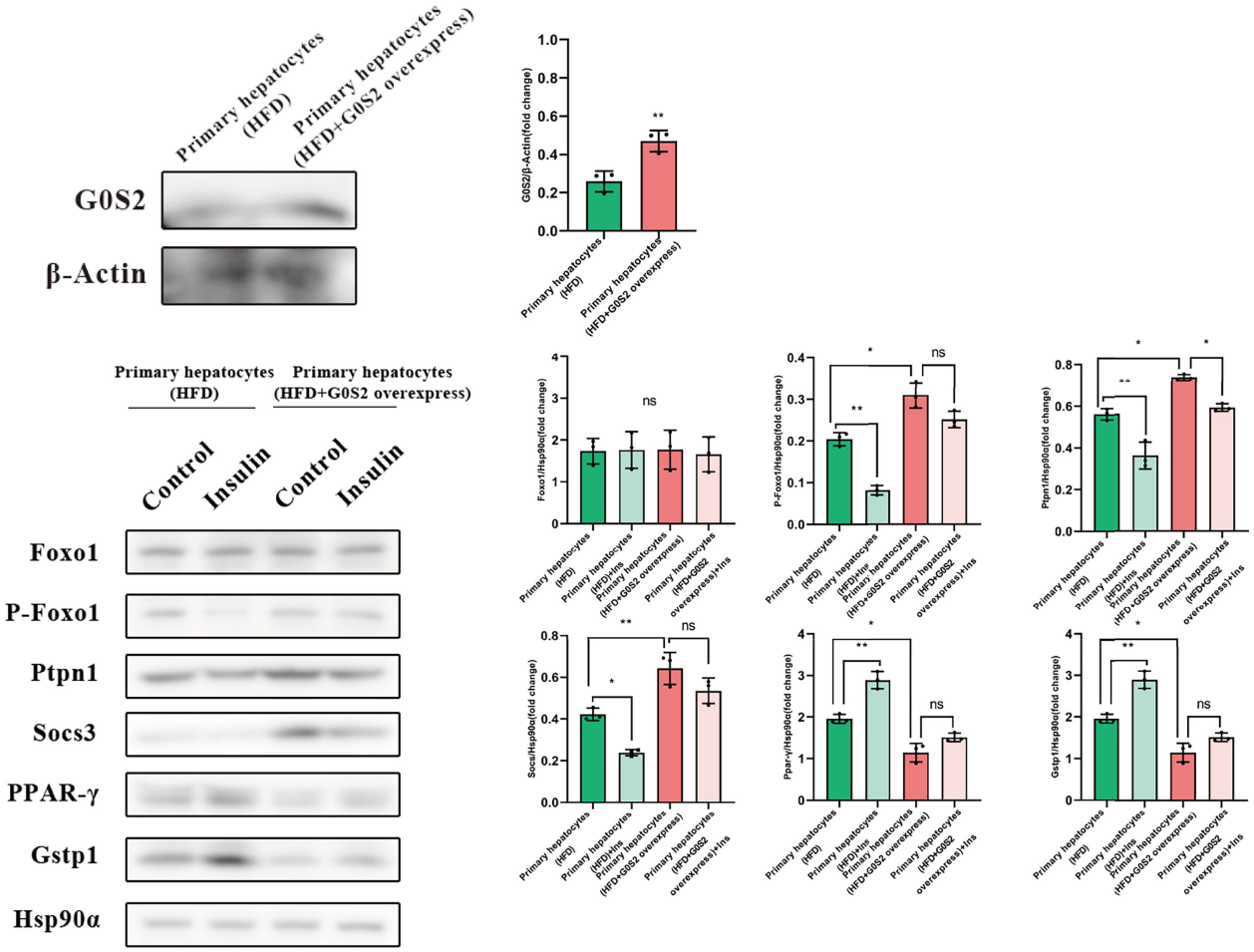

FIGURE 8
The proteins involved in insulin resistance were evaluated using western blotting analysis in primary mouse hepatocytes. Downregulation of the phosphatases Foxo1, Socs3, and Ptpn1, as well as upregulation of Gstp1 and PPAR-γ, were determined in primary mouse hepatocytes isolated from *G0S2*-overexpressing mice (HFD+G0S2 overexpression) by western blotting. *P<0.05, **P<0.01, ns P>0.05.

insulin receptors and dephosphorylation of tyrosine residues on insulin receptors substrates, leading to insulin resistance and finally to diabetes (60–62). A study revealed that by inhibiting Ptpn1 expression and promoting phosphorylation of insulin receptor, microRNA-206 impaired hepatic lipogenesis and exerted the beneficial effect of preventing hepatic steatosis (63). Our study results are consistent with the above observations in that it suggests that inhibition of Ptpn1 expression mediates the beneficial effect of G0S2 on NAFLD and diabetes.

Our study results demonstrated that the levels of Gstp1 and PPAR-γ were significantly down-regulated after overexpression of G0S2. Previous studies ruled out that Gstp1 is closely involved in the inhibition of cell apoptosis and regulation of cell oxidative stress (64, 65). The tumor necrosis factor-related receptor 2 (TRAF2) interacts with apoptosis signal regulating kinase 1 (ASK1), and the interaction between them could be abolished by binding Gstp1 to TRAF2 (66). Gstp1 regulated the ASK1-MEK-JNK/p38 pathway negatively and inhibited cell apoptosis (67). Another research on humans showed that participants with Gstp1 AG genotypes showed stronger associations between insulin resistance markers who were exposed to air pollution (68).

PPAR agonists, lipid sensors that modulate whole-body energy metabolism, have been used to treat dyslipidemia and diabetes for decades. PPAR-γ increases systemic insulin sensitivity by increasing adipocyte differentiation and fatty acid uptake and storage in lipid droplets (69). PPAR-γ deficiency in adipose tissue causes metabolic dysfunction in mice (70). Under conditions of energy deficiency, PPAR-γ on Lys 268 and Lys 293 was deacetylated by SIRT 1. Regulation of PPAR-γ can protect mice from HFD-induced insulin resistance (71–73). Notably, thermogenesis was enhanced in the mouse model of Kdm2a deficiency in macrophages, and the obesity induced by HFD was prevented by enhancing H3K36me2 at the PPAR-γ locus. The upregulation of PPAR-γ may highlight a new mechanism by which G0S2 helps improve insulin sensitivity in NAFLD and diabetes.

There are some limitations to this study. For example, there was no control group of mice on normal chow diet. Based on the absence of these groups as control, the results of our study should be

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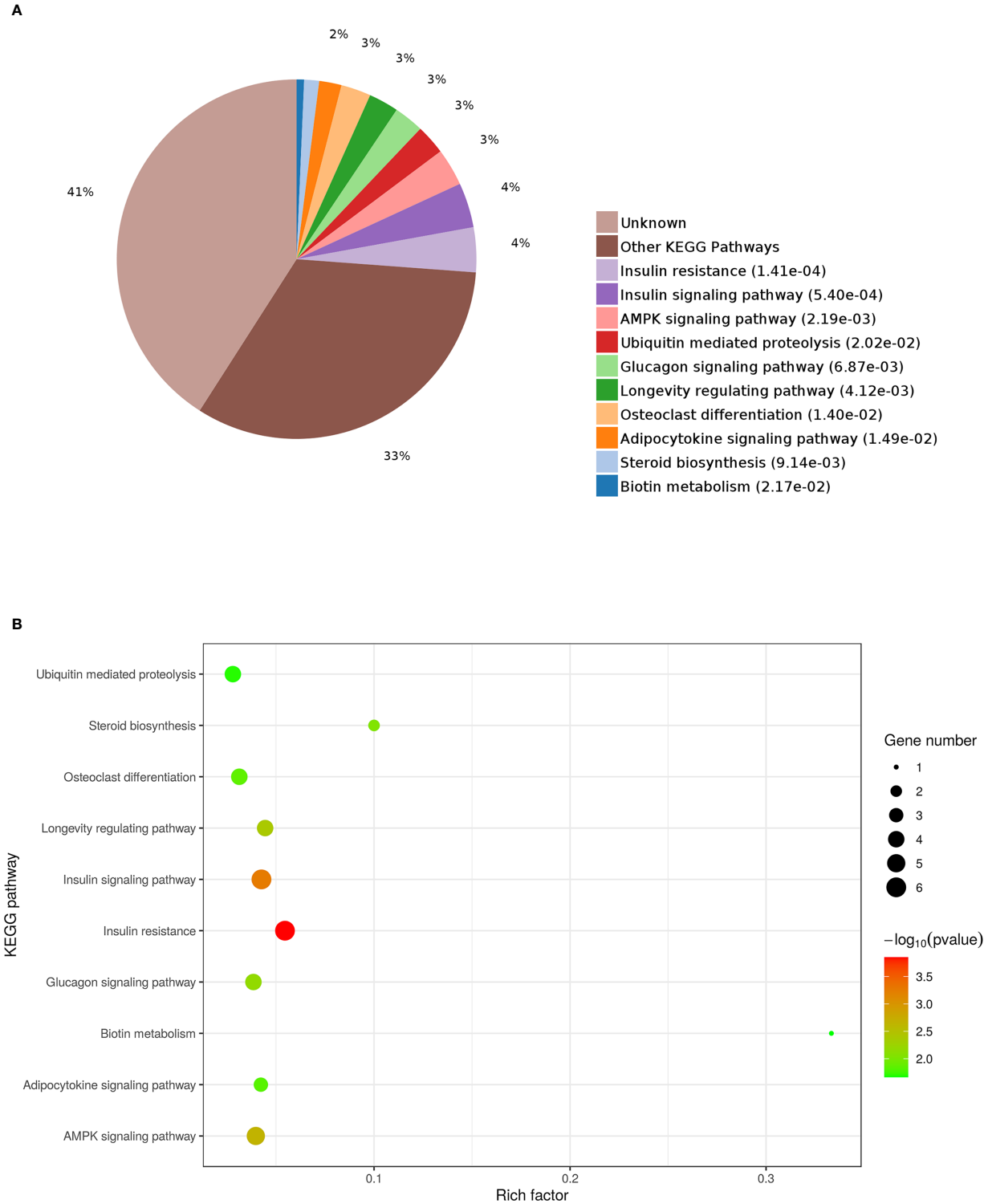

FIGURE 9
Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEPs. **(A)** Pie chart of DEP-enriched KEGG pathways. **(B)** KEGG pathway enrichment distribution of DEPs.

interpreted with caution, and further investigations are needed. Another limitation is that we did not test the effects of G0S2 gene deletion to determine whether such deletion is sufficient to improve insulin resistance.

In conclusion, we focused our study on the effect of G0S2 on insulin resistance. Insulin resistance is a key contributor to the pathogenesis of NAFLD, diabetes, and fatty and other metabolic diseases. Our research demonstrates that the expression patterns of

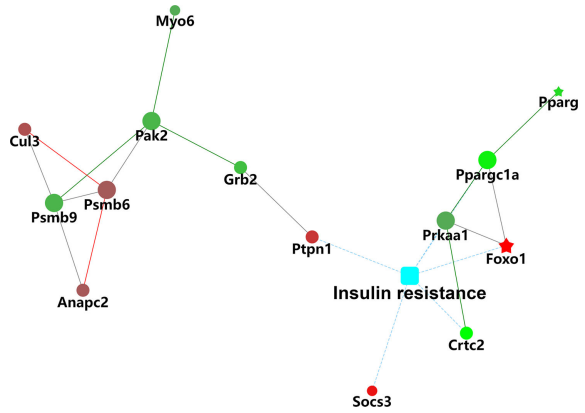

FIGURE 10
Protein–protein interaction (PPI) network analysis of differentially insulin resistance-associated proteins.

several proteins associated with insulin signaling pathway are consistent with the change of insulin resistance after overexpression of G0S2. These observations might uncover the molecular mechanisms of metabolic diseases and provide novel insights into potential therapeutic targets for NAFLD, diabetes, and other metabolic diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

All methods are implemented in conformity with relative instructions and regulations. All surgeries were performed under sodium pentobarbital anesthesia to minimize pain. The present study was approved by the Ethics Committee of Shandong Provincial Hospital (NSFC: NO.2019-131). All methods were performed following the ARRIVE guidelines.

Author contributions

DW was the experimental designer and executor of the experimental study, completed the data analysis, and wrote the first draft of the paper. SM was the conceptualizer and leader of the project, and directed the experimental design, data analysis, and paper writing and revision. ZZ, WS, YY, and MJ contributed to experiment and data analyze. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Response to pioglitazone in non-alcoholic fatty liver disease patients with *vs*. without type 2 diabetes: A meta-analysis of randomized controlled trials

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Background: Pioglitazone is considered a potential therapy for non-alcoholic fatty liver disease (NAFLD). However, different effects of pioglitazone on NAFLD have been demonstrated in diabetic and non-diabetic patients. Herein, a meta-analysis of randomized, placebo-controlled trials was carried out to indirectly compare pioglitazone in NAFLD patients with *vs*. without type 2 diabetes.

Methods: Randomized controlled trials (RCTs) of pioglitazone *vs*. placebo involving NAFLD patients with or without type 2 diabetes/prediabetes collected from databases were enrolled into this analysis. Methodological quality was employed to evaluate the domains recommended by the Cochrane Collaboration. The analysis covered the changes in histology (fibrosis, hepatocellular ballooning, inflammation, steatosis), liver enzymes, blood lipids, fasting blood glucose (FBS), homeostasis model assessment-IR (HOMA-IR), weight and body mass index (BMI) before and after treatment, and adverse events.

Results: The review covered seven articles, with 614 patients in total, of which three were non-diabetic RCTs. No difference was found in patients with *vs*. without type 2 diabetes in histology, liver enzymes, blood lipids, HOMA-IR, weight, BMI, and FBS. Moreover, no significant difference was revealed in adverse effects between NAFLD patients with diabetes and without DM, except the incidence of edema that was found to be higher in the pioglitazone group than in the placebo group in NAFLD patients with diabetes.

Conclusions: Pioglitazone could exert a certain effect on alleviating NAFLD, which was consistent between non-diabetic NAFLD patients and diabetic NAFLD patients in improving histopathology, liver enzymes, and HOMA-IR and reducing

blood lipids. Furthermore, there were no adverse effects, except the incidence of edema which is higher in the pioglitazone group in NAFLD patients with diabetes. However, large sample sizes and well-designed RCTs are required to further confirm these conclusions.

KEYWORDS

pioglitazone, nonalcoholic fatty liver disease, randomized controlled trials, diabetes mellitus, nonalcoholic steatohepatitis

Introduction

The overall prevalence of non-alcoholic fatty liver disease (NAFLD) is globally estimated at 25%–40%, which has been considered a major disease burden worldwide with a rising trend (1). Non-alcoholic steatohepatitis (NASH) will be developed in approximately 25% of NAFLD patients, of whom one-fourth will develop liver failure and hepatocellular carcinoma (HCC) with higher rates of progression to cirrhosis (2–4). Indeed, a study in the US has already demonstrated that NAFLD is the most common risk factor for HCC (24%), in contrast to HCV (23%) and hepatitis B (19.3%) (5). NAFLD could exhibit a close correlation with metabolic syndrome, a range of risk factors for type 2 diabetes mellitus, and end-stage vascular disease, with cardiovascular disease being the most common burden of death in patients with NAFLD (6). Lifestyle interventions, such as calorie restriction and exercise therapy, are demonstrated to play a central role in treating NAFLD, which, however, are difficult to achieve and maintain. Despite several pharmacologic interventions to treat NAFLD, there is still no approved drug for its effective treatment (3, 7).

Pioglitazone as a peroxisome proliferator-activated receptor (PPAR) agonist could increase plasma adiponectin levels, which are associated with insulin sensitivity improvement, exerting anti-inflammatory and antifibrotic effects on NAFLD (8). Della et al. discovered that treatment with pioglitazone at low dosage significantly improved liver inflammation and alleviated insulin resistance in NAFLD patients with type 2 diabetes mellitus (T2DM) (9). Bril et al. found that pioglitazone discontinuation in patients with biopsy-proven NASH was associated with biochemical worsening of the disease, and pioglitazone therapy in patients with NASH should be considered as a long-term treatment (10). These studies suggest that pioglitazone has a certain role in the treatment of NAFLD. As a result, pioglitazone may be recommended for treating NAFLD as verified by the improvement of liver histology and some biochemical indexes in several studies (11–15). These studies have explored the efficacy of pioglitazone in NAFLD patients, primarily by comparing the effect of pioglitazone and all other drugs for NAFLD together. Furthermore, these studies have not compared NAFLD patients with T2DM to non-diabetic patients, and there are varying opinions among these studies. For this reason, it is of significance to investigate whether pioglitazone will exert different effects between diabetic and non-diabetic individuals, so as to treat different types of NAFLD more efficiently.

This meta-analysis was carried out to compare the efficacy and safety of pioglitazone in treating NAFLD with *vs.* without T2DM. Nevertheless, few studies have compared pioglitazone with placebo in patients with NAFLD between T2DM and normal glucose tolerance; therefore, we conducted this study to try to replenish this gap.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; CNKI, China National Knowledge Infrastructure; RCTs, randomized controlled trials; FBS, fasting blood glucose; BMI, body mass index; AST, aspartate transaminase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; NASH, non-alcoholic steatohepatitis; PPARγ, peroxisome proliferator-activated receptor-γ; T2DM, type 2 diabetes mellitus; ORs, odds ratios; MDs, mean differences; CIs, confidence intervals; OR, odds ratio; TC, total cholesterol; FFAs, free fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance.

Materials and methods

Retrieval strategy

The major databases PubMed, Embase, Web of Science, WangFang Data, CNKI, and Medline were systematically searched for literature to retrieve eligible studies without language restriction by two reviewers from inception to May 2022, and additional information or raw data were asked by the corresponding authors through email. The keywords "nonalcoholic steatohepatitis" OR "nonalcoholic fatty liver disease" OR "NASH" OR "NAFLD" AND "pioglitazone" were employed. At the same time, a wide scanning of relevant references listed in the retrieved articles was also conducted to seek other articles of possible eligibility. The research selection process is provided in Figure 1.

Inclusion and exclusion criteria

Randomized controlled trials of pioglitazone *vs.* placebo involving patients with NAFLD confirmed by liver biopsy or ultrasound, with or without T2DM/prediabetes, were included.

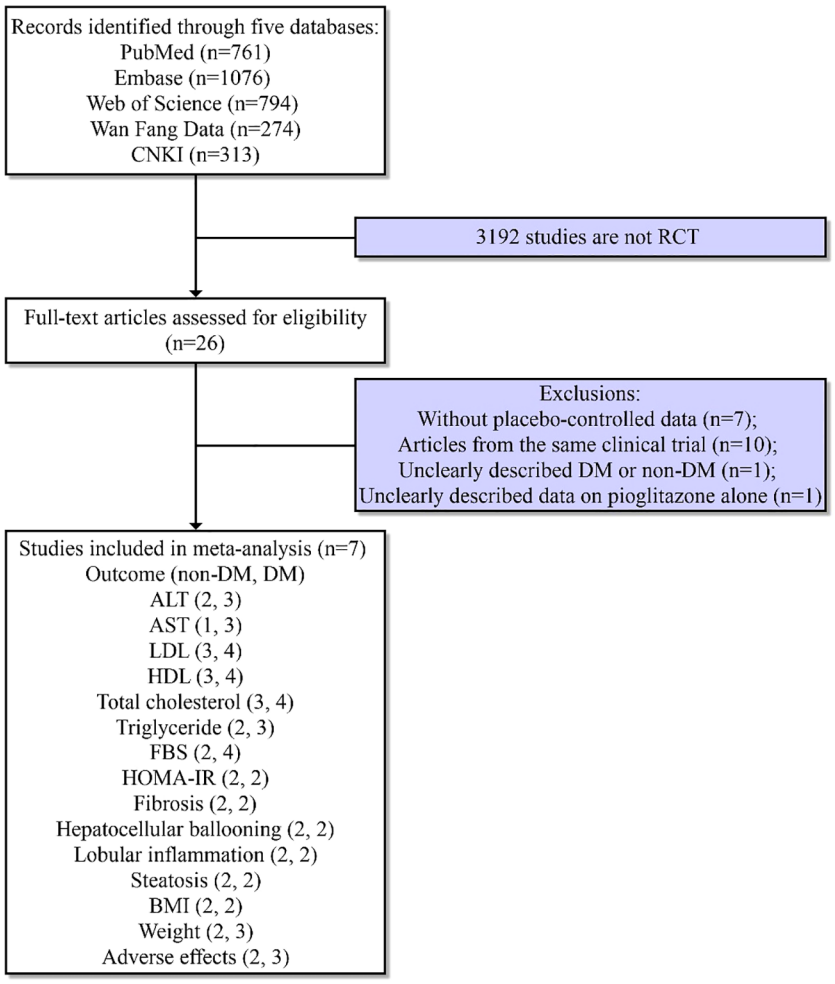

FIGURE 1
Flowchart of study information.

The exclusion criteria were as follows: i) non-randomized placebo-controlled trials; ii) trials without raw data; iii) leading articles, abstracts, letters, animal experiments, case reports, meta-analysis, expert opinion, conference papers, and book sections; iv) no clear validity of whether NAFLD patients were complicated with diabetes; v) patients with severe renal failure, heart failure, malignant tumor, or secondary hepatic fat accumulation such as viral hepatitis or significant alcohol consumption; and vi) trials that did not present data on pioglitazone alone.

Methodological quality assessment

Each randomized controlled trial was evaluated for methodological quality using Cochrane Collaboration's tool (16), which involved sequence generation, allocation hiding, the blinding method in the selection of participants and personnel and result evaluators, processing of data results, and the lack of other deviation sources, determining the high, low, or unclear deviation risk of the research. The assessment of the enrolled studies is presented in Figure 2.

Outcome measures

The primary outcomes referred to histological variables such as fibrosis, steatosis, inflammation, and hepatocellular ballooning, and the secondary outcomes included changes in alanine transaminase (ALT), aspartate aminotransferase (AST), FBS, blood lipids, HOMA-IR, weight, and BMI. In addition, the impact on adverse events was evaluated.

Data extraction

Data were extracted by two reviewers independently and summarized into a standardized spreadsheet in duplicate after the studies have been confirmed to meet the predetermined criteria. Disagreements were resolved by negotiated solutions or mutual discussion, and the quality of the trials was assessed by kappa statistics scoring. The following variables were extracted from each study: i) general information (name of the first author, year, study design, presence of diabetes); ii) treatment details (dosage, frequency, duration, lifestyle changes throughout the trial); iii)

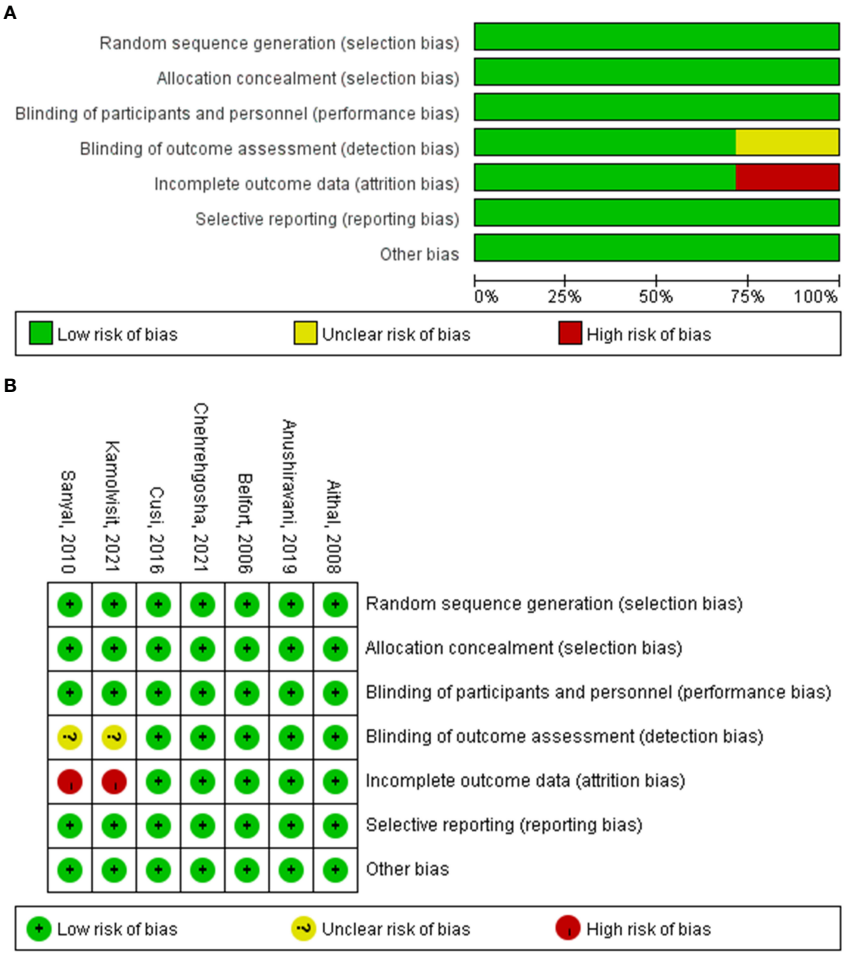

FIGURE 2
Methodological quality **(A)** and risk of bias **(B)** for trials included in systematic review.

histological variables (baseline and at the end of the study): fibrosis, steatosis, inflammation, and hepatocellular ballooning; iv) laboratory and anthropometric tests (baseline and at the end of the study), covering ALT, AST, blood lipids, FBS, HOMA-IR, weight, and BMI; and v) adverse events.

Data analysis

All data were analyzed on R software v3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). The "Meta" package was employed in the meta-analysis. Mean differences were calculated by the following formula: (mean value of treatment at baseline − mean value of treatment at the end of the study) − (mean value of control at baseline − mean value of control at the end of the study). The mean differences for the intervention and control groups were either directly provided by the research results or calculated by the mean values before and after treatment. To calculate the SD of the change in means for those studies, it was imputed applying a modified method by Follmann et al.: SDchange in means = sq root [(SDpre)2 + (SDpost)2 − (2(q)·SDpre·SDpost)] (17). The change in means (SDchange in means) was obtained using the SD of the preintervention mean (SDpre) and the SD of the postintervention mean (SDpost) as well as the within-participant correlation (q) of the outcome measures. Sensitivity analysis was conducted to exclude studies that influence the stability of research results and to assess heterogeneity. Publication bias was evaluated with funnel plot analysis and Egger's and Begg's tests. The level of statistical significance was 0.05, and the statistical heterogeneity across studies was represented by I^2 statistics. Improvement was determined by a reduction of 1 point or more in the pathology score. The fixed-effects model will be employed in the statistical analysis when $P \geq 0.05$ and $I^2 \leq 50\%$; otherwise, the random-effects model was applied. Dichotomous and continuous variables were expressed as odds ratios (ORs), mean differences (MDs), and 95% confidence intervals (CIs), respectively.

Result

Study characteristics

After the primary screening, 26 studies were included for the subsequent full-text review until May 2022. Seven articles (9, 18–23)

without placebo-controlled data, 10 articles (24–33) from the same clinical trial, one article (34) without a clear statement of whether NAFLD patients were complicated with diabetes, and one article (35) that did not present data on pioglitazone alone were removed. Ultimately, a total of seven studies (11, 12, 36–40) deemed eligible were included, covering 614 patients, three of which (36–38) were non-diabetic RCTs, each being extracted for outcomes. The subjects of four studies included patients with NASH, and three studies included patients with NAFLD. The mean age of the patients with diabetes or prediabetes *vs.* without diabetes or prediabetes was found to be 51.1 ± 8.4 *vs.* 49.3 ± 11 years, and the male sex distribution was 59.3% *vs.* 49.1%. The main characteristics of the RCTs involved in the network meta-analysis are summarized in Table 1 and Supplementary Table 1. The flowchart in Figure 1 describes the selection process of the literature and the final selection of the studies. The dose of pioglitazone ranged from 15 to 45 mg/day, and the duration of pioglitazone or placebo treatment ranged from 3 to 24 months.

Study quality assessment

The risk of bias (such as selection bias, performance bias, detection bias, attrition bias, and reporting bias) was assessed using Cochrane Collaboration's tool. All data were derived from randomized studies. The probability of bias was estimated and considered low in most studies and domains (Figure 2).

Changes in liver histology with pioglitazone

The histological changes of the liver were significantly improved in NAFLD patients who received pioglitazone therapy (fibrosis: I^2 = 0, OR = 1.81, 95% CI: 1.15 - 2.83, P = 0.01; hepatocellular ballooning: I^2 = 0, OR = 2.71, 95% CI: 1.71 - 4.31, P < 0.01; lobular inflammation: I^2 = 0, OR = 2.94, 95% CI: 1.89 - 4.59, P < 0.01; steatosis: I^2 = 40%, OR = 4.04, 95% CI: 2.59 - 6.30, P < 0.01; Figure 3). No significant differences in primary outcomes were found in NAFLD patients with diabetes compared with those without diabetes who received pioglitazone therapy (fibrosis: χ^2 = 0.02, P = 0.90; hepatocellular ballooning: χ^2 = 0.68, P = 0.41; lobular inflammation: χ^2 = 0.31, P = 0.57; steatosis: χ^2 = 0.78, P = 0.38; Figure 3).

The subgroup comparison results revealed no obvious superiority of pioglitazone therapy in fibrosis both in NAFLD patients with diabetes and without diabetes (with DM: OR = 1.87, 95% CI: 0.94 - 3.72, P = 0.08; without DM: OR = 1.76, 95% CI: 0.97 - 3.19, P = 0.06; Figure 3). However, these results suggest that pioglitazone may play a role in the treatment of liver fibrosis, with significant improvements in hepatocellular ballooning (with DM: OR = 3.40, 95% CI: 1.68 - 6.88, P < 0.01; without DM: OR = 2.29, 95% CI: 1.24 - 4.24, P < 0.01; Figure 3), lobular inflammation (with DM: OR = 3.43, 95% CI: 1.70 - 6.92, P < 0.01; without DM: OR = 2.65, 95% CI: 1.49 - 4.71, P < 0.01; Figure 3), and steatosis (with DM: OR = 5.16, 95% CI: 2.56 - 10.39, P < 0.01; without DM: OR = 3.02, 95% CI: 1.01 - 8.97, P = 0.05; Figure 3) compared with placebo.

Changes in liver enzymes with pioglitazone

AST and ALT were confirmed to be significantly decreased in NAFLD patients who received pioglitazone therapy (AST: I^2 = 51%, MD = −6.56, 95% CI: (−11.18) - (−1.94), P < 0.01; ALT: I^2 = 71%, MD = −14, 95% CI: (−23.75) - (−4.26), P < 0.01; Supplementary Figure 1). No significant differences were found in both AST and ALT between NAFLD patients with diabetes and those without diabetes who received pioglitazone therapy (AST: χ^2 = 0.19, P = 0.66; ALT: χ^2 = 0.16, P = 0.69; Supplementary Figure 1).

The subgroup comparison indicated no significant improvements in both AST and ALT in NAFLD patients without

TABLE 1 Patient and trial characteristics of the included studies.

Study	*N*	Intervention, dose	Comparator(s)	Duration	Diabetes or prediabetes	NASH or NAFLD	Country	NASH/NAFLD assessment in results
Aithal (36),	74	Pioglitazone, 30 mg/day	Placebo	12 months	No	NASH	United Kingdom	Histology
Anushiravani (37),	60[a]	Lifestyle + pioglitazone, 15 mg/day	Lifestyle + placebo	3 months	No	NAFLD	Iran	Ultrasound
Sanyal (38),	163[a]	Pioglitazone, 30 mg/day	Placebo	24 months	No	NASH	America	Histology
Belfort (11),	47[a]	Hypocaloric diet + pioglitazone, 45 mg/day	Hypocaloric diet + placebo	6 months	Yes	NASH	America	Histology
Cusi (12),	101	Pioglitazone, 45 mg/day	Placebo	18 months	Yes	NASH	America	Histology
Kamolvisit (39),	98	Pioglitazone, 45 mg/day	Placebo	18 months	Yes	NAFLD	Thailand	Ultrasound
Chehrehgosha (40),	71[a]	Pioglitazone, 30 mg/day	Placebo	6 months	Yes	NAFLD	Iran	Ultrasound

[a]Represents patients in the trial arms of interest only.

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diabetes who received pioglitazone therapy compared with those who received placebo [AST: MD = −5.5, 95% CI: (−11.33) - 0.33, P = 0.06; ALT: MD = −17.79, 95% CI: (−38.14) - 2.57, P = 0.09; Supplementary Figure 1], while there was a significant reduction in AST in patients with diabetes [MD = −7.48, 95% CI: (−14.27) - (−0.7), P = 0.03; Supplementary Figure 1], but not in ALT [MD = −12.74, 95% CI: (−26.33) - 0.84), P = 0.07; Supplementary Figure 1].

Changes in metabolism with pioglitazone

HDL and HOMA-IR were confirmed to be significantly improved in NAFLD patients who received pioglitazone therapy; however, the levels of LDL, total cholesterol, triglycerides, and FBS showed no significant changes compared with the placebo groups. No significant differences were found in NAFLD patients with diabetes compared with those without diabetes who received pioglitazone therapy in terms of HDL, LDL, total cholesterol, triglycerides, HOMA-IR, and FBS (HDL: I^2 = 96%, χ^2 = 0.00, P = 0.99; LDL: I^2 = 0%, χ^2 = 0.23, P = 0.63; total cholesterol: I^2 = 0%, χ^2 = 0.91, P = 0.34; triglycerides: I^2 = 40%, χ^2 = 1.53, P = 0.22; HOMA-IR: I^2 = 92%, χ^2 = 1.30, P = 0.25; FBS: I^2 = 81%, χ^2 = 2.42, P = 0.12; Figure 4 and Supplementary Figure 2).

The subgroup comparison results showed significant improvements in HDL, LDL, total cholesterol, and triglycerides with pioglitazone therapy than with placebo in patients without diabetes [HDL: MD = 2.98, 95% CI: 2.64 - 3.31, P < 0.01; LDL: MD = −2.22, 95% CI: (−3.48) - (−0.96), P < 0.01; total cholesterol: MD = −1.76, 95% CI: (−3.14) - (−0.37), P = 0.01; triglycerides: MD = −13.07, 95% CI: (−15.47) - (−10.66), P < 0.01; Figure 4], while there were no significant improvements in both FBS and HOMA-IR [FBS: MD = −6.16, 95% CI: (−22.14) - 9.81, P = 0.45; HOMA-IR: MD = −0.43, 95% CI: (−2.06) - 1.2, P = 0.60; Supplementary Figure 2]. However, no significant improvements were found in NAFLD patients with diabetes in HDL, LDL, and total cholesterol [HDL: MD = 1.87, 95% CI: (−0.77) - 4.52, P = 0.16; LDL: MD = −3.59, 95% CI: (−8.97) - 1.79, P = 0.19; total cholesterol: MD = −4.54, 95% CI: (−10.08) - 1.00, P = 0.11; Figure 4]. Significant improvements were revealed in triglycerides, FBS, and HOMA-IR in NAFLD patients with diabetes [triglycerides: MD = −38.61, 95% CI: (−76.17) - (−1.06), P = 0.04; FBS: MD = −21.84, 95% CI: (−23.06) - (−20.63), P < 0.01; HOMA-IR: MD = −1.82, 95% CI: (−3.57) - (−0.07), P = 0.04; Figure 4 and Supplementary Figure 2].

Changes in weight and BMI with pioglitazone

Weight and BMI showed no significant differences in patients who received pioglitazone therapy and those who received a placebo. No significant differences were found in both weight and BMI between NAFLD patients with diabetes and those without diabetes who received pioglitazone therapy (weight: I^2 = 0%, χ^2 = 1.15, P = 0.28; BMI: I^2 = 0%, χ^2 = 0.07, P = 0.79; Supplementary Figure 3).

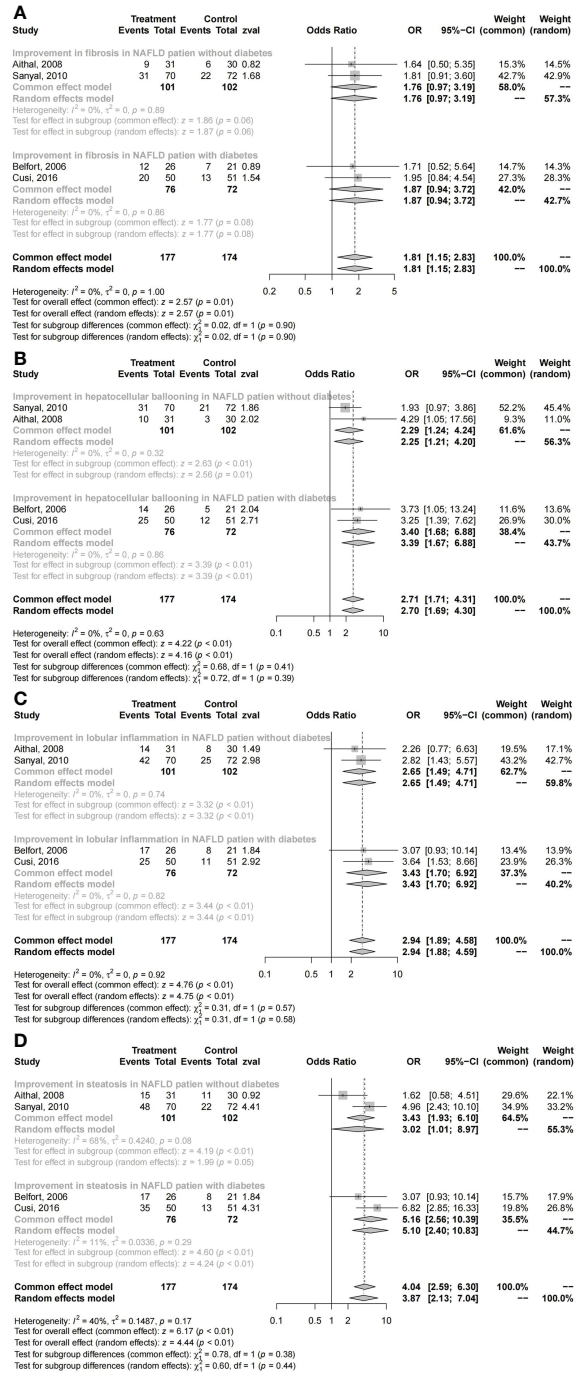

FIGURE 3
Changes in histology with pioglitazone: **(A)** fibrosis, **(B)** hepatocellular ballooning, **(C)** lobular inflammation, and **(D)** steatosis.

The subgroup comparison results revealed significant increases in both weight and BMI compared with the placebo groups in patients without diabetes (weight: MD = 4.15, 95% CI: 2.14 - 6.17, P < 0.01; BMI: MD = 0.84, 95% CI: 0.03 - 1.65, P = 0.04; Supplementary Figure 3). No significant difference in BMI [MD = 0.64, 95% CI: (−0.58) - 1.87, P = 0.30] or weight [MD = 1.77, 95% CI: (−2.09) - 5.63, P = 0.37; Supplementary Figure 3] was found in NAFLD patients with diabetes.

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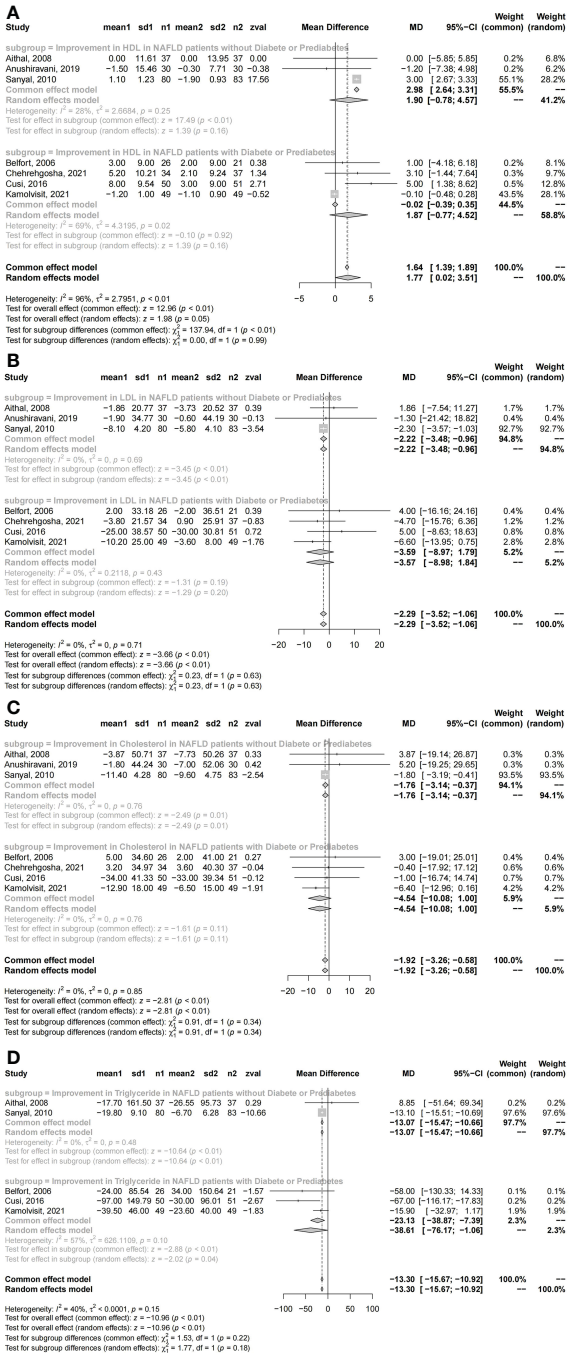

FIGURE 4
Changes in metabolism with pioglitazone: **(A)** HDL, **(B)** LDL, **(C)** total cholesterol, and **(D)** triglycerides.

Adverse effects of pioglitazone compared with placebo

No significant differences were revealed in terms of adverse effects between NAFLD patients with diabetes and those without diabetes who received pioglitazone therapy (I^2 = 61%, χ^2 = 3.44, P = 0.06; Supplementary Figure 4).

No significant difference was found in terms of adverse effects between pioglitazone and placebo in NAFLD patients with or without diabetes. The mean differences and 95% CI for patients with diabetes and without diabetes with NAFLD were calculated as follows: DM: OR = 1.61, 95% CI: 0.82 - 3.16, P = 0.17; without DM: OR = 0.47, 95% CI: 0.16 - 1.42, P = 0.18 (Supplementary Figure 4). The incidence of edema was significantly increased in the pioglitazone group than in the placebo group in NAFLD patients with DM. No statistical significance was found in specific adverse effects comparing the pioglitazone group with the corresponding placebo group (Table 2).

Sensitivity analysis and publication bias

We conducted a sensitivity analysis and publication bias analysis on the research studies with significant heterogeneity. Running the sensitivity analysis by excluding some high-risk studies showed a remarkable effect on the results of the analysis. Excluding the studies of Chehrehgosha et al. (40) in the ALT analysis, Anushiravani et al. (37) in the HDL analysis, Kamolvisit et al. (39) in the FBS analysis, and Cusi et al. (12) in HOMA-IR changes the substantiation of the corresponding results of the meta-analysis (Supplementary Figure 5). The analysis of the funnel plot for publication bias is shown in Supplementary Figure 6. Furthermore, Begg's test showed no publication bias in ALT, FBS, HDL, and HOMA-IR analysis (all P > 0.05).

Discussion

The present guidelines state the promising role of pioglitazone in liver histology in NASH patients as confirmed by liver biopsy, whether or not suffering from T2DM; however, the safety of long-term treatment should also be considered (41, 42). Tokushige et al. (43) recommend pioglitazone for NASH patients with insulin resistance. A prospective study (44) aiming at adults with biopsy-proven NASH (49 with prediabetes and 52 with T2DM) suggested pioglitazone for NASH patients with prediabetes as well as for NASH patients with T2DM to achieve metabolic and histologic benefits. However, this head-to-head observational study may lead to erroneous results with inconsistent baselines. Previous meta-analyses (13, 45–48) have explored the efficacy of pioglitazone in the treatment of NAFLD, primarily by comparing the effect of pioglitazone and all other drugs for NAFLD together and obtaining similar conclusions that pioglitazone has effects on NAFLD patients with T2DM or non-diabetes. Furthermore, studies have not compared NAFLD patients with T2DM to NAFLD patients without diabetes. As a result, no convincing conclusions about pioglitazone in the treatment of NAFLD patients without diabetes can be indeed drawn. In order to obtain a better understanding of the effects of pioglitazone in non-diabetes and diabetes NAFLD, RCTs on pioglitazone in the treatment of diabetes or non-diabetes NAFLD were searched and compared with placebo, so as to achieve an indirect comparison of pioglitazone in the treatment of NAFLD with *vs.* without diabetes, comprehending the efficacy and adverse effects of pioglitazone in the treatment of NAFLD patients.

TABLE 2 Reported adverse events and withdrawals during the treatment period.

Adverse Events	NAFLD with DM			NAFLD without DM		
	Placebo (n = 141)	Pioglitazone (n = 152)	P	Placebo (n = 120)	Pioglitazone (n = 111)	P
Cardiovascular	9	4	0.116	14	10	0.507
Gastrointestinal	17	14	0.429	7	4	0.423
Hypoglycemic	9	5	0.213	8	15	0.081
Neurologic	6	9	0.516	6	2	0.173
Gynecologic	2	2	0.94	0	1	0.225
Urologic	4	7	0.423	0	0	-
Edema	3	12	0.02	0	0	-
Musculoskeletal	21	23	0.955	4	4	0.911
Hepatotoxicity	1	0	0.226	6	4	0.601
Bone fractures	0	0	-	5	3	0.541
Cancer	1	0	0.226	0	0	-
Total number of withdrawals	3	2	0.591	7	6	0.888

The improvement of liver fibrosis is of crucial significance for the treatment of NAFLD as it is associated with higher rates of cirrhosis as well as overall mortality (1, 2). Mahady et al. (49) have stated that pioglitazone can optimize histological variables, such as fibrosis, hepatocellular ballooning, lobular inflammation, and steatosis. As Musso et al. (45) stated, pioglitazone can contribute to reversing advanced fibrosis in NASH, even in non-diabetic patients. However, the article has not compared the effects of non-diabetes NAFLD with diabetes NAFLD, but only compared pioglitazone with different drugs. We demonstrated the outcomes of pioglitazone in NAFLD patients on improvements in fibrosis, hepatocellular ballooning, lobular inflammation, and steatosis, which were similar to the results of the placebo group. The subgroup comparison results revealed the association of pioglitazone with significant improvements in hepatocellular ballooning, lobular inflammation, and steatosis both in NAFLD patients with diabetes and without diabetes compared with placebo. Though no significant improvements in fibrosis were found both in NAFLD patients with diabetes and without diabetes, it may be related to the relatively limited sample size, and both groups have trends of improvement.

Van et al. (50) reported that pioglitazone can improve liver biochemistry in mice deficient in phosphatidylethanolamine N-methyltransferase by activating PPARγ, which redirects the flux of fatty acids toward the adipose tissue away from the liver. Mahady et al. (49) concluded that thiazolidinediones can improve liver biochemistry by lowering ALT. In this review, we discovered the same effects of pioglitazone on improvements in both ALT and AST compared with diabetes NAFLD. The subgroup comparison results showed significant reductions in AST only in patients with diabetes (P = 0.003), while improvement was exhibited in the liver enzymes in both groups. The absence of statistical significance may be attributed to the high heterogeneity, limited sample size, and the calculated SD value.

The effect of pioglitazone on blood lipids varies among patients with NAFLD. Aithal et al. (36) confirmed the inhibitory role of pioglitazone in LDL but not in TC and HDL. Anushiravani et al. (37) concluded that pioglitazone can reduce LDL and TC. Pioglitazone can elevate plasma adiponectin levels, which is conducive to improving insulin sensitivity. We observed no significant differences in HDL, LDL, total cholesterol, triglycerides, FBS, and HOMA-IR between NAFLD patients with diabetes and those without diabetes who received pioglitazone therapy. Subgroup analysis showed a reduction of blood lipids to some extent in NAFLD patients with or without diabetes by pioglitazone. The higher baseline FBS values and greater room for improvement of patients with diabetes may affect the statistical results.

Pioglitazone serves as a prominent regulator of adipocyte differentiation and adipogenesis, which can lead to weight gain and obesity with chronic stimulation (51). Similar to previous results (42, 49, 51), in terms of variations in weight and BMI, we revealed significant differences in the two indexes between non-diabetes patients treated with pioglitazone and those with a placebo. However, an increase in weight can be found in NAFLD patients with diabetes, and the results showed no significant difference. The increase in weight caused by pioglitazone may be related to water–sodium retention and increased fat content (52, 53). These results still need to be studied with a larger sample size.

Drug safety is one of the key factors in the practicability of a drug. As a hypoglycemic drug, the application of pioglitazone in non-diabetes patients remains controversial. Some studies (54, 55) have suggested the contributed development of bladder cancer by the long-term use of pioglitazone, but others (56, 57) argued otherwise. A meta-analysis (58) revealed the increased risk of

congestive heart failure by the use of glitazones, and another article (59) indicated its contribution to the increased risks of bone fracture. However, the patients involved in these articles are mainly diabetes patients, who require a long-term administration of pioglitazone, and the observed patients are the same. In the meta-analysis, pioglitazone could be well tolerated, and no major adverse events were found in the relevant literature. We noticed no significant adverse effects between NAFLD patients who received pioglitazone therapy and those who received a placebo. No statistical significance was found in the specific adverse effects of most groups compared with the corresponding placebo group, including cancer, congestive heart failure, and bone fracture. The incidence of edema was found to be higher in the pioglitazone group than in the placebo group in NAFLD patients with diabetes. Although pioglitazone has the risk of causing water and sodium retention (60), however, the higher risk of edema in the diabetes group is more likely due to the combination of insulin use in most diabetes patients. Some studies suggest that pioglitazone combined with insulin has a significantly higher probability of edema than pioglitazone alone (60). Although pioglitazone may be associated with water and sodium retention, it can also reduce the risk of myocardial infarction and ischemic stroke (61). A small sample size and a relatively short follow-up time may not reveal the entire spectrum of side effects; thus, the side effects of pioglitazone on NAFLD patients require a larger sample size and a longer follow-up time to get relatively true results.

The limitations of the article are related to the research design and the biochemical and histological parameters. In terms of the research design, the doses of pioglitazone medication varied among studies (15 (37), 30 (36, 38–40), and 45 mg/day (11, 12)), as well as the treatment courses (3 (37), 6 (11, 40), 12 (36, 39), 18 (12), and 24 months (38)). Some studies implemented strict diet (11, 12, 36, 37, 39) and exercise (36, 39, 40) regimens, while some did not provide any information about lifestyle (38). In addition, the inclusion criteria were also inconsistent among studies: some trials enrolled only type 2 diabetics (40), while others also included prediabetics. Some prediabetic NAFLD patients may be included in the non-diabetic NAFLD patients. The proportion of gender differences between diabetes and non-diabetes patients was relatively different. As for the explanation of biochemical parameters, some articles did not cover the research indicators, accompanied by inconsistent units of results, and some studies did not list an average of changes before and after treatment, resulting in insufficiently accurate results. Due to the limitation of the number of studies, we included NASH and NAFLD for analysis. The involvement of both NAFLD (37, 39, 40) and NASH in the present study (11, 12, 36, 38) also enhanced the heterogeneity of the research.

In conclusion, this systematic review suggests the same efficacy of pioglitazone in non-diabetic and diabetic NAFLD patients in alleviating histopathology, liver enzymes, and HOMA-IR and reducing blood lipids. Furthermore, it did not elicit extra adverse effects. Large sample sizes and well-designed RCTs are required to further confirm these conclusions.

Data availability statement

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

Author contributions

YJ, MX and GL designed the study. ZW and HD performed the research and carried out the statistical analysis. YR, YZ, CM and HC wrote the manuscript. JT, CX and ML read and checked the paper. All authors co ntributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1111430/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Changes of liver function with pioglitazone **(A)** AST, **(B)** ALT.

SUPPLEMENTARY FIGURE 2
Changes in metabolism with Pioglitazone **(A)** HOMA-IR, **(B)** FBS.

SUPPLEMENTARY FIGURE 3
Changes in weight and BMI with pioglitazone **(A)** weight, **(B)** BMI.

SUPPLEMENTARY FIGURE 4
Adverse effects with pioglitazone.

SUPPLEMENTARY FIGURE 5
Sensitivity analysis in NAFLD patients with Diabete or Prediabetes based on **(A)** ALT, **(B)** FBS, **(C)** HDL, **(D)** HOMA-IR.

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Fatty liver mediates the association of hyperuricemia with prediabetes and diabetes: a weighting-based mediation analysis

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Background: Fatty liver, obesity, and dyslipidemia are associated with prediabetes or diabetes risk, and hyperuricemia co-exists. The present study evaluated the role of multiple mediators, namely, fatty liver, body mass index (BMI), and dyslipidemia, in the association between hyperuricemia and diabetes status.

Methods: Baseline data from the ongoing Fuqing cohort (5,336 participants) were analyzed to investigate the association of hyperuricemia with diabetes status using a multinomial logistic regression model. Furthermore, causal mediation analysis with the weighting-based approach was performed to estimate hyperuricemia's total natural direct effect (tnde), total natural indirect effect (tnie), and total effect (te) on prediabetes and diabetes risk, mediating jointly *via* fatty liver, BMI, and dyslipidemia.

Results: In multinomial analysis without considering mediators' effects, hyperuricemia was associated with a higher risk of prediabetes only (odds ratio: 1.25; 95% CI: 1.09–1.43; $p < 0.001$). When fatty liver, BMI, and dyslipidemia were considered as multiple mediators in the association, hyperuricemia was linked to both prediabetes [tnde: 1.11, 95% CI: 1.04–1.11; tnie: 1.07, 95% CI: 1.05–1.09; and overall proportion mediated (pm): 42%, 95% CI: 27%–73%] and diabetes risk (tnde: 0.96, 95% CI: 0.82–1.14; tnie: 1.25, 95% CI: 1.18–1.33; and pm: 100%, 95% CI: 57%–361%). Hyperuricemia showed significant tnde, te, and tnie, mediated by fatty liver jointly with dyslipidemia (pm = 17%) or BMI (pm = 35%), on prediabetes risk.

Conclusion: Hyperuricemia could increase prediabetes or diabetes risk, partially mediated by fatty liver, BMI, and dyslipidemia. Fatty liver is the crucial mediator in the association between hyperuricemia and prediabetes.

KEYWORDS

hyperuricemia (HUA), prediabetes, diabetes, multiple mediators, China

Highlights

• Fatty liver disease singly and combined with body mass index and/or dyslipidemia could mediate the association between hyperuricemia and diabetes. Therefore, fatty liver disease is a crucial mediator in this association.

• The present findings suggest further randomized control trials are needed to consider treatment options for asymptomatic hyperuricemia with higher BMI, dyslipidemia, and fatty liver to prevent prediabetes and diabetes risk.

• Clinicians should be cautious of hyperuricemic patients with higher BMI, dyslipidemia, and fatty liver to avoid the future risk of developing diabetes.

Introduction

Type 2 diabetes mellitus (T2DM) is a leading public health burden as the incidence and prevalence are substantial worldwide and even increasing. The International Diabetes Federation estimated that the number of T2DM patients worldwide was 463 million in 2019, and age-adjusted prevalence was 8.3% and expected to increase to 9.6% by 2045 among the age group 20–79 years (1). A recent national representative diabetes survey reported that the weighted prevalence of total diabetes, self-reported diabetes, newly diagnosed diabetes, and prediabetes was 12.8% [95% confidence interval (CI) 12.0%–13.6%], 6.0% (5.4%–6.7%), 6.8% (6.1%–7.4%), and 35.2% (33.5%–37.0%), respectively, among adults living in China (2). A varied range of risk factors, such as socioeconomic, dietary, lifestyle, environmental, and genetic factors, are under consideration for prediabetes and diabetes in different populations worldwide (3).

In recent decades, the incidence and prevalence of high serum uric acid (SUA) have increased worldwide. Although high SUA is causally linked to gout, evidence shows that it is also related to several chronic diseases, including kidney disease, diabetes, and cardiovascular diseases (4, 5). Several studies identified high SUA as an independent risk factor for T2DM, particularly among the Western population (6, 7); however, epidemiological studies reported conflicting results among the Asian population (8–10). For instance, recent cohort studies in China demonstrated that high SUA was linked to an increased risk of T2DM only in women (8, 11).

Overweight/obesity, dyslipidemia, and hypertension often co-exist with T2DM (12, 13) and are also related to high SUA levels (14). A national health survey showed a significant association between elevated SUA levels and the increased prevalence of abdominal obesity, hypertriglyceridemia, and hyperglycemia in the US population (15). A previous study reported that obesity could significantly mediate the association between hyperuricemia and diabetes risk (16), and body mass index (BMI) and dyslipidemia were significant mediators in the association only in women (11). However, the causal relationship between hyperuricemia and prediabetes or diabetes has yet to be explored. It is still unclear whether the increased prediabetes and diabetes risk due to elevated SUA is *via* multiple mediators like obesity, fatty liver, and dyslipidemia. Therefore, we aim to determine the mediating mechanism of their relationship *via* fatty liver disease, high BMI, and dyslipidemia. Also, our study evaluates both single and possible combinations of the mediators' effects on prediabetes and diabetes with the weighting-based mediation model approach.

Methods

Design and setting

The Fuqing cohort aims to investigate the natural history and risk factors of chronic non-communicable diseases, including cancer, diabetes, and fatty liver, among the Chinese population residing in the Southeast coastal region of China. The present study was based on the baseline data collected from the Fuqing cohort participants, which began on 14 July 2020. Seven thousand and nine individuals aged 35 to 75 years old and residing in the 23 rural villages of Gaoshan town were recruited for the study until 31 June 2021.

Participants

The current analysis excluded subjects with self-reported diabetes and undergoing-treatment diabetes or hyperuricemia cases, and a detailed description of the selection of study participants is presented in Supplementary Figure 1. Finally, the dataset for analysis included 5,336 participants (1,870 men and 3,466 women; median age of 57 years). Each participant was interviewed by trained staff using a structured electronic questionnaire, including socio-demographics, lifestyle and dietary habits, history of selected diseases and medication use, and family history of selected diseases. The interview was tape-recorded. The response rate of the Fuqing cohort was 48% for study participants. The ethical committee of Fujian Medical University approved this study [2017-07] and [2020-58], and all participants provided written informed consent before participation in the study.

Laboratory testing

Each serum sample was measured on an automatic biochemical analyzer (TBA-120FR, TOSHIBA, Japan) with reagents from DiaSys Co., Ltd (Golzheim, Germany). SUA was measured using an enzymatic colorimetric test with the uricase-peroxidase method, and its concentration was measured in mg/dl (1 mg/dl = 59.48 mmol/L). Serum total cholesterol (TC) and triglycerides (TG) were measured using a chromatographic enzymic method in the analyzer. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured using a homogeneous method. Serum creatinine was measured using a kinetic test.

Definition of outcome and exposure

Participants whose fasting blood glucose levels ≥ 7 mmol/L and/or glucose level after 2 h of oral glucose tolerance test (OGTT) ≥ 11.1 mmol/L and/or glycated hemoglobin A1c (HbA1c) ≥ 6.5% and/or the use of anti-diabetic drugs were classified as having type 2 diabetes, and non-diabetic individuals whose fasting blood glucose levels ≥ 5.6 mmol/L to < 7 mmol/L, or glucose level after 2 h of OGTT ≥ 7.8 mmol/L to < 11.1 mmol/L, or HbA1c ≥ 5.7 to < 6.5% were classified as prediabetes, according to American Diabetes Association criteria (17). Hyperuricemia was defined as SUA concentration > 7.0 mg/dl (416.4 μmol/L) for men or > 6.0 mg/dl (356.9 μmol/L) for women (18).

Definition, measurements, and classification of mediators

SUA is related to BMI and diabetes, and obesity is considered a mediator in the association between hyperuricemia and diabetes (16). A recent study showed that high BMI and dyslipidemia significantly mediated the association in women (11). Elevated SUA is significantly associated with hyperlipidemia (19) and a higher percentage of fat accumulation in the liver (20). Atherogenic dyslipidemia likely causes incident diabetes (21), and the fatty liver condition is an independent predictor of T2DM in several studies (22). Therefore, we considered high BMI, dyslipidemia, and fatty liver as mediators through which hyperuricemia could increase prediabetes or diabetes risk.

Height was measured, to the nearest 0.1 cm, without shoes, and weight was measured with an electronic bulk composition meter (580515, TANITA Corporation, Japan), to the nearest 100 g, without shoes and with light clothes. BMI was calculated as weight (in kilograms) divided by height (in meters squared). Dyslipidemia was defined as having either or a combination of serum TC ≥ 6.2 mmol/L, LDL-C ≥ 4.1 mmol/L, HDL-C < 1 mmol/L, TG ≥ 2.2 mmol/L, and self-reported use of lipid-lowering medication, according to the 2007 Chinese guidelines on the prevention and treatment of dyslipidemia (23). Likewise, non-alcoholic fatty liver disease (NAFLD) was diagnosed by experienced doctors using ultrasound images (ALOKA Prosound α7, Japan) and was divided into normal, mild, and moderate-to-severe.

Definition and classification of covariates

The trained staff took the participants' blood pressure measurements from their relaxed right arm, which was supported by a table with an electronic sphygmomanometer (OMRON U30 sphygmomanometer, OMRON Healthcare Co, Japan). Each participant was measured twice, and the average of the two measurements was used in the analysis. If the difference between the two measurements were > 5 mmHg, the third measurement was conducted and calculated as the average of two measurements with similar values. Participants whose average blood pressure levels were ≥ 140/90 mmHg or under anti-hypertensive medication were categorized as having hypertension (24). Smoking was categorized into never, past, and current smokers. Likewise, alcohol drinking was classified into never, past, and current users. Physical activities were measured as a metabolic equivalent (MET) per day. The estimated glomerular filtration rate (eGFR) was calculated using blood creatinine value; the calculation method was based on the chronic disease epidemiology method (25).

Causal mediation analysis

Causal inference methods for mediation analysis are an extension of the traditional approach. First, in the presence of exposure–mediator interaction, total effect (te) is decomposed into direct and indirect effects (controlled or natural) from a potential counterfactual outcomes framework; it develops estimations of these quantities that are not model specific. Second, causal mediation elucidates the primary assumptions to estimate direct and indirect effects, providing clarity to the no unmeasured confounding assumptions. Under the causal mediation approach, sensitivity analyses can be conducted to examine the robustness of findings to violations of these assumptions.

The controlled direct effect (cde) is the effect derived by the contrast between the counterfactual outcome if the individual were exposed at A = a and the counterfactual outcome if the same individual were exposed at A = a*, with the mediator set to a fixed level M = m. The natural direct effect (nde) is the contrast between the counterfactual outcome if the individual were exposed at A = a and the counterfactual outcome if the same individual were exposed at A = a*, with the mediator assuming whatever value it would have taken at the reference value of the exposure A = a*. The pure natural direct effect (pnde) is in the absence of reference interaction while the total natural direct effect (tnde) is the effect including reference interaction. The natural indirect effect (nide) is intuitively defined as the effect of the mediator in the absence of exposure. This effect is the contrast between the counterfactual outcome if the mediator assumed whatever value it would have taken at a value of the exposure A = a and the counterfactual outcome if the mediator assumed whatever value it would have taken at a reference value of the exposure A = a*. The pure natural indirect (pnie) effect is in the absence of mediator interaction, while the total natural indirect effect (tnie) is the effect including mediator interaction. The total effect not only is equal to the sum of the indirect and direct effects but also includes interaction if it exists. Proportion mediated (pm) is defined as the ratio of the total natural indirect effect to the total effect.

Several causal mediation analysis approaches are implemented, including the regression-based approach, the weighting-based approach, the inverse-odds-ratio-weighting approach, the natural effect model, the marginal structural model, and the g-formula approach. A regression-based method estimates the direct and indirect effects under a parametric assumption. It requires the model for the outcome, and the models for each of the mediators

are correctly specified; no model for the exposure is needed in the regression approach (26). In contrast, the weighting approach specifies correctly the model for the outcome and the model for the exposure; no models for the mediators are needed. In the regression approach, the model for the outcome, and the models for each of the mediators are required to be correctly specified, whereas no model for the exposure is needed (26). Although this approach deals with when the outcome is binary rather than continuous, it can be used if the mediators are binary (or if some are binary and some are continuous). This weighting approach can be used for any type of outcome, including non-rare binary outcomes; it can also be used regardless of whether there are exposure–mediator or mediator–mediator interactions (26). However, as with other weighting approaches, it works best when the exposure is binary or discrete with only a few levels. If there is a missingness problem in the outcome dataset, natural effect models can deal with it within the counterfactual framework (27).

Some more causal mediation approaches work in their principle and the assumptions under which effect values are calculated for time-varying variables. For example, the marginal structural model is designed to control for the effect of confounding variables that change over time and are affected by previous treatment (28). The parametric g-formula approach can accommodate both mediation and time-varying exposures, mediators, and confounders (29); thus, it constitutes a general approach to mediation analysis with time-varying exposures and mediators.

Statistical analyses

Continuous and categorical variables are presented as mean values ± standard deviation and frequencies with percentages, respectively. An independent two-sample *t*-test was used to test differences among participants with and without hyperuricemia for continuous variables. The difference in distribution for categorical variables was tested using χ^2 test. Multinomial logistic regression was performed to examine the association between hyperuricemia and the risk of prediabetes or diabetes, adjusting for potential confounding covariates, namely, age in years, sex (male/female), BMI, fatty liver (none/mild/moderate-to-severe), hypertension (yes/no), dyslipidemia (yes/no), eGFR, alcohol drinking (current/past/never), smoking (current/past/never), and physical activity metabolic equivalent (MET) per day. We used a weighting-based approach because of several reasons (1): our study mediators were binary, ordinal, and continuous (2); our outcome variable (prediabetes or diabetes) was not a rare disease (3); there were unequal distribution of covariates between those with hyperuricemia and without hyperuricemia.

We estimated cde, pnde, tnde, pnie, tnie, te, and pm; the mathematical formula for calculation has been explained in Supplementary File 1. The point estimate of each causal effect was obtained by imputing counterfactuals directly. The standard deviations of bootstrapped results are the standard errors of causal effects, and the percentiles of bootstrapped results get the causal effects' confidence intervals. A two-tailed *p*-value of 5% was considered statistically significant. We performed mediation analysis with the "CMAverse" R package, and all other statistical analyses were executed in R statistical software using its base packages.

Results

Prevalence and general characteristics

The proportions for prediabetes and diabetes were 44.3% and 11.5%, respectively, after excluding the previously diagnosed cases of diabetes and the participants under antidiabetic medication. We observed 48.5% and 12.9% prediabetes and diabetes among hyperuricemic while 42.2% and 10.8% prediabetes and diabetes, respectively, among normouricemic individuals; the difference in the distribution was significant ($\chi^2 = 34.3$, $p < 0.001$). The baseline characteristics of hyperuricemia status are presented in Table 1. Men and older participants had a higher chance of having hyperuricemia. Participants with higher BMI, hypertension, dyslipidemia, fatty liver, and lower eGFR were more likely to be hyperuricemic. Likewise, physical activity, smoking, and alcohol drinking were significantly associated with hyperuricemia. We evaluated correlation among exposure, mediators, and confounders with correlation matrix (Supplementary Figure 2) and principal component analysis (Supplementary Figure 3).

Multinomial analysis

We constructed two multinomial regression models: model 1 adjusted for covariates only (age, sex, hypertension, eGFR, alcohol drinking, smoking, and physical activity), and model 2 additionally adjusted for mediators (BMI, fatty liver, and dyslipidemia). In model 1, SUA level was significantly associated with increased risks for both prediabetes and diabetes. The estimates were attenuated mostly for diabetes in model 2 after further adjusting for mediators; the significant results were constrained to overall and women only. Likewise, hyperuricemic individuals had significantly increased risks of prediabetes or diabetes overall in model 1, while the estimates were attenuated in model 2 and only significant for prediabetes. Stratified analyses by sex showed similar patterns, but significant findings were mostly observed among women. With SUA quintile categories, significant associations were observed for prediabetes and diabetes in model 1, which became attenuated mainly in model 2, especially for diabetes. Stratified analyses by sex showed similar patterns, and again, significant findings were mostly found among women (Table 2). Furthermore, we stratified the analysis by age into middle-aged adults (<55 years) and older adults (55 years and over), and significant prediabetes risk was observed for the fifth quintile of SUA compared to the first quintile in the overall population (Supplementary Table 1).

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TABLE 1 Characteristics of participants by hyperuricemia status.

Variable	Category	Participants (%) or mean (SD)	Hyperuricemia		
			No (%)	Yes (%)	*p*-value
Age (years)		56.6 (9.8)	56.3 (9.8)	57.1 (9.9)	<0.001
Sex	Male	1,870 (35.0)	1,071 (30.1)	799 (44.9)	<0.001
	Female	3,466 (65.0)	2,484 (69.9)	982 (55.1)	
BMI (kg/m^2)		24.0 (3.2)	23.5 (3.0)	24.9 (3.3)	<0.001
Hypertension	No	2,931 (55.1)	2,061 (58.1)	870 (49.0)	<0.001
	Yes	2,388 (44.9)	1,484 (41.9)	904 (51.0)	
Dyslipidemia	No	3,557 (66.7)	2,512 (70.7)	1,045 (58.7)	<0.001
	Yes	1,779 (33.3)	1,043 (29.3)	736 (41.3)	
Fatty liver	No	3,610 (68.6)	2,651 (75.4)	959 (54.9)	<0.001
	Mild	1,213 (23.0)	674 (19.1)	539 (30.8)	
	Moderate-to-severe	441 (8.4)	192 (5.5)	249 (14.3)	
eGFR (ml/min/1.73 m^2)		96.5 (11.7)	98.1 (10.7)	93.4 (12.9)	<0.001
Physical activity (MET/day)		14.0 (13.0)	14.0 (13.1)	14.0 (12.6)	<0.001
Smoking	Never	3,935 (73.8)	2,742 (77.2)	1,193 (67.0)	<0.001
	Ex-smoker	467 (8.8)	263 (7.4)	204 (11.5)	
	Daily	928 (17.4)	546 (15.4)	382 (21.5)	
Alcohol drinking	Never	4,754 (89.2)	3,224 (90.8)	1,530 (85.9)	<0.001
	Former	170 (3.2)	102 (2.9)	68 (3.8)	
	Current	408 (7.6)	225 (6.3)	183 (10.3)	

SD, standard deviation; BMI, body mass index; eGFR, estimated glomerular filtration rate; MET, metabolic equivalent per day; kg, kilogram; m^2, meter squared; ml, milliliter; min, minute.

Mediation analysis

A directed acyclic graph was constructed considering fatty liver, BMI, and dyslipidemia as mediators in the association between hyperuricemia and diabetes status (Figure 1). All estimates (tnde: 1.11; 95% CI: 1.04, 1.18; tnie: 1.07; 95% CI: 1.05, 1.09; te: 1.18; 95% CI: 1.10, 1.25) were significant for prediabetes risk linked to hyperuricemia while only tnie (1.25; 95% CI: 1.18, 1.33) and te (1.25; 95% CI: 1.05, 1.49) were significant for diabetes risk. The corresponding pm were 42% (95% CI: 27%, 73%) and 100% (95% CI: 57%, 361%) for prediabetes and diabetes, respectively. In sex-wise subgroup analysis, hyperuricemia showed significant tnde only for prediabetes in women and tnie for prediabetes or diabetes risk in both sexes. However, pm was 48% (p = 0.008) in men for prediabetes but not significant in men for diabetes while 35% (p < 0.001) and 96% (p = 0.020) for prediabetes and diabetes in women, respectively (Table 3). In subgroup analysis among those less than 55 years (middle-aged adults) and equal to or over 55 years (older adults), tnie and pm were significant for prediabetes and diabetes in middle-aged adults. In further age–sex stratification, hyperuricemic middle-aged men and women had statistically significant tnie and pm for prediabetes and diabetes while tnie and pm were significant only in the prediabetes men (Supplementary Table 2).

In addition, we evaluated the effect of each mediator and the possible combination of mediators (Supplementary Table 3). Hyperuricemia showed significant tnde, te, and tnie, including pm, mediated by fatty liver jointly with dyslipidemia (pm = 17%) or BMI (pm = 35%), on prediabetes risk. In contrast, for diabetes risk, the only significant indirect effect was observed mediated by fatty liver disease singly or jointly with either BMI or dyslipidemia, while other mediation parameters were insignificant.

Effect modification with mediators

Hyperuricemia and SUA quintiles (p for linear trend = 0.001) were significantly associated with prediabetes among individuals with mild fatty liver disease compared to those with no fatty liver (p for interaction 0.172 for hyperuricemia and 0.073 for SUA quintiles). Hyperuricemia demonstrated a relatively higher prediabetes risk among people with fatty liver and normal blood lipid levels than individuals with no fatty liver and no dyslipidemia (p for interaction 0.047). Compared to the lower SUA quintile, the highest SUA quintile showed significant prediabetes risk among people with fatty liver and normal lipid levels (p for linear trend 0.017 and interaction 0.010) and fatty

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TABLE 2 Multinomial logistic analysis for prediabetes and diabetes risk in association with serum uric acid.

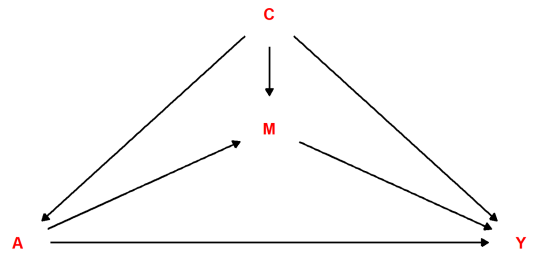
		Model 1		Model 2	
		Prediabetes OR (95% CI)	Diabetes OR (95% CI)	Prediabetes OR (95% CI)	Diabetes OR (95% CI)
Uric acid (continuous)					
Overall		1.003 (1.002, 1.004)***	1.004 (1.003, 1.005)***	1.002 (1.001, 1.003)***	1.001 (1.000, 1.003)*
Men		1.002 (1.001, 1.003)***	1.003 (1.001, 1.004)**	1.001 (1.000, 1.002)*	1.001 (0.999, 1.003)
Women		1.004 (1.003, 1.005)***	1.005 (1.004, 1.007)***	1.002 (1.001, 1.003)***	1.002 (1.000, 1.004)*
Hyperuricemia (yes *vs*. no)					
Overall		1.52 (1.33, 1.73)***	1.65 (1.35, 2.02)***	1.25 (1.09, 1.43)***	1.13 (0.91, 1.40)
Men		1.38 (1.12, 1.70)***	1.32 (0.94, 1.85)	1.19 (0.96, 1.48)	1.03 (0.73, 1.47)
Women		1.57 (1.32, 1.87)***	1.81 (1.40, 2.34)***	1.29 (1.07, 1.54)**	1.18 (0.90, 1.56)
Uric acid (higher quartile *vs*. the lowest quartile)					
Overall	Q2	1.34 (1.11, 1.62)***	1.44 (1.06, 1.96)*	1.24 (1.02, 1.50)*	1.26 (0.91, 1.73)
	Q3	1.37 (1.13, 1.66)***	1.67 (1.22, 2.27)***	1.16 (0.95, 1.41)	1.21 (0.88, 1.67)
	Q4	1.60 (1.31, 1.96)***	2.00 (1.46, 2.75)***	1.27 (1.03, 1.56)*	1.28 (0.92, 1.78)
	Q5	2.26 (1.82, 2.82)***	2.88 (2.05, 4.05)***	1.58 (1.26, 1.99)***	1.44 (1.00, 2.07)*
p for linear trend				<0.001	0.075
Men	Q2	1.19 (0.86, 1.62)	1.29 (0.76, 2.18)	1.10 (0.80, 1.52)	1.15 (0.67, 1.96)
	Q3	1.01 (0.73, 1.38)	1.53 (0.93, 2.54)	0.89 (0.64, 1.24)	1.26 (0.75, 2.12)
	Q4	1.32 (0.96, 1.83)	1.46 (0.86, 2.49)	1.11 (0.80, 1.55)	1.09 (0.63, 1.88)
	Q5	1.82 (1.31, 2.54)***	1.72 (0.99, 2.99)	1.41 (1.00, 1.98)*	1.05 (0.59, 1.87)
p for linear trend				0.08	0.837
Women	Q2	1.18 (0.94, 1.50)	1.20 (0.81, 1.77)	1.10 (0.87, 1.40)	1.03 (0.69, 1.53)
	Q3	1.31 (1.03, 1.66)*	1.65 (1.13, 2.41)**	1.14 (0.89, 1.45)	1.28 (0.86, 1.88)
	Q4	1.49 (1.17, 1.89)***	1.74 (1.18, 2.55)***	1.22 (0.95, 1.56)	1.13 (0.75, 1.69)
	Q5	2.09 (1.63, 2.68)***	2.72 (1.86, 3.98)***	1.53 (1.18, 1.98)***	1.40 (0.93, 2.10)
p for linear trend				0.001	0.120

OR, odds ratio; CI, confidence interval; Q, quintile; vs, versus.
*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
Model 1 (without adjustment for mediators) adjusted with age, smoking, alcohol drinking, log of physical activity, and in overall group also adjusted with sex.
Model 2 (with adjustment for mediators) further adjusted with fatty liver, body mass index, and dyslipidemia.

liver and dyslipidemia (*p* for linear trend 0.010 and interaction 0.182) than people with no fatty liver and no dyslipidemia. Hyperuricemia showed a higher prediabetes risk among people with fatty liver and non-obesity than people with no fatty liver and non-obesity (*p* for interaction 0.138). Compared to the lowest SUA quintile, the highest SUA quintile demonstrated significant prediabetes risk among people with fatty liver and non-obesity (*p* for linear trend 0.002 and interaction 0.169) and both fatty liver and obesity (*p* for linear trend 0.005 and interaction 0.987) compared to people with no fatty liver and non-obesity. Significant diabetes risk was observed with the highest SUA quintile compared to the lowest among individuals with fatty liver and obesity (*p* for linear trend 0.019 and interaction 0.019) than people with no fatty liver and non-obesity. The above results indicated that increased prediabetes risk was greater among people with fatty liver disease (Supplementary Tables 4–6).

Sensitivity analysis

The mediation estimates were likely influenced by unmeasured confounders, such as environmental exposure to toxicants, dietary factors, and a family history of diabetes; therefore, we performed a sensitivity analysis considering the effect of unmeasured confounders in the association. Overall, we observed high E-values for prediabetes and diabetes risk in men and women, which indicated that only relatively



A (exposure): hyperuricemia (yes/no)
M (mediator): body mass index, fatty liver, dyslipidemia
Y (outcome): diabetes status (normal/prediabetes/diabetes)
C (confounders not affected by the exposure): sex, age (years), hypertension, estimated glomerular filtration rate, alcohol drinking, smoking, physical activity

FIGURE 1
Directed acyclic graph for the combined mediating effect of fatty liver, body mass index, and dyslipidemia on the association between hyperuricemia and diabetes.

strong unmeasured confounders could change the reported effects (Supplementary Table 7).

Discussion

In a multinomial regression model without considering mediators in the association, we observed that hyperuricemia was significantly associated with a higher risk of prediabetes independent of age, sex, BMI, dyslipidemia, fatty liver, hypertension, eGFR, smoking, alcohol drinking, and physical activity. Furthermore, the association remained significant for prediabetic risk only in women; however, we did not find a significant association between hyperuricemia and increased diabetes risk.

In addition, we investigated the mediation mechanism of association between hyperuricemia and diabetes status: individual and the combined effects of BMI, dyslipidemia, and fatty liver were evaluated. We observed that BMI, dyslipidemia, and fatty liver jointly mediated the association. In the analysis considering these three mediators, hyperuricemia significantly increased the risk directly and indirectly for prediabetes or diabetes; the corresponding pm was 42% ($p < 0.001$) and 100% ($p = 0.008$), respectively. In sex-wise subgroup analysis, these mediators modulated the association significantly for prediabetes or diabetes risk in women (pm = 35% and 96%, respectively), while men had significant pm only for prediabetes (pm = 48%).

An elevated SUA level has been reported with an increased risk for diabetes and prediabetes in the Western population. For instance, cohort studies in the US (6), the Netherlands (7), and Germany (30) showed that hyperuricemia was an independent risk factor for prediabetes or diabetes. A meta-analysis also revealed a higher diabetes risk among hyperuricemic subjects, providing strong evidence that a higher SUA level is independent of other established risk factors for developing T2DM in middle-aged and older people (31).

In contrast, among the Asian population, including the Chinese people, mixed results were revealed (8–11). Using the multinomial logistic regression model, we observed that hyperuricemia was an independent risk factor for prediabetes but not diabetes among adults (aged between 35 and 75 years). The significant risk for prediabetes and not diabetes may be because our analysis excluded diagnosed cases of diabetes and individuals under antidiabetic medication. However, the finding agreed with a study that concludes that serum uric acid is more closely linked to early-phase mechanisms in the development of T2DM than late-phase mechanisms (7). The reported differences in the relative risk among the different gender and populations were probably partly due to the study's sample size, disease classification, lifestyle, dietary habits, and exposure to environmental conditions that interact with their genetic background. Also, there might be differences in the burden of comorbidities like hypertension, fatty liver, obesity, dyslipidemia, and kidney disease, which directly or indirectly were associated with hyperuricemia and diabetes prevalence.

In subgroup analysis, we demonstrated that hyperuricemic women had a higher chance of having prediabetes only. Recent large cohort studies in the Chinese population reported a higher risk of diabetes in hyperuricemic women but not men (8, 11). Likewise, women with higher uric acid were reported at a higher prediabetes and diabetes risk among the Japanese (9) and Korean populations (10). Elevated SUA levels independently increase prediabetes or diabetes risk among the younger population (6), indicating the potential causal role of SUA at an early age. We also stratified by age group into middle-aged adults (<55 years) and older adults (≥ 55 years) and observed that middle-aged adult women with hyperuricemia were at higher risk of diabetes. The reason might be the different biological pathways, including hormonal differences and the effect of confounders involved in the disease progress (hyperuricemia to diabetes).

By performing weighting-based mediation analysis, we found that higher BMI, fatty liver, and dyslipidemia jointly mediated the association between hyperuricemia and diabetes status. Although the indirect effects remained significant in men and women, the proportion mediated was significant for prediabetes and diabetes in women while only for prediabetes in men. Thus, the finding suggested that the effect of mediators was prominent in women with diabetes risk. In the further stratified analysis of the middle-aged and elderly population, we found that middle-aged women with hyperuricemia were more likely to have prediabetes or diabetes than their counterparts. In our study, excess diabetes risk in middle-aged women (<55 years) might be due to a larger proportion of women during the menopausal stage who might suffer from hormonal changes leading to higher uric acid levels (32). A previous study also revealed a significant correlation between SUA levels and metabolic syndrome, and the association was significant in premenopausal women compared to postmenopausal ones (33).

In addition, we evaluated the effect of individual mediators and the possible combination of mediators. When a single mediator was considered, the mediation effect was too small and insignificant. In opposition to our finding, Han et al. reported that BMI as a single mediator significantly mediated the association between hyperuricemia and diabetes; the mediation proportion was 20% (16). A recent cohort study demonstrated that high BMI and dyslipidemia partially mediated the association in Chinese adult women (11). However, both studies reported their findings

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TABLE 3 Prediabetes and diabetes causal risk associated with hyperuricemia based on weighted model jointly mediated by dyslipidemia, body mass index, and fatty liver.

Mediators' parameter	Prediabetes		Diabetes	
	Estimate	95%CI	Estimate	95%CI
Overall				
Controlled direct effect	1.17	(1.07, 1.29)**	1.05	(0.87, 1.29)
Pure natural direct effect	1.10	(1.03, 1.18)**	1.00	(0.84, 1.19)
Total natural direct effect	1.11	(1.04, 1.18)***	0.96	(0.82, 1.14)
Pure natural indirect effect	1.06	(1.04, 1.09)***	1.30	(1.23, 1.37)***
Total natural indirect effect	1.07	(1.05, 1.09)***	1.25	(1.18, 1.33)***
Total effect	1.18	(1.10, 1.25)***	1.25	(1.05, 1.49)**
Proportion mediated (%)	42	(27, 73)***	100	(57, 361)**
Men				
Controlled direct effect	1.17	(0.95, 1.40)	1.01	(0.71, 1.41)
Pure natural direct effect	1.08	(0.96, 1.20)	0.97	(0.73, 1.26)
Total natural direct effect	1.11	(0.99, 1.22)	0.92	(0.68, 1.23)
Pure natural indirect effect	1.04	(1.01, 1.08)*	1.19	(1.10, 1.28)***
Total natural indirect effect	1.07	(1.03, 1.10)***	1.13	(1.03, 1.24)**
Total effect	1.16	(1.03, 1.27)**	1.10	(0.82, 1.43)
Proportion mediated (%)	48	(22, 197)**	128	(-695, 849)
Women				
Controlled direct effect	1.16	(1.05, 1.29)**	1.06	(0.81, 1.32)
Pure natural direct effect	1.11	(1.03, 1.20)**	1.01	(0.80, 1.21)
Total natural direct effect	1.11	(1.03, 1.20)**	0.99	(0.80, 1.19)
Pure natural indirect effect	1.06	(1.03, 1.08)***	1.31	(1.21, 1.42)***
Total natural indirect effect	1.05	(1.02, 1.09)***	1.28	(1.18, 1.40)***
Total effect	1.17	(1.09, 1.27)***	1.30	(1.04, 1.56)*
Proportion mediated (%)	35	(15, 67)***	96	(54, 337)*

CI, confidence interval; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
Adjusted for age, sex, hypertension, estimated glomerular filtration rate, smoking, alcohol drinking, log of physical activity.

analyzing diabetes as a binary variable. A possible combination of two mediators, especially fatty liver with either BMI or dyslipidemia, significantly increased the effect of hyperuricemia on prediabetes risk, indicating that fatty liver condition has a crucial mediating role in the association. The previous study claimed a potentially causal impact of NAFLD on diabetes (34), which supported the idea that fatty liver was a primary mediator in the association.

The previous clinical and experimental studies have shown that higher uric acid mediates vascular changes leading to renal ischemia and renin–angiotensin system stimulation, promoting hypertension, hypertriglyceridemia, and hepatic steatosis through pro-oxidative mechanisms and ultimately the development of insulin resistance and decreased release of insulin leading to T2DM (35), which supports our proposed multiple mediation mechanism. Higher uric acid also augments reactive oxygen species production leading to the loss of transcription factors needed for insulin gene expression, eventually decreasing insulin production and secretion (36).

The present study has several limitations. First, we used cross-sectional data for the cause–effect analysis, which has several inherent study design drawbacks. Second, the traditional non-instrumental variable method for mediation analysis has its methodological problem, including bias due to confounding between exposure, mediator, and outcome. Simplifying some mediators like fatty liver and dyslipidemia into categorical variables introduced measurement error, which biases the indirect effect and thus mediated proportion towards the null. Therefore, the actual mediated proportion of the association between hyperuricemia and prediabetes or diabetes mediated by biological fatty liver and dyslipidemia might be higher than that reported in our study. Furthermore, we analyzed the effect of multiple

mediators in the association between hyperuricemia and diabetes status without considering the time effect and the confounder affected by exposures (hyperuricemia).

The previous research showed that persistent hyperuricemia at baseline to follow-up could better predict diabetes risk and cross-lag analysis shows the reverse relation of diabetes to the SUA level (8). Therefore, we analyzed the data excluding self-reported diabetes and individual undergoing-treatment for diabetes and hyperuricemia, possibly removing the effect of reverse causality. We used weighting-based mediation analysis to better predict the causal estimation in the scenario where some mediators like fatty liver and dyslipidemia were simplified into categorical variables. Considering that the estimates were likely to be influenced by unmeasured confounders like family history of diabetes, environmental exposure to toxicants, and dietary factors, we performed a sensitivity analysis that showed relatively large E-values, indicating that considerable unmeasured confounding would be needed to explain away an effect estimate.

Hyperuricemia is associated with higher prediabetes and diabetes risk among the Chinese population, partially mediated by higher BMI, dyslipidemia, and fatty liver. Increased diabetes and prediabetes risks were more prominent in women and middle-aged adults. Among the mediators considered, fatty liver jointly with either dyslipidemia or higher BMI had a robust mediating effect in the association. The findings suggest that further randomized controlled trials are needed to consider treatment options for asymptomatic hyperuricemia, with higher BMI, dyslipidemia, and fatty liver to prevent prediabetes and diabetes risk. Finally, the clinician should be cautious of hyperuricemic patients with higher BMI, dyslipidemia, and fatty liver to avoid the future risk of developing diabetes.

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 ethical committee of Fujian Medical University approved this study [2017-07] and [2020-58]), and all participants provided written informed consent before participation in the study. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TB and WY contributed to the conception and design of the work. TB, RF, SD, JG and JMG contributed to the task's acquisition, analysis, or interpretation of data. TB drafted the manuscript. TB, RF, SD, JG, and WY critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1133515/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Flowchart diagram of selection of study subjects.

SUPPLEMENTARY FIGURE 2
Correlation matrix for exposure, mediators, confounders, mediators, and outcome variables under study.

SUPPLEMENTARY FIGURE 3
Principal component analysis of the study variables (exposure, confounders, mediators, and outcome).

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The change of non-alcoholic fatty liver disease is associated with risk of incident diabetes

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Background & aims: The effect of change in non-alcoholic fatty liver disease (NAFLD) status on incident diabetes has not been well studied. We aimed to investigate the association of NAFLD development and remission with the risk of incident diabetes during a median of 3.5-year follow-up.

Methods: A total of 2690 participants without diabetes were recruited in 2011-2012 and assessed for incident diabetes in 2014. Abdominal ultrasonography was used to determine the change of NAFLD. 75 g oral glucose tolerance test (OGTT) was performed to determine diabetes. NAFLD severity was assessed using Gholam's model. The odds ratios (ORs) for incident diabetes were estimated by logistic regression models.

Results: NAFLD was developed in 580 (33.2%) participants and NAFLD remission occurred in 150 (15.9%) participants during a median of 3.5-year follow-up. A total of 484 participants developed diabetes during follow-up, including 170 (14.6%) in consistent non-NAFLD group, 111 (19.1%) in NAFLD developed group, 19 (12.7%) in NAFLD remission group, and 184 (23.2%) in sustained NAFLD group. The development of NAFLD increased the risk of incident diabetes by 43% (OR, 1.43; 95%CI, 1.10-1.86) after adjustment for multiple confounders. Compared with sustained NAFLD group, remission of NAFLD reduced the risk of incident diabetes by 52% (OR, 0.48; 95%CI, 0.29-0.80). The effect of NAFLD alteration on incident diabetes was not changed after adjustment for body mass index or waist circumference, change of body mass index or waist circumference. In NAFLD remission group, participants with non-alcoholic steatohepatitis (NASH) at baseline were more likely to develop diabetes (OR, 3.03; 95%CI, 1.01-9.12).

Conclusions: NAFLD development increases the risk of incident diabetes, whereas NAFLD remission reduces the risk of incident diabetes. Moreover, presence of NASH at baseline could attenuate the protective effect of NAFLD remission on incident diabetes. Our study suggests that early intervention of NAFLD and maintenance of non-NAFLD are important for prevention of diabetes.

KEYWORDS

non-alcoholic fatty liver disease, incident diabetes, obesity, type2 diabetes mellitus (T2DM), prevention

Introduction

Type 2 diabetes mellitus (T2DM) poses a serious challenge for human health due to complicated cardiovascular diseases and mortality (1). The prevalence of diabetes is rapidly increased (2, 3), therefore, it is urgent to identify risk factors for incident diabetes in order to prevent major complications. Accumulating evidence has demonstrated that non-alcoholic fatty liver disease (NAFLD) is emerging as a leading cause of chronic liver disease worldwide in the past two decades (4). The close association of NAFLD and diabetes has been well determined. In patients with diabetes the prevalence of NAFLD is as high as 40-70% (5) and NAFLD patients are usually accompanied with impaired glucose metabolism as well (6, 7). A long-term effect of NAFLD on incident T2DM risk has been reported. A 19-year cohort study reported that the risk of T2DM was increased by 11.7 folds in NAFLD subjects as compared to the general population (8). Sinn Dong Hyun reported that NAFLD subjects with either normal weight or overweight/obesity was an independent risk for incident diabetes (9). Of note, the co-existence of NAFLD and diabetes results in worse hepatic injury, as the presence of diabetes accelerates the progression of simple fatty liver to steatohepatitis, cirrhosis, and hepatocellular carcinoma (10). Moreover, unfavorable extrahepatic disease risks should be highlighted. The co-existence of NAFLD in patients with diabetes leads to an increased risk of chronic kidney disease (1.87-fold), cardiovascular disease (1.96-fold), and cardiovascular mortality (3.46-fold), imposing a heavy burden on global healthcare systems (11–14).

NAFLD can be dynamic across the lifespan, changing from remission to worsening. As the pathophysiology of the association between NAFLD development and incident diabetes has been well illustrated, which involves insulin resistance, increased lipogenesis, overproduced hepatic glucose, and dysregulated hepatokines thus contributing to β-cell dysfunction, the change in NAFLD status might modify the risk of diabetes (15, 16). Several previous studies have proved that the risk of incident diabetes was increased with the development of fatty liver and worsening of fatty liver (17). However, the effect of remission of NAFLD on incident diabetes has not been well studied. As NAFLD could be ameliorated by clinical intervention (18, 19), targeting the effect of the change in NAFLD, especially the improvement of NAFLD might be important for diabetes prevention.

In the present study, we explored whether the development and remission of NAFLD increased and reduced the risk of incident diabetes in a prospective cohort.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; OGTT, oral glucose tolerance test; ORs, odds ratios; NASH, non-alcoholic steatohepatitis; T2DM, type 2 diabetes mellitus; BMI, body mass index; WC, waist circumference; TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; SD, standard deviations; ANOVA, one-way analysis of variance.

Materials and methods

Subjects and study design

Our cohort study was conducted in the Chongming District, Shanghai and the detailed information about study design, eligibility criteria, and sampling has been described previously (20). In brief, a total of 9930 participants received a baseline survey from 2011 to 2012 and 7707 participants completed the follow-up survey in 2014. In our present study, 3577 subjects who had complete baseline and follow-up information were included. Those individuals with diabetes at baseline (n=771), a history of known liver disease including viral or autoimmune hepatitis, liver cancer, or cirrhosis (n=35), abusing alcohol (alcohol consumption >140 g/week in men or >70 g/week in women, n=75), or missing information of fatty liver (n=6) were excluded. Finally, 2690 participants were included for this analysis. Our prospective cohort study was approved by the Ethical Committee of Zhongshan Hospital, Fudan University, and each participant was provided with a written informed consent.

Clinical and laboratory evaluation

Standard questionnaires were employed to obtain the information about demographic characteristics, lifestyles, history of diseases and medication on site conducted by trained investigators. Body weight and height were obtained in light clothes and bare feet to the nearest 0.1 kg and 0.1 cm, respectively. Body mass index (BMI) was derived from weight in kilograms divided by square of height in meters. Waist circumference (WC) was measured at the level of umbilicus in a standing position. Blood pressure was measured on non-dominant arm at a seated position, three times consecutively with 1-min rest and 10-min interval using an automated electronic sphygmomanometer (OMRON Model HEM-752 FUZZY' Omron Co., Dalian, China). The average value of three readings was used. Current smokers were defined as participants regularly consuming cigarettes (duration> 6 months) right before the survey. Former smokers were defined as participants with a history of cigarettes consuming for longer than 6 months and having quitted smoking at the time of survey. Similarly, current drinkers were defined as participants regularly consuming alcohol (duration > 6 months) right before the survey. Former drinkers were defined as participants with a history of alcohol consuming for longer than 6 months and having quitted drinking at the time of survey.

Blood samplings were done two times, one at baseline and another at the 3.5-year follow-up. Fasting venous blood samples were collected after at least 10-h fasting. Serum triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), alanine aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) were measured on the auto analyser (Modular E170, Roche).

Diabetes definition

A 75g oral glucose tolerance test (OGTT) was conducted and blood samples at 0h and 2h after glucose load were collected. Fasting blood glucose (FBG) and 2-h post-load glucose levels were measured using glucose oxidase method on an auto analyser (Modular P800, Roche). Serum insulin was measured by an electrochemiluminescence assay (Modular E170, Roche). The homeostasis model assessment of insulin resistance index (HOMA_IR) was calculated as fasting insulin (μIU/ml) × fasting glucose (mmol/L)/22.5. Glycated hemoglobin (HbA1c) was measured by high-performance liquid chromatography. According to American Diabetes Association 2010 criteria, diabetes mellitus was defined as 1) self-reported doctor-diagnosed diabetes or taking antidiabetic medications, and/or 2) FBG levels ≥ 7.0 mmol/L and/or, 3) 2h post-load glucose levels ≥ 11.1 mmol/L, and/or 4) HbA1c concentration ≥ 6.5% (48mmol/mol). In the absence of unequivocal hyperglycemia, diagnosis requires two abnormal test results from the same sample or in two separate test samples.

NAFLD definition

NAFLD was diagnosed by ultrasonography with exclusion of a history of known liver diseases. Liver ultrasonography was operated by two specialists who were blinded to clinical data using a high-resolution B-mode tomographic ultrasound system (Esaote Biomedica SpA, Italy) equipped with a 3.5-MHz probe. Fatty liver was defined as the presence of at least two of the following three findings: 1) diffusely increased echogenicity of the liver relative to kidney; 2) ultrasound beam attenuation; 3) poor visualization of intrahepatic structures. The definitions for NAFLD development were absence of NAFLD at baseline and presence of NAFLD at the end of follow-up, NAFLD remission presence of NAFLD at baseline and absence of NAFLD at the end of follow-up, consistent non-NAFLD absence of NAFLD at baseline till the end of follow-up and sustained NAFLD presence of NAFLD at baseline till the end of follow-up. Non-invasive NAFLD scores was used to assess the non-alcoholic steatohepatitis (NASH). Gholam's model was calculated as 2.627 * ln AST + 2.13 for diabetics, with a cut-off for predicting NASH of 8.22 (21, 22).

Statistical analysis

Normally distributed continuous variables were presented as means with standard deviations (SDs), whereas skewed distributed continuous variables were presented as geometrical median and interquartile range. Continuous variables were compared by student *t* tests and one-way analysis of variance (ANOVA), whereas skewed distributed variables were compared by Mann Whitney U and Kruskal Wallis tests. Categorical variables were expressed as proportions and compared across groups using chi-square tests or fisher exact test. The unadjusted and multivariate adjusted logistic regression analyses were performed to investigate the odds ratios of new development and remission of NAFLD on the risk of incident diabetes. In the NAFLD remission group, logistic regression analysis was further performed to compare the risk of incident diabetes between subjects with or without steatohepatitis at baseline. Statistical analyses were performed on SPSS version 26 (IBM Corp., Armonk, NY). A two-sided p value less than 0.05 was considered as statistical significance.

Results

Baseline characteristics of participants with and without incident type 2 diabetes

The present study included 2690 participants free of diabetes at baseline from 2011 to 2012, and followed up in 2014. Diabetes developed in 484 subjects (18.0%). The baseline characteristics of participants by incident diabetes at follow-up were shown in Table 1. Participants who developed diabetes were older ($p = 0.006$), had higher BMI and WC, higher concentrations of TC, TG (all $p < 0.0001$) and LDL-C ($p = 0.01$) at baseline. The incidence of diabetes was 21.5% in subjects with presence of NAFLD at baseline and 16.1% in subjects without NAFLD at baseline (21.5% VS 16.1%, $p < 0.0001$).

The association of NAFLD alteration with incident diabetes

Table 2 showed the change of NAFLD during 3.5-year follow-up. Of 1746 non-NAFLD subjects at baseline, 580 (33.2%) participants developed NAFLD and 1166 (66.8%) was consistently free of NAFLD throughout the follow-up. Of 944 NAFLD subjects at baseline, 150 participants (15.9%) had NAFLD remission and 794 (84.1%) participants had sustained NAFLD. We then investigated the association of NAFLD alteration and incident diabetes. 170 of 1166 (14.6%) participants with consistent non-NAFLD developed diabetes, whereas 184 of 794 (23.2%) participants with sustained NAFLD developed diabetes. In contrast, 111 of 580 (19.1%) subjects with NAFLD development developed diabetes, and 19 of 150 (12.7%) subjects with NAFLD remission developed diabetes.

The risk for incident diabetes according to NAFLD alteration by logistic regression analysis

Table 3 showed the baseline clinical and biochemical characteristics according to NAFLD alterations during 3.5-year follow-up. The subjects with consistent non-NAFLD were younger by age, had lower BMI, WC, blood pressure, plasma glucose, TG, and higher HDL-C, whereas sustained-NAFLD group was older and had higher BMI, WC, blood pressure, plasma glucose, insulin resistance, and more adverse lipid

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TABLE 1 Baseline characteristics of participants with and without incident type 2 diabetes: demographics and laboratory values.

	Non-Diabetes (n=2206)	Incident Diabetes (n=484)	P value
Age, y	55 ± 8	56± 8	0.006
Gender (male/female)	586/1620 (27%)	150/334 (31%)	0.048
Smoking status, n (%)			
Current smoker	178 (8.1%)	33 (6.8%)	0.36
Former smoker	70 (3.2%)	11 (2.3%)	
Never smoker	1958 (88.8%)	440 (90.9%)	
Drinking status, n (%)			
Current drinker	74 (4.4%)	15 (3.7%)	0.69
Former drinker	230 (15.6%)	50 (14.9%)	
Never drinker	1264 (79.9%)	253 (81.4%)	
BMI, kg/m^2	24.1 ± 3.3	24.7± 3.4	<0.0001
WC, cm	82.2 ± 9.5	84.0 ± 10.1	<0.0001
SBP, mmHg	126 ± 17	131± 17	<0.0001
DBP, mmHg	79 ± 10	81 ± 10	<0.0001
Lipids			
Total cholesterol, mmol/L	4.40 ± 0.99	4.57 ± 1.05	<0.0001
Triglycerides, mmol/L	1.22 (0.89-1.74)	1.36 (0.97-1.92)	<0.0001
LDL-C, mmol/L	2.48 ± 0.74	2.58± 0.76	0.01
HDL-C, mmol/L	1.19 ± 0.30	1.20 ± 0.32	0.75
NAFLD at baseline (%)			
Yes	741 (33.6%)	203 (41.9%)	<0.0001
No	1465 (66.4%)	281 (58.1%)	

Data are presented as mean ± SD, number and percentage, or median (IQR). Continuous variables were compared by student t tests, skewed distributed variables were compared by Mann Whitney U tests, categorical variables were compared by chi-square tests. A two-sided p value < 0.05 was considered as statistical significance. BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease.

metabolism at baseline (all p < 0.0001). There were no significant differences in smoking or drinking status across four groups.

Then logistic regression analyses were performed to study the effect of NAFLD alteration on the risk of incident diabetes (Figure 1). After adjustment for age, gender, smoking and drinking status, subjects with NAFLD development had a significantly higher risk for diabetes as compared with sustained non-NAFLD group (OR, 1.43; 95%CI, 1.10-1.86). The risk was not changed after further adjustment for BMI (OR, 1.36; 95%CI, 1.03-1.79) or WC (OR, 1.38; 95%CI, 1.05-1.81). After adjustment for age, gender, smoking and drinking status, subjects with remission of NAFLD had a significantly decreased risk for diabetes as compared with sustained NAFLD (OR, 0.48; 95%CI, 0.29-0.80). The decreased risk was not changed after further adjustment for BMI (OR, 0.49; 95%CI, 0.30-0.83) or WC (OR, 0.49; 95%CI, 0.29-0.82). Since change in NAFLD status is always accompanied with change of BMI or WC, and meanwhile BMI and WC have strong associations with incident diabetes, therefore we assessed the risk after adjustment for BMI change and WC change in the existing model, respectively. The results showed that the association of change of NAFLD status with incident diabetes was independent of the change of BMI and WC (Figure 2).

The association between baseline NAFLD severity and risk of incident diabetes in NAFLD remission group

In order to investigate what might contribute to the incidence of diabetes in subjects whose NAFLD remitted, we then calculated the Gholam's model to assess their NAFLD severity at baseline. 27 of 150 (18.0%) subjects were identified with NASH at baseline. The incidence of diabetes in subjects with NASH at baseline was obviously higher than in those without NASH at baseline (25.9% VS 9.8%, p=0.048) (Supplemental Table 1). In the age, gender adjusted- logistic model, presence of NASH at baseline increased risk of incident diabetes in participants with NAFLD remission (OR, 3.08; 95%CI, 1.05-8.99). After further adjustment for smoking

TABLE 2 (A) NAFLD status at baseline and follow-up.

Baseline NAFLD status	Follow-up NAFLD status		P value
	No NAFLD (n=1316)	NAFLD (n=1374)	
No NAFLD (n=1746)	1166/1746 (66.8%)	580/1746 (33.2%)	<0.0001
NAFLD (n=944)	150/944 (15.9%)	794/944 (84.1%)	

Data are presented as number and percentage. P values was compared among groups using chi-square test. P value < 0.05 was defined as statistically significant.

(B) Incident diabetes according to baseline and follow-up NAFLD status

NAFLD status at baseline and follow-up	No. of cases/total	Incidence rate	P value
Sustained non-NAFLD	170/1166	14.6%	0.015
New NAFLD	111/580	19.1%	
NAFLD remission	19/150	12.7%	0.004
Sustained NAFLD	184/794	23.2%	

Data are presented as number and proportion. P values were compared across groups sustained non-NAFLD VS new NAFLD; NAFLD remission VS sustained NAFLD using chi-square tests. P value < 0.05 was defined as statistically significant.

and drinking status, and baseline BMI, the association persisted (OR, 3.03; 95%CI, 1.01-9.12) (Table 4).

Discussion

NAFLD is indicative of intrahepatic triglyceride accumulation and strongly associated with diabetes (15) and cardiovascular disease (23). Previous studies have indicated that NAFLD patients were more likely to have impaired glucose regulation and to develop type 2 diabetes (5, 6). Park SK et al. have revealed that, compared to non-NAFLD participants, mild to moderate NAFLD patients increased the risk of incident diabetes by 42% and moderate to severe NAFLD increased the risk of incident diabetes by 158% in 5-year follow-up. The associations were independent of age, BMI, smoking status, regular exercise or family history of diabetes (24). Given that liver fat content is variable, NAFLD status can change from remission to worsening. As the pathophysiology of the interplay between NAFLD and incident diabetes has been elucidated, the change in NAFLD status might modify the risk of incident diabetes. However, the association of the change of NAFLD status, especially the NAFLD remission with incident diabetes has not been well studied.

Our present study showed that new development of NAFLD increased the incident diabetes, in accordance with previous studies (25, 26). Yamazaki H et al. reported that NAFLD remission reduced the risk of incident diabetes (25), whereas, the association was not observed by Sung KC et al., probably due to they adopted different controls, the former focused on whether NAFLD remission reduced the risk of incident diabetes, and the latter focused on whether people had an increased risk of diabetes even if NAFLD resolved (27). In our study, NAFLD remission markedly decreased the incident diabetes compared with sustained NAFLD. Since NAFLD status was changeable, and NAFLD remission reduced risk of incident diabetes, targeting the improvement of NAFLD might be important to prevent diabetes. NAFLD could be ameliorated by lifestyle intervention, including lifestyle modification and physical exercise, medications, and bariatric surgery as well (18, 19, 28, 29). Petersen KF et al. reported that 8% of body weight loss by caloric restriction could reverse NAFLD and hepatic insulin resistance and further normalized plasma glucose levels in patients with diabetes (30). Taylor R et al. demonstrated that removal of excess intrahepatic fat *via* substantial weight loss can normalize hepatic insulin responsiveness, which was required remission in human type 2 diabetes (31). They revealed that both fatty liver and diabetes were closely associated with hepatic insulin resistance and speculated that fatty liver played a central role in the progression of diabetes (32).

Our data indicated that remission of NAFLD reduced the risk of incident diabetes, which might be explained by: 1) the improvement of hepatic insulin resistance; 2) alteration of hepatokine production, such as a reduction of fetuin A levels (33). Liver fat content is an important regulator of hepatic insulin sensitivity, and hepatic insulin sensitivity was found to be a strong predictor of glucose tolerance. And decreased liver fat is always accompanied by a decrease in serum Fetuin A levels. Fetuin A can induce insulin resistance by interruption of insulin receptors and activation of toll-like receptors (34).

However, there were still a proportion of subjects developing diabetes even though their NAFLD remitted. A meta-analysis in 501,022 adult individuals showed that patients with more 'severe' NAFLD were also more likely to develop incident diabetes (17). Similarly, we found in participants with NAFLD remission, those predicted to have NASH at baseline were more likely to develop diabetes. This indicated that increased severity of NAFLD

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TABLE 3 Baseline characteristics of the cohort stratified by NAFLD status at baseline and at follow up.

	NAFLD status				
	Sustained non-NAFLD	New NAFLD	Remission of NAFLD	Sustained NAFLD	P for trend
Age, y	55 ± 8	54 ± 8	56 ± 8	56 ± 7	<0.0001
Gender (male/female, male%)	343/823 (29%)	132/448 (23%)	37/113 (25%)	224/570 (28%)	0.023
Smoking status, n (%)					
Current smoker	97 (8.3%)	45 (7.8%)	8 (5.3%)	61 (7.7%)	0.78
Former smoker	40 (3.4%)	17 (2.9%)	4 (2.7%)	20 (2.5%)	
Never smoker	1029 (88.3%)	518 (89.3%)	138 (92.0%)	713 (89.8%)	
Drinking status, n (%)					
Current drinker	50 (4.3%)	24 (4.1%)	4 (2.7%)	38 (4.8%)	0.90
Former drinker	177 (15.2%)	94 (16.2%)	21 (14.0%)	125 (15.7%)	
Never drinker	939 (80.5%)	462 (79.7%)	125 (83.3%)	631 (79.5%)	
BMI, kg/m^2	22.4 ± 2.5	24.5 ± 2.7	25.1 ± 2.7	26.7 ± 3.2	<0.0001
WC, cm	78 ± 8	83± 8	86 ± 9	89 ± 8	<0.0001
SBP, mmHg	124 ± 18	126 ± 17	127 ± 17	131 ± 17	<0.0001
DBP, mmHg	77 ± 10	79 ± 9	80 ± 10	82 ± 10	<0.0001
Lipids					
Total cholesterol, mmol/L	4.37 ± 0.99	4.34 ± 0.99	4.55 ± 1.12	4.56 ± 1.00	<0.0001
Triglycerides, mmol/L	1.01 (0.78-1.40)	1.25 (0.92-1.82)	1.34 (0.98-1.81)	1.64 (1.20-2.31)	<0.0001
LDL-C, mmol/L	2.46 ± 0.74	2.43 ± 0.69	2.62 ± 0.85	2.58 ± 0.75	<0.0001
HDL-C, mmol/L	1.27 ± 0.32	1.15 ± 0.28	1.15 ± 0.29	1.11 ± 0.26	<0.0001
FBG, mmol/L	5.52 ± 0.51	5.56 ± 0.53	5.73 ± 0.54	5.72 ± 0.53	<0.0001
2h-BG, mmol/L	6.56 ± 1.62	7.03 ± 1.54	7.00 ± 1.68	7.59 ± 1.58	<0.0001
HbA1c, %	5.66 ± 0.37	5.71 ± 0.36	5.75 ± 0.35	5.81 ± 0.35	<0.0001
HbA1c, mmol/mol	38 ± 4.1	39 ± 4.0	39 ± 3.8	40 ± 3.8	<0.0001
HOMA_IR	1.32 (0.96-1.71)	1.63 (1.31-2.19)	1.79 (1.29-2.33)	2.33 (1.74-2.93)	<0.0001

Data are presented as mean ± SD, number and percentage, or median (IQR). Continuous variables were compared by one-way analysis of variance (ANOVA). Skewed distributed variables were compared by Kruskal Wallis tests. Categorical variables were compared by chi-square tests. A two-sided p value < 0.05 was considered as statistical significance. BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease.

(ie. NASH) at baseline could attenuate the protective effect of NAFLD remission. Therefore, early intervention of NAFLD is important.

Strengths and limitations

The strengths of the present study are as follows. First, we focused on change in NAFLD status as effects of alcohol consuming and other liver diseases were ruled out. We conducted a well-designed longitudinal cohort and reported the effect of NAFLD status change, including new development and remission of NAFLD on incident diabetes in a 3.5-year Chinese cohort population for the first time. Third, standardized collection of covariates allowed for adjustment for potential confounders. We also have some limitations. First, the study was performed in middle-aged and older Chinese population and cannot be generalized to adolescent or other ethnical populations. Second, NAFLD was determined by ultrasonography, which had limited sensitivity to detect low-level liver fat, limiting the generalizability of our study to earlier stages of NAFLD. NASH were assessed by non-invasive score instead of gold-standard hepatic biopsy. Third, diagnoses of diabetes and NAFLD were only made at baseline and the 3.5-year follow-up, so it might not differentiate which one developed first, and an annual screening for incident diabetes could be helpful.

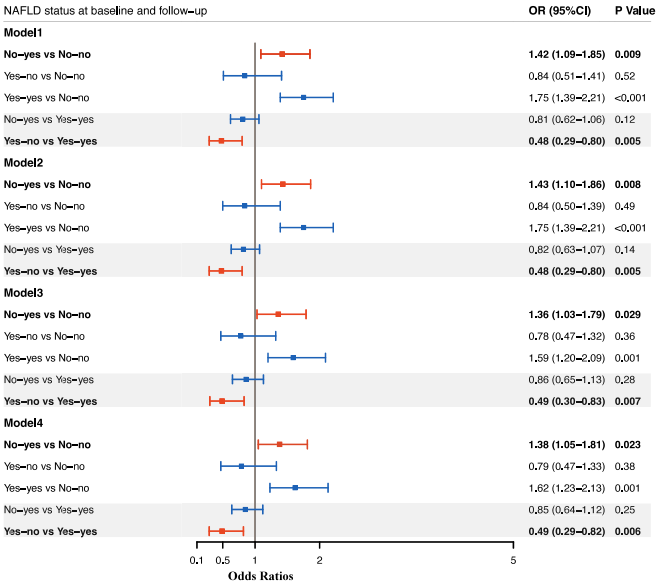

FIGURE 1
Odds ratios for incident diabetes according to change in NAFLD status between baseline and follow-up. Data are presented as Odds ratios (ORs), and the corresponding 95%CI in each group. Logistic regression models were used to estimate the ORs, 95% CIs, and P values. Model 1: adjusted for age, gender; Model 2: adjusted for age, gender, smoking and drinking status; Model 2: adjusted for age, gender, smoking and drinking status, baseline BMI; Model 4: adjusted for age, gender, smoking and drinking status, baseline WC. No-yes: absence of NAFLD at baseline but presence of NAFLD at follow-up; No-no: absence of NAFLD at baseline till the follow-up; Yes-no: presence of NAFLD at baseline but absence of NAFLD at follow-up; Yes-yes: presence of NAFLD at baseline till the follow-up.

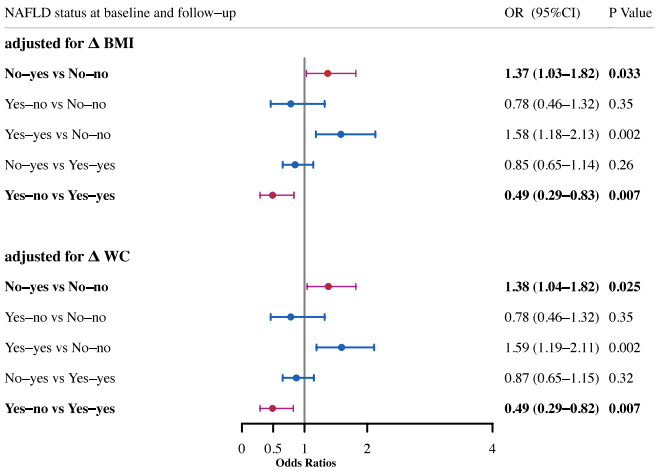

FIGURE 2
Odds ratios for incident diabetes after adjustment for change of BMI and WC. All adjusted for age, sex, smoking and drinking status, baseline BMI and baseline WC.

TABLE 4 Odds ratios for incident diabetes according to Gholam's model assessment at baseline in the NAFLD remission group.

NAFLD severity at baseline	No. of cases/controls	Model 1		Model 2	
		OR (95% CI)	P value	OR (95% CI)	P value
Gholam's <8.22	7/20	Ref.		Ref.	
Gholam's >8.22	12/111	3.08 (1.05-8.99)	0.040	3.03 (1.01-9.12)	0.048

Model1: adjusted for age, gender;
Model2: adjusted for age, gender, smoking and drinking status, baseline BMI.

Conclusion

In conclusion, the change of NAFLD is associated with the change of risk of diabetes. NAFLD development increases the risk of incident diabetes, whereas NAFLD remission decreases the risk of incident diabetes, after adjustment for multiple potential confounders. Moreover, presence of NASH at baseline could attenuate the protective effict of NAFLD remission on incident diabetes. Therefore, our study indicates that early intervention of NAFLD and maintenance of non-NAFLD are important for prevention of diabetes.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Ethical Committee of Zhongshan Hospital, Fudan University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LQ and XL contributed to the conception and design of the study. ZY, and YF contributed to the acquisition of data. CC, and YZ analyzed the data. CC wrote the manuscript. QS reviewed and revised the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1108442/full#supplementary-material

SUPPLEMENTARY TABLE 1
Changes in clinical parameters of participants stratified by NAFLD status at baseline and at follow up.

SUPPLEMENTARY TABLE 2
Incident diabetes according to Gholam's model assessment at baseline in the NAFLD remission group. Data are presented as number and percentage. P values was compared among groups using chi-square test. P value < 0.05 was defined as statistically significant.

SUPPLEMENTARY TABLE 3
Incident diabetes according to BARD score assessment at baseline in the NAFLD remission group. Data are presented as number and percentage. P values was compared among groups using chi-square test. P value < 0.05 was defined as statistically significant.

SUPPLEMENTARY TABLE 4
Incident diabetes according to BAAT score assessment at baseline in the NAFLD remission group. Data are presented as number and percentage. P values was compared among groups using chi-square test. P value < 0.05 was defined as statistically significant.

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Non-alcoholic fatty liver disease and incidence of microvascular complications of diabetes in patients with type 2 diabetes: a prospective cohort study

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Objective: To investigate the association between non-alcoholic fatty liver disease (NAFLD) and liver enzymes with the incidence of microvascular complications (neuropathy, retinopathy, and nephropathy) in a cohort of Iranian patients with type 2 diabetes.

Methods: For a total population of 3123 patients with type 2 diabetes, a prospective study was designed for 1215 patients with NAFLD and 1908 gender and age-matched control patients without NAFLD. The two groups were followed for a median duration of 5 years for the incidence of microvascular complications. The association between having NAFLD, the level of liver enzymes, aspartate aminotransferase to platelet ratio index (APRI), Fibrosis-4 (FIB-4) value, and the incidence risk of diabetic retinopathy, neuropathy, and nephropathy were assessed through logistic regression analysis.

Results: NAFLD was found to be associated with incidence of diabetic neuropathy and nephropathy (Odds ratio: 1.338 (95% confidence interval: 1.091-1.640) and 1.333 (1.007-1.764), respectively). Alkaline-phosphatase enzyme was found to be associated with higher risks of diabetic neuropathy and nephropathy ((Risk estimate: 1.002 (95% CI: 1.001-1.003) and 1.002 (1.001-1.004), respectively)). Moreover, gamma-glutamyl transferase was associated with a higher risk of diabetic nephropathy (1.006 (1.002-1.009). Aspartate aminotransferase and alanine aminotransferase were inversely associated with the risk of diabetic retinopathy (0.989 (0.979-0.998) and 0.990 (0.983-0.996), respectively). Furthermore, ARPI_T (1), ARPI_T (2), and ARPI_T (3) were shown to be associated with NAFLD (1.440 (1.061-1.954), 1.589 (1.163-2.171), and 2.673

(1.925, 3.710), respectively). However, FIB-4 score was not significantly associated with risk of microvascular complications.

Conclusion: Despite the benign nature of NAFLD, patients with type 2 diabetes should be always assessed for NAFLD to ensure early diagnosis and entry into proper medical care. Regular screenings of microvascular complications of diabetes is also suggested for these patients.

KEYWORDS

type 2 diabetes, non-alcoholic fatty liver disease, diabetic neuropathy, diabetic retinopathy, diabetic nephropathy

Introduction

Non-alcoholic fatty liver disease (NAFLD) occurs commonly in patients with type 2 diabetes mellitus, with a prevalence of 55%–68% (1) due to the frequent occurrence of insulin resistance and obesity in patients with type 2 diabetes (2).

There is now growing evidence that independent of other known risk factors and especially in patients with type 2 diabetes, NAFLD, could be associated with an increased risk of macrovascular complications. Several observational studies and some meta-analyses have documented that NAFLD, especially its advanced forms, is strongly associated with fatal and non-fatal cardiovascular events, as well as with specific cardiac complications, including sub-clinical myocardial alteration and dysfunction, heart valve diseases and cardiac arrhythmias. Importantly, across various studies, these associations remained significant after adjustment for established cardiovascular risk factors and other confounders (3–7).

Furthermore, It has been shown that NAFLD increases the risks for the development of type 2 diabetes and/or its progression (8, 9). Therefore, NAFLD could increase the risk for type 2 diabetes organ-specific complications as well and consequently the incidence of type 2 diabetes complications such as nephropathy, retinopathy, and neuropathy with NAFLD is an emerging concept (10).

Several population-based studies have revealed different types of associations between the incidence of microvascular complications of type 2 diabetes and NAFLD. A report from India revealed increased prevalence of microvascular complications including nephropathy and neuropathy in patients with type 2 diabetes and fatty liver disease (10). However, a cross sectional study of the Korean population reported that prevalence of diabetic nephropathy and retinopathy were lower in patients with type 2 diabetes who had NAFLD (11). Conversely, Targher et al. (12, 13) reported that NAFLD is independently associated with an increased prevalence of both diabetic nephropathy and retinopathy in patients with type 2 diabetes. Altogether, convincing epidemiological evidence have supported a strong association between the presence and severity of NAFLD, and the risk of chronic microvascular diabetes complications (14).

Since results of the previous studies are controversial, once again the present study aimed to investigate the association of NAFLD, liver enzymes, and Fibrosis-4 (FIB-4) index [a non-invasive fibrosis scoring systems to checkup liver fibrosis (15, 16)] with the incidence of microvascular complications (neuropathy, retinopathy, and nephropathy) in a cohort of Iranian patients with type 2 diabetes.

Materials and methods

Study population

In this prospective cohort study, 3123 patients with a history of type 2 diabetes enrolled and were followed for median of 5 years. The participants had all previously attended the endocrinology clinic of Vali-Asr Hospital, a medical center affiliated with Tehran University of Medical Sciences. The study group was chosen based on comprehensive exclusion criteria which consisted of having a history of glaucoma, vitreous surgery, cataract on eye examination, kidney disease (Creatinine (Cr)> 2 mg/dl) or low estimated glomerular filtration rate (eGFR) <30 cc/min), hypothyroidism, familial hypercholesterolemia, liver dysfunction, epilepsy, and hemoglobinopathy. Women taking oral contraceptives or hormone replacement therapy and pregnant women were also excluded. Additionally, patients with type 1 diabetes, gestational diabetes, diabetes due to pancreatic cancer, pancreatitis and other metabolic conditions were excluded. Patients with a history of alcohol use, viral hepatitis, hepatotoxicity-inducing drugs usage, autoimmune hepatitis, and/or rapid weight loss were excluded from the study. Baseline biochemical tests of the patients such as cholesterol levels and other lipid and glycemic indices were measured (Table 1).

NAFLD was defined as the presence of definite hepatic steatosis on ultrasound scan in the absence of a secondary cause for hepatic steatosis. The participants were divided into two study groups based on the presence of NAFLD at the start of the study: 1215 patients with NAFLD and 1908 without NAFLD. The presence of NAFLD was diagnosed based on the observation of definite hepatic steatosis on abdominal ultrasound performed by an expert radiologist (i.e., grades 2 or 3 hepatic steatosis, defined based on marked and diffuse hepatic hyperechogenicity relative to the renal parenchyma, ultra-

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TABLE 1 Baseline characteristics of the study population based on the presence of fatty liver.

	With fatty liver				Without fatty liver				P-value
	total	female	male	p-value	total	female	male	p-value	
Age (year)	51.34 ± 12.86	52.02 ± 12.98	50.56 ± 12.70	**0.005**	57.54 ± 28.91	56.40 ± 34.87	59.59 ± 12.04	**0.008**	**<0.001**
Diabetes duration (year)	9.44 ± 7.58	9.76 ± 7.69	9.12 ± 7.47	0.068	9.50 ± 8.58	8.86 ± 8.29	10.58 ± 8.96	**<0.001**	0. 797
SBP (mmHg)	126.23 ± 27.17	125.12 ± 16.97	127.50 ± 35.40	**0.030**	128.98 ± 31.60	127.72 ± 17.39	131.26 ± 47.41	**0.007**	**0.001**
DBP (mmHg)	78.76 ± 8.75	78.41 ± 8.85	79.15 ± 8.63	**0.037**	75.61 ± 10.57	74.85 ± 10.73	76.99 ± 10.13	**<0.001**	**<0.001**
FBS (mg/dL)	139.40 ± 55.17	134.43 ± 52.56	145.14 ± 55.54	**<0.001**	144.45 ± 56.55	139.95 ± 56.94	152.60 ± 54.96	**<0.001**	**0.001**
2hPP (mg/dL)	192.03 ± 86.73	181.11 ± 85.14	203.82 ± 86.93	**<0.001**	190.31 ± 88.78	178.55 ± 85.18	211.40 ± 91.22	**<0.001**	0.506
Hb AIC (%)	7.05 ± 1.69	6.89 ± 1.66	7.23 ± 1.71	**<0.001**	7.51 ± 14.63	7.51 ± 18.20	7.51 ± 1.67	0.995	0.134
Chl (mg/dL)	186.94 ± 43.97	191.95 ± 43.10	181.14 ± 44.28	**<0.001**	172.41 ± 44.75	177.16 ± 44.64	163.79 ± 43.68	**<0.001**	**<0.001**
HDL (mg/dL)	44.85 ± 11.63	47.68 ± 11.55	41.58 ± 10.87	**<0.001**	46.11 ± 12.13	48.10 ± 12.57	42.51 ± 10.38	**<0.001**	**<0.001**
LDL (mg/dL)	107.55 ± 35.16	110.42 ± 35.32	104.23 ± 34.71	**<0.001**	97.23 ± 33.62	99.66 ± 34.13	92.85 ± 32.25	**<0.001**	**<0.001**
TG (mg/dL)	183.54 ± 119.48	175.03 ± 95.02	193.36 ± 142.02	**<0.001**	155.65 ± 90.17	156.32 ± 86.30	154.44 ± 96.84	0.619	**<0.001**
Cr (mg/dL)	0.98 ± 0.21	0.90 ± 0.20	1.07 ± 0.20	**<0.001**	0.97 ± 0.24	0.91 ± 0.21	1.09 ± 0.26	**<0.001**	0.147
eGFR (ml/min)	49.00 ± 23.67	49.20 ± 31.54	48.83 ± 18.04	0.981	50.45 ± 20.59	48.82 ± 23.46	51.22 ± 17.84	0.884	0.860
UA (mg/dL)	5.36 ± 1.95	5.09 ± 2.14	5.66 ± 1.70	**<0.001**	4.78 ± 2.07	4.58 ± 1.71	5.15 ± 2.57	**<0.001**	**<0.001**
BMI (Kg/m^2)	32.37 ± 6.48	33.70 ± 7.10	30.74 ± 5.20	**<0.001**	28.67 ± 5.17	29.41 ± 5.63	27.54 ± 4.14	**<0.001**	**<0.001**
AST	28.79 ± 17.11	27.17 ± 14.20	29.45 ± 17.41	**0.001**	19.33 ± 10.70	18.80 ± 8.61	20.09 ± 12.01	**0.002**	**<0.001**
ALT	40.38 ± 24.62	35.19 ± 20.63	43.39 ± 24.14	**<0.001**	21.67 ± 11.65	20.82 ± 11.25	23.18 ± 9.68	**<0.001**	**<0.001**
ALKP	166.84 ± 82.75	174.56 ± 89.15	158.46 ± 74.34	**<0.001**	141. 41 ± 69.54	143.05 ± 68.80	138.50 ± 70.79	0.183	**<0.001**
GGT	38.54 ± 50.51	35.94 ± 53.82	40.99 ± 47.14	0.185	27.82 ± 25.08	24.29 ± 20.19	32.19 ± 29.52	**0.002**	**<0.001**
Retinopathy	157 (8.96)	82 (4.68)	75 (4.28)	0.616	235 (10.37)	126 (5.56)	109 (4.81)	**0.002**	0.134
Neuropathy	228 (17.62)	116 (8.96)	112 (8.66)	0.942	296 (14.47)	157 (7.67)	139 (6.80)	**0.001**	**0.015**
Nephropathy	108 (8.67)	51 (4.09)	57 (4.58)	0.420	126 (6.50)	52 (2.68)	74 (3.82)	**<0.001**	**0.022**

SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; 2hPP, Two-Hour Postprandial Glucose; Hb AIC, hemoglobin A1C; Chl, cholesterol; HDL, High density lipoproteins; LDL, low density lipoproteins; TG, triglycerides, Cr, creatinine; eGFR, Estimated Glomerular Filtration Rate; UA, uric acid; BMI, body mass index; AST, aspartate aminotransferase; ALT, Alanine transaminase; ALKP, Alkaline phosphatase, GGT, gamma-glutamyl transferase.
Bold values report statistically significant difference.

sound beam attenuations and/or poor or no visualization of the diaphragm and intrahepatic vessels/structures, with or without focal fatty sparing consistent with the evidence of severe hepatic steatosis). Three hundred and fifty (350) patients who developed NAFLD over the duration of the study were excluded. During the study period, the status of possible chronic causes of non-NAFLD were constantly recorded. Patients with positive serology tests for hepatitis B, and C viruses surface antigens, and other causes of chronic liver diseases such as autoimmune hepatitis, hemochromatosis, Wilson's disease, primary biliary cirrhosis, and sclerosing cholangitis were excluded from the current study, based on physical examinations and blood tests (i.e. antinuclear and anti-smooth muscle antibody, iron studies, ceruloplasmin, and urinary copper). After obtaining written informed consent from the participants, the incidence of microvascular complications was investigated in both groups. The study was reviewed and approved by the ethics committee of Tehran University of Medical Sciences.

Physical examinations

For each participant, baseline demographic data such as weight, height, blood pressure, diabetes duration, the usage of anti-hypertensive and lipid lowering drugs were recorded by trained medical staff. Patients' weight was measured using a portable digital scale with a precision of 0.1 kg, after they were asked to wear light clothing. An inflexible measurement tape with a precision of 0.1 cm was used to measure height with the subjects being asked to stand erect and remove their socks and shoes. Using the height and weight data, each individual's body mass index (BMI) was then measured (Kg/m^2). The subjects' blood pressure was measured three times, after a ten-minute seated rest and within five-minute intervals. To measure blood pressure, a calibrated Omron M7 digital sphyg142 manometer (Hoofddorp, The Netherlands) with appropriately sized cuffs which covered at least 80% of the subjects' right arm was used. First reading was discarded due to possible imprecision and the second and third readings were averaged to calculate the mean value of systolic (SBP) and diastolic blood pressure (DBP). The participants were asked to stand still in a relaxed position, placing both feet together on a flat surface for waist circumference (WC) measurements; one layer of clothing was accepted. A non-stretchable measuring tape was used to measure WC as the smallest horizontal girth between the costal margins and the iliac crest at minimal respiration. During the interview process, demographic information, smoking habits, and medication usage status were obtained.

Laboratory analysis

Ten ml of blood sample was drawn from each individual who was asked to fast for 12 to 14 hours over night. The samples were kept at a temperature of 4 to 8 °C in cold biochemistry tubes and were later sent to the appropriate calibrating laboratories where they were instantly centrifuged (1500 rmp, for 10 min, at standard room temperature of 21 °C). The extracted serum was stored in the temperature of -70) (17). Laboratory evaluations were done on the extracted serum stored at a temperature of -70°C. In randomly selected urine samples, the measure of urinary albumin excretion was performed using urinary albumin-to-creatinine ratio (UACR). Urinary albumin concentrations were evaluated by an immunoturbidimetric commercial kit (Randox, Antrim, UK). The Chronic Kidney Disease Epidemiology Collaboration Equation was used to calculate the estimated glomerular filtration rate (eGFR).

Employing high-performance liquid chromatography (A1C, DS5 Pink kit; Drew, Marseille, France), Glycated hemoglobin (HbA1c) was measured. Fasting plasma glucose (FPG) and two-hour postprandial (2HPP) glucose were measured using colorimetric methods by the glucose oxidase test. Serum lipid concentrations [triglycerides(TG), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) (18), and total cholesterols (Chl) (19)] were measured using enzymatic methods. The kits used in this study were approved by the central reference laboratory in Tehran, Iran (17). Enzymatic photometry was used to analyze serum liver enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP), and gamma glutamyl transferase (GGT). Based on respective standardized criteria, an ALT level >30 IU/L in women and >40 IU/L in men, AST level >30 IU/L in women and >36 IU/L in men, ALKP levels of greater than 306 U/L in both genders and GGT levels greater than 60 U/L for men and greater than 40 IU/L in women were considered elevated. IFCC (International Federation of Clinical Chemistry and LaboratoryMedicine) method was employed to measure levels of ALT, AST and GGT (ALT intra-assay CV = 3.7%, AST intra-assay = 2.5% and GGT intra-assay CV = 2.2%) (20). The level of ALKP was measured according to Deutsche Gesellschaft für Klinische Chemie (DGKC) method (21). The liver enzyme measurements were performed using commercial Parsazmun kits (Tehran, Iran) and Hitachi 704 automatic analyzer (Tokyo, Japan).

A non-invasive diagnostic test for NAFLS is aspartate aminotransferase to platelet ratio index (APRI) which is calculated as (AST level/AST upper level of normal/platelet count) × 100 (22).

The FIB-4 index, a marker of hepatic fibrosis, was calculated by the following formula:

$\{age\ [years] \times AST\ [U/L]/[platelet\ count\ (10^9/L) \times ALT\ (U/L)^{1/2}]\}$ (23)

Assessment of complications

To identify the microvascular complications associated with diabetes, the International Classification of Diseases, Tenth Revision (ICD-10) was used. Diabetic neuropathy was identified using specific codes E10.4, E11.4, E12.4, E13.4, and E14.4. Diabetes-related chronic microvascular complications were identified according to the International Classification of Diseases, Tenth Revision (ICD-10). The specific codes used were: E10.3, E11.3, E12.3, E13.3 and E14.3 for diabetic retinopathy; E10.2, E11.2, E12.2, E13.2 and E14.2 for diabetic nephropathy; E10.4, E11.4, E12.4, E13.4 and E14.4 for diabetic neuropathy (24, 25). Based on Macular Edema Disease Severity Scale, patients with moderate to severe maculopathy who required laser therapy were also considered as patients with retinopathy (26, 27).

Statistical analysis

To statistically analyze the recorded data, version 25 of SPSS software was employed. Kolmogorov–Smirnov and Shapiro-Wilk normality tests, P-P plot, and histograms were used to test for the normality of the study population. The tested variables were discovered to be normal and the null hypothesis was rejected. Univariable analysis of potential categorical and continuous risk factor variables was performed using t-test and chi-square test, respectively. Mean ± standard deviation (SD) was used to report continuous, and proportions were used to report values for categorical variables. Logistic regression was conducted to

ascertain the effects of NAFLD, liver enzymes and FIB-4 on microvascular complications and APRI on NAFLD. The results were adjusted for age, sex, and duration of diabetes, 2hpp, FBS, Cr, and BMI. Multifocal logistic regression and 4 models group of covariates used for evaluation of relationship between NAFLD and diabetic microvascular complications. A p-value < 0.05 was considered statistically significant.

Results

Characteristics of the study population

The level of ALKP, liver enzymes (ALT, AST, GGT) as well as the presence of non-alcoholic fatty liver based on the ultrasound findings were assessed and statistically analyzed as possible predictors of microvascular complications. The baseline characteristics of the study population were summarized in Table 1. Overall, the patients with fatty liver tended to be significantly younger compared to those without (Age: 51.34 ± 12.86 vs. 57.54 ± 28.91, p-value<0.001), have lower levels of FBS, SBP, shorter drug duration and HDL, and BMI, DBP, cholesterol (Chl), LDL, Tg, and uric acid (UA) (Table 1). Moreover, 17.62% and 8.67% of the NAFLD patients had neuropathy and nephropathy, respectively, which were significantly higher than patients without NAFLD (p-value: 0.015 and p-value: 0.022, respectively). However, the prevalence of retinopathy incidence was not significant between the patients with and without NAFLD.

Association of gender and baseline characteristics and microvascular complications of diabetes

Considering the importance of the role of gender in the occurrence of non-alcoholic fatty liver, statistical analysis was performed to investigate the role of gender in NAFLD determinants. In the group of fatty liver, females were older significantly (52.02 ± 12.98 vs. 50.56 ± 12.70, p-value=0.005), had lower SBP, DBP, drug duration, FBS, 2hpp, HbA1C, TG, Cr, uric acid, AST, ALT and higher Chol, HDL, LDL, BMI and ALKP, although there were no statistically significant in the prevalence of microvascular complications in fatty liver group based on gender (Table 1).

Association of serum liver enzymes and NAFLD with microvascular complications of diabetes

Tables 2 summarizes the association between liver enzyme levels and incidence of the three outlined complications. ALKP increased the incidence risk of diabetic neuropathy and nephropathy (Odds ratio (OR):1.002 (95% confidence interval (CI) 1.001-1.003), and 1.002 (1.001-1.004), respectively). Moreover, GGT was a risk factor for the incidence risk of diabetic nephropathy (1.006 (1.002-1.009)). AST and ALT were inversely associated with the risk of diabetic retinopathy (0.989 (0.979-0.998) and 0.990 (0.983-0.996), respectively). Tertiary multivariate adjusted model was used to adjust for duration of diabetes, fasting blood sugar level, sex, age, 2hpp, creatinine, and BMI. As shown in Table 3, different parameters were used in 3 models to investigate the relationship between NAFLD and microvascular complications of type 2 diabetes. The results of these models are in general agreement with the baseline model and indicate that the incidence risk of diabetic neuropathy and nephropathy were significantly increased in patients with NAFLD in base line model and even after adjusting for various confounding variables (OR: 1.338 (95% CI: 1.091-1.640) and 1.333 (1.007-1.764), respectively). On the contrary, the incidence of the other complication, retinopathy, was not found to be significantly associated with the presence of NAFLD in patients.

Association of APRI and NAFLD

Table 4 summarizes the association between APRI and NAFLD. ARPI_T(1), ARPI_T(2), and ARPI_T(3) values were significantly increased in patients with NAFLD after adjusting for confounding variables (1.440 (1.061-1.954), 1.589 (1.163-2.171), and 2.673 (1.925, 3.710), respectively). Tertiary multivariate adjusted model was used to adjust for duration of diabetes, fasting blood sugar level, sex, age, 2hpp, creatinine, and BMI.

TABLE 2 Association between incidence of diabetic microvascular complications with liver enzymes.

	Retinopathy			Neuropathy			Nephropathy		
	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
AST	0.989	0.979-0.998	0.02	1.000	0.994-1.006	0.993	1.001	0.992-1.011	0.794
ALT	0.990	0.983-0.996	0.003	0.995	0.989-1.001	0.079	1.001	0.994-1.009	0.769
ALK-P	1.001	1.000-1.003	0.079	1.002	1.001-1.003	0.001	1.002	1.001-1.004	0.008
GGT	1.002	0.999-1.005	0.267	1.000	0.997-1.004	0.926	1.006	1.002-1.009	0.001

Data was adjusted for age, sex, duration of diabetes, fasting blood sugar, 2hPP, Cr, and BMI in the tertiary multivariate adjusted model. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALKP, alkaline phosphatase; GGT, gamma glutamyl transferase.

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TABLE 3 Association between NAFLD and diabetes-related microvascular complications among patients with type 2 diabetes [OR 95%CI].

	Model 1	Model 2	Model 3
Retinopathy	0.799 (0.624-1.023)	0.848 (0.660-1.089)	0.899 (0.713-1.134)
Neuropathy	1.492 (1.199-1.856)	1.540 (1.234-1.923)	1.338 (1.091-1.640)
Nephropathy	1.290 (0.963-1.726)	1.253 (0.933-1.684)	1.333 (1.007-1.764)

Model 1: Baseline model.
Model 2: Model adjusted for age and sex.
Model 3: Model adjusted for gender, age, diabetes duration, FBS, 2hPP, Cr, and BMI
NAFLD, non-alcoholic fatty liver disease.

Association of FIB_4 and microvascular complications

Table 5 shows the relationship between the severity of liver fibrosis (measured through the FIB-4 index) and the occurrence of microvascular complications of diabetes. In the third tertile of FIB-4 compared to the first one, the risk of complications such as retinopathy and neuropathy is higher (1.116 (0.741-1.681) compared to 1.399 (0.335-5.168) and 1.003 (0.683-1.473) compared to 1.399 (0.371-5.276), respectively; while this trend is reversed in nephropathy (1.087 (0.678-1.744) compared to 0.741 (0.192-2.86). However, the mentioned findings are not statistically significant.

Discussion

This prospective cohort study investigated the association of NAFLD and liver enzymes with the incidence of microvascular complications (retinopathy, nephropathy, and neuropathy). After adjustment for confounding factors, NAFLD was a precipitating factor of nephropathy and neuropathy in patients with type 2 diabetes. On the contrary, NAFLD was not a risk factor of retinopathy in patients with type 2 diabetes after adjustment for confounding factors. Although it was found that alkaline-phosphatase increases the incidence risk of diabetic neuropathy and nephropathy and GGT is an increasing risk factor for the incidence risk of diabetic nephropathy. However, other liver enzymes as well as FIB-4 levels were not associated with microvascular complications.

We observed that the incidence risk of diabetic neuropathy is significantly associated with NAFLD. In line with our study, a recent meta-analysis by Greco et al. (28) demonstrated that the prevalence of diabetic neuropathy significantly increased in patients with type 2 diabetes and NAFLD. Moreover, a recent observational study also conformed the association between diabetic neuropathy and NAFLD by measuring NAFLD fibrosis score and FIB-4 (29). Mantovani et al. (30) also suggested that NAFLD exacerbates hepatic and peripheral insulin resistance, presents with a predisposition to atherogenic dyslipidemia, and results in the activation of several pro-fibrogenic mediators, procoagulant, proinflammatory, and pro-oxidant. This can have a crucial role in neuropathy pathology. For instance, several studies established that atherogenic dyslipidemia can directly promote nerve damage via lipotoxicity of free fatty acids and, indirectly, via free fatty acids which can stimulate a systemic inflammatory cytokine cascade and elevate insulin resistance (31, 32). Many other studies also indicated that pro-inflammatory and pro-oxidant mediators have an essential role in neuropathy pathology (32).

TABLE 4 Association between incidence of NAFLD and APRI.

	NAFLD		
	Odds ratio	95% CI	P-value
APRI_T(1)	1.440	1.061-1.954	0.019
APRI_T(2)	1.589	1.163-2.171	0.004
APRI_T(3)	2.673	1.925-3.710	0.001

Data was adjusted for age, sex, duration of diabetes, fasting blood sugar, and 2hPP in the tertiary multivariate adjusted model. T, tertile. APRI, aspartate aminotransferase to platelet ratio index. NAFLD, non-alcoholic fatty liver disease.

Our results showed that the incidence risk of diabetic nephropathy is significantly correlated with NAFLD. In line with our results Casoinic et al. (33) discovered NAFLD to be positively correlated with microalbuminuria, which is a marker of the early stage of nephropathy in patients with type 2 diabetes. A report from India also revealed an increased prevalence of microvascular complications including nephropathy in patients with type 2 diabetes who had fatty liver disease (10). Wen et al. reported that the presence of kidney disease and retinopathy was higher in the "indeterminate risk" and "high risk" groups than in the "low risk" group of NAFLD, after adjusting for the same covariates. They also found that the presence of diabetic kidney disease significantly increased with high NAFLD fibrosis score (34). Another study on Iranian population also reported that NAFLD was not found to increase the risk of diabetic nephropathy (35). Furthermore, Targher et al. (12, 36) reported that NAFLD is independently associated with an increased prevalence of chronic kidney disease in patients with type 2 diabetes. Jia et al. (37) reported a positive association between NAFLD and serum uric acid, tumor necrosis factor-α (TNF-α), insulin resistance index, omentin-1, free fatty acids, homocysteine, and visceral fat area. Any of the above factors combined with NAFLD can elevate nephropathy risk in patients with type 2 diabetes. They established that NAFLD patients showed insulin resistance and elevated visceral fat area, which are the usual components of the metabolic syndrome, a crucial contributor to the progression and development of nephropathy (38, 39). Several studies also indicated a positive correlation between insulin resistance and nephropathy (40, 41).

TABLE 5 Association between incidence of microvascular complications and FIB-4.

	Retinopathy			Neuropathy			Nephropathy		
	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
FIB-4_T1	Reference	–	–	Reference	–	–	Reference	–	–
FIB-4_T2	1.116	0.741-1.681	0.600	1.003	0.683-1.473	0.988	1.087	0.678-1.744	0.728
FIB-4_T3	1.399	0.335-5.168	0.695	1.399	0.371-5.276	0.620	0.741	0.192-2.860	0.741

Data was adjusted for age, sex, duration of diabetes, fasting blood sugar, and 2hPP in the multimodal logistic regression. FIB4, Fibrosis 4 score; T, Tertile.

In contrast to our results, a cross-sectional study of the Korean population reported that the prevalence of nephropathy was lower in patients with type 2 diabetes and NAFLD (11). In addition, Afarideh et al. reported that NAFLD was inversely associated with the prevalence of diabetic nephropathy in the Iranian population (42).

In contrast to our findings Lv et al. reported that NAFLD negatively correlated with the risks of nephropathy, retinopathy and neuropathy (43). Moreover, in contrast to several previously conducted studies (11, 12, 43, 44), our study failed to demonstrate any associations between diabetic retinopathy. However, similar to our results among the Western population and independent of gender, age, ethnicity, serum HDL-C, serum triglycerides, waist circumference, SBP, and A1C, NAFLD was found not to be associated with the presence of retinopathy in the US general population with or without diabetes (45).

These discrepancies in the findings of the studies might be attributed to differences in baseline characteristics of the participants. Furthermore, the ethnic differences for pathophysiological characteristics of patients with type 2 diabetes might also be responsible for the differences between our findings and those of the mentioned studies.

Our result showed ALKP had a significant association with incidence of neuropathy and nephropathy. Also, GGT had a significant association with nephropathy. An inverse association between ALT and AST were also observed with incidence of diabetic retinopathy. Similarly, Afarideh et al. (42), established that ALT had an inverse association with diabetic neuropathy and retinopathy. Similar to our result, a retrospective study reported that elevated ALKP level is associated with nephropathy in patients with type 2 diabetes (46). Circulating ALKP degrades pyrophosphate, which is an endogenous anti-calcification factor in the arterial wall. So, high levels of ALKP can promote arterial calcification and lead to cardiovascular disease (47). Increased arterial stiffness led to elevated systemic blood pressure in the defective glomerular capillaries, with low resistance, and exacerbated intraglomerular hypertension and hyperfiltration, and eventually, nephrosclerosis (46). Therefore, the ALP-diabetic nephropathy association identified in our study may support the role of arterial calcification in the progression of kidney disease (48). Our study failed to demonstrate associations between the level of other liver enzymes and incidence of microvascular complications; However, a recent study by Kim et al. (29) found that the levels of ALT and AST is higher in diabetic neuropathy in patients with NAFLD. In another study, Lin et al. (49) showed that neuropathy is directly associated with GGT in a Chinese population with diabetes.

Despite the existence of conflicting studies on the relationship between microvascular complications of diabetes and NAFLD, several systematic reviews, meta-analyses and umbrella reviews have been conducted to investigate this relationship. It is concluded that NAFLD is a multi-system disease that does not only affect the liver tissue, but it causes many important complications in several organs and increases the mortality of diabetic patients, which is one of the important reasons for the increase in the mortality of coronary artery disease and also diabetic nephropathy (50). Diabetic neuropathy also increases as one of the complications of diabetes following the occurrence of NAFLD in diabetic patients (28), but Dandan Song's meta-analysis did not report a relationship between diabetic retinopathy and NAFLD (50), and the results of these complications were in line with our study.

In the present study, APRI had significant association with NAFLD. In line with our results, a recent systematic review reported that APRI risk stratify morbidity and mortality in patients with NAFLD (51). Also a recent cross-sectional study in Iran showed that APRI can significantly detect fibrosis in NAFLD (52). Also, a retrospective cohort study in Canada evaluated the prognostic values of non-invasive diagnostic tests such as APRI against liver histology and hepatic venous pressure gradient (HVPG) in NAFLD patients. Their results showed that APRI can predict outcomes of NAFLD patients and it could be used to monitor, risk stratify, and find targeted interventions (53). Furthermore, a prospective study in Brazil demonstrated that is a very accurate in identifying NAFLD (54).

To the best of our knowledge, this is the first prospective cohort study with a population-based sample in Middle East and North Africa (MENA) region to identify the association of NAFLD and liver enzymes with the incidence risk of microvascular complications (retinopathy, neuropathy, and nephropathy) in patients with type 2 diabetes. Another strength of the current study is the sufficient sample size, exact ultrasound grading by single expert operator, as well as the exclusion of other causes of liver disease to assess the presence of NAFLD in patients with type 2

diabetes related microvascular complications.

However, there were some limitations to our study. First, since the present study population was a cohort of patients cared for at a single center, caution should be taken when extrapolating the results to all patients with type 2 diabetes. It should be considered that the majority of the participants were typical patients with type 2 diabetes commonly encountered in outpatient clinics and the present study granted a high degree of consistency regarding, ultrasonographic findings, laboratory data, and the assessment of microvascular diabetic complications. Second, estimated GFR was used rather than a more precise measure of kidney function, e.g., iothalamate clearance. Third, due to the NAFLD definition in the present study based on definite signs of hepatic steatosis (grade 3 hepatic steatosis on abdominal ultrasound), our results may not apply to patients with earlier hepatic steatosis stages on ultrasound or those individuals with sonographically undetectable NAFLD. Fourth, in our database one of the ignored data was consumption of some oral anti-diabetic agents like pioglitazone, so in the next studies, researchers should consider this issue. Moreover, the lack of a liver biopsy which is the gold standard method for diagnosis of NAFLD as well as differentiating it from non-alcoholic stetohepatitis (NASH) (55) can be deemed as the most significant limitation of the present study. Lack of Vibration-controlled Transient Elastography and fibro scan are also other limitations of the current study. However, due to the aggressive nature of liver biopsy, in this study similar to most previous studies, ultrasound was preferred for the diagnosis of NAFLD.

Conclusion

The present prospective cohort study found that NAFLD, as diagnosed by characteristic sonographic features, was associated with an increased incidence of diabetic nephropathy and neuropathy. Additionally, according to our data, ALKP, GGT, were associated with increased risks of microvascular complication of diabetes, while ALT and AST were shown to be inversely associated with the incidence of diabetic retinopathy. Future studies are required to assess possible mechanisms related to the underlying pathophysiological basis of these associations.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Tehran University of Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ND, FD, and FM performed the data analysis; AE conceived the article; NY, AP, MD, and MP drafted the manuscript; SR and IK provided manuscript revisions; and MN clinically revised manuscript. All authors contributed to the article and approved the submitted version.

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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Uncoupling hepatic insulin resistance – hepatic inflammation to improve insulin sensitivity and to prevent impaired metabolism-associated fatty liver disease in type 2 diabetes

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Diabetes mellitus is a metabolic disease clinically-characterized as acute and chronic hyperglycemia. It is emerging as one of the common conditions associated with incident liver disease in the US. The mechanism by which diabetes drives liver disease has become an intense topic of discussion and a highly sought-after therapeutic target. Insulin resistance (IR) appears early in the progression of type 2 diabetes (T2D), particularly in obese individuals. One of the co-morbid conditions of obesity-associated diabetes that is on the rise globally is referred to as non-alcoholic fatty liver disease (NAFLD). IR is one of a number of known and suspected mechanism that underlie the progression of NAFLD which concurrently exhibits hepatic inflammation, particularly enriched in cells of the innate arm of the immune system. In this review we focus on the known mechanisms that are suspected to play a role in the cause-effect relationship between hepatic IR and hepatic inflammation and its role in the progression of T2D-associated NAFLD. Uncoupling hepatic IR/hepatic inflammation may break an intra-hepatic vicious cycle, facilitating the attenuation or prevention of NAFLD with a concurrent restoration of physiologic glycemic control. As part of this review, we therefore also assess the potential of a number of existing and emerging therapeutic interventions that can target both conditions simultaneously as treatment options to break this cycle.

KEYWORDS

insulin resistance, type 2 diabetes, NAFLD, NASH, hepatic insulin resistance

Introduction

Diabetes mellitus is a metabolic disease clinically-characterized as acute and chronic hyperglycemia (1). It is emerging as one of the common conditions associated with incident liver disease in the US. The spectrum of liver disease ranges from mild transaminitis to non-alcoholic fatty liver disease (NAFLD). NAFLD encompasses non-alcoholic steatosis (fatty liver) without inflammation (normal transaminases), non-alcoholic steatohepatitis (NASH) without fibrosis, NASH with fibrosis eventually progressing to cirrhosis, hepatocellular carcinoma and liver failure culminating in death (1, 2). In clinical practice, most patients with NAFLD are asymptomatic with possible hepatomegaly. They are diagnosed when liver enzymes ALT and/or AST are elevated, or steatosis is detected on abdominal imaging. It is a diagnosis of exclusion, and normal liver enzymes do not eliminate a diagnosis of NAFLD (3–7). Worldwide, the pooled prevalence of NAFLD (umbrella term of macrovesicular fat deposition) is 25.24% (8). In the US, a comparison of 3 cycles of the National Health and Nutrition Examination Survey (NHANES) based on transaminitis alone, demonstrated a steady increase in the prevalence of NAFLD from 5.5% in 1988 to 11% in 2008. The inclusion of steatosis with normal transaminases may account for an even higher prevalence (9). The prevalence of NAFLD's closely associated metabolic counterparts such as essential hypertension, obesity and diabetes has trended up as well (10). Studies in multiple countries have demonstrated that NAFLD has a higher prevalence in men. Prevalence in women increases with age, while it remains stable in men. Sex hormones, menopausal status and obesity are major contributing factors to this disparity (11).

The mechanisms by which diabetes drives liver disease have become a topic of intense discussion and highly sought-after therapeutic targets. Traditionally, diabetes has been classified into type 1 (T1D) and type 2 (T2D). T1D begins as an autoimmune process culminating in an autoimmune inflammation-mediated, selective impairment of the pancreatic beta cells and overt hyperglycemia. T2D, instead, is characterized by peripheral insulin resistance (IR) compensated for by the production of more insulin culminating in overt hyperglycemia. Accumulating evidence suggests that these seemingly divergent conditions share many etiopathogenetic and clinical features other than just hyperglycemia. Thus, latent autoimmune diabetes of adults (LADA) presents features of both T1D and T2D and IR is seen in overweight T1D patients (12). On the other hand, some T2D patients exhibit pancreatic autoimmunity (13).

Evolution of hepatic IR in T2D and T2D-associated NAFLD

Broadly-understood, IR is coupled to impaired insulin action at multiple points in the signaling cascade in the main glucose-utilizing, insulin-responsive tissues, particularly skeletal muscle, adipose, and the liver. These as well as possible pressure points of therapeutic interest are illustrated in Figure 1. These include the action of lipid mediators, cellular stress, mitochondrial abnormalities, and leukocyte-derived soluble molecules (14). Lipid-induced IR has been observed in the liver (15) as the consequence of high fat diet (HFD) or lipolysis, where the concentration of FFA exceeds that of the intracellular fatty acid oxidation and storage rate, as demonstrated in humans and rodent models (14). Increased concentrations of diacylglycerol (DAG) also lead to IR by impairing insulin signaling (14). For example, plasmalemmal accumulation of intrahepatic DAG stimulates protein kinase Cε and inhibitory insulin receptor kinase phosphorylation on threonine (16, 17) resulting in IR. These results were consistent in rodent models and humans. In addition to protein kinase Cε, increased activity of the δ enzymatic isoform in livers of obese humans has been observed to cause hepatic IR (18). Human study outcomes and rodent models have shown that activation of other protein kinase C isoforms (δ, ε, ν, θ) have been implicated in DAG release and IR onset or progression (14). Non-FFA-derived lipids are another species implicated in the onset of hepatic IR in humans exhibiting NASH. A number of studies in humans revealed elevated intra-hepatic FFA concentrations concurrent with hepatic oxidative stress and inflammation (19). While ceramides have also been implicated in hepatic IR under obese conditions and T2D evolution, this has been well-reviewed elsewhere (20) and remains outside the topic of the current review. While HFD-facilitated elevations in circulating FFAs and lipids as a basis of IR is strongly-supported by many lines of animal and human investigation (21), not all situations of IR are a consequence of this. Cellular stress, instead, is a better predictor of IR in the obese state. Endoplasmic reticulum (ER) stress, particularly, is a common finding in the liver among obese men and women (22, 23). Nevertheless, exposure to HFD in rodents leads to an expansion of lipid deposition inside the liver followed by hepatic IR even in the absence of peripheral fat accumulation and peripheral IR. Under such diet conditions, insulin signaling has been shown to be impaired, partly due to activation of PKCε and JNK1 (24). Estrogen has a protective role against hepatic steatosis and insulin resistance by decreasing triglyceride synthesis and increasing hepatic FFA oxidation (25). Circulating 17-beta estradiol also suppresses hepatic gluconeogenesis *via* FoxO1 signaling, independent of IRS-1 and IRS- 2 (26). In mice IRS-2 is transcriptionally-attenuated as a function of sterol-regulatory element binding protein (SREBP) activation and FoxO suppression (27–31). This is possibly a consequence of hyperinsulinemia-induced downregulation of IRS-2 facilitating hepatic IR (32, 33). Further, growing evidence indicates that hepatic DAG accumulation potentiates hepatic IR (34) and DAG levels inside hepatocyte lipid droplets were particularly-informative predictors of IR in humans (35).

The paradox of increased hepatic lipogenesis in the presence of hepatic IR

One of the molecular pathways of insulin signaling is the activation of Akt which, as it suppresses hepatic gluconeogenesis,

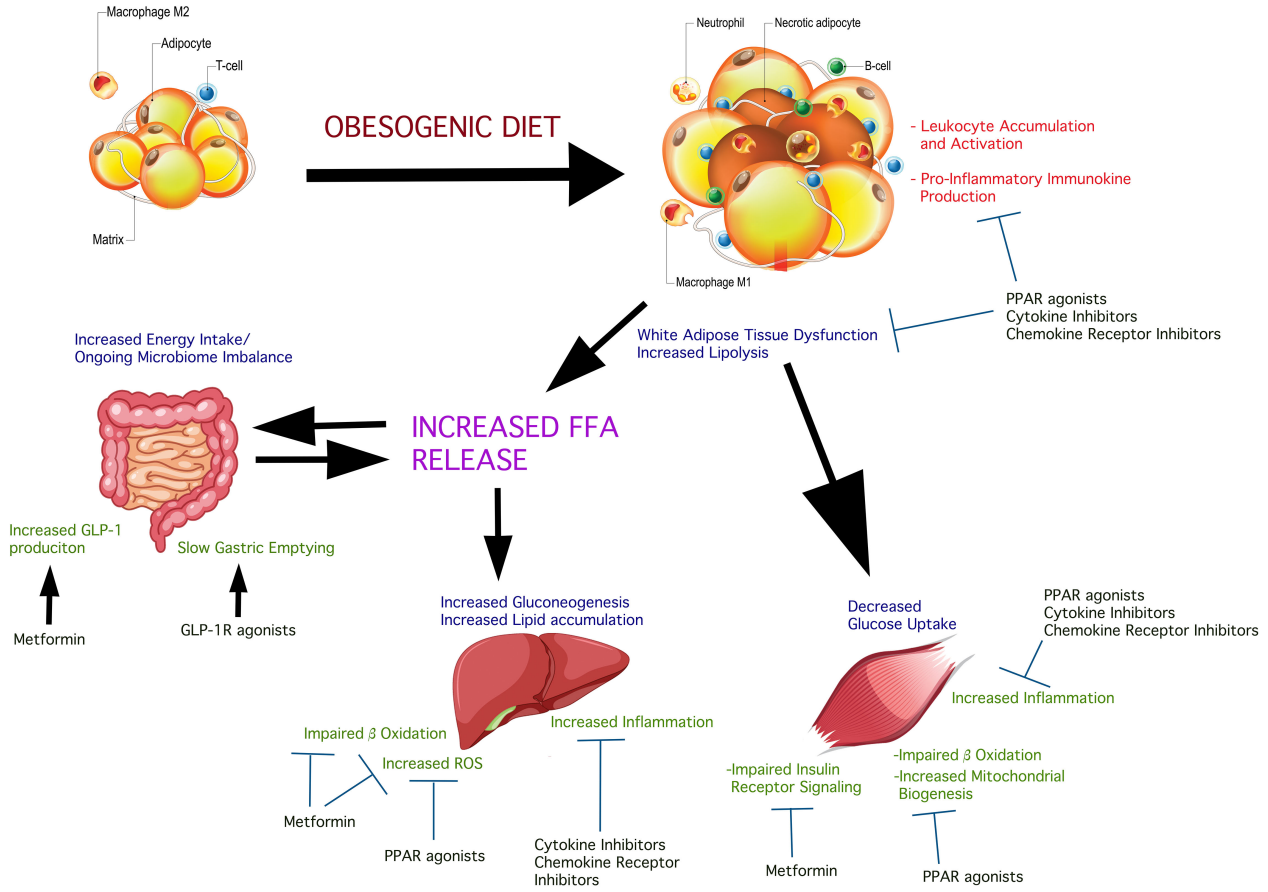

FIGURE 1
Insulin-sensitive inter-organ effects of obesogenic diets in the deterioration of insulin sensitivity and possible treatment pressure points. Obesogenic diets promote a state of systemic low-grade inflammation which contributes to, and is response to pathologic changes in glucolipometabolism in the main insulin-sensitive tissues and organs. The intestinal microbiome is altered causing changes in the complement of short-chain fatty acids produced. These are released into the circulation affecting insulin sensitivity and potentiate systemic and insulin-sensitive tissue inflammation. Obesogenic diet causes adipose hypertrophy and expansion, resulting in the conversion of resident M2 macrophages into pro-inflammatory M1 macrophage. Concurrent core adipose hypoxia creates an environment that signals "danger". This initiates the accumulation of neutrophils and other leukocytes which become activated, further potentiating local inflammation. The net result is peripheral insulin resistance, consequent to insulin receptor signaling impairment due to the action of immunokines produced by the accumulating pro-inflammatory leukocytes. IL-6, for example, acting *via* the IL-6 receptor on adipocytes, impairs insulin-stimulated phosphorylation of signaling components downstream of the insulin receptor tyrosine kinase. Skeletal muscle is susceptible to expanding fat and accumulation of lipid droplets, as well as the effects of circulating FFA on hepatocytes cause the accumulation of leukocytes and their activation, with a net effect of insulin signaling impairment. The different classes of agents shown in the Figure have shown variable beneficial effects on insulin sensitivity. Combination approaches could simultaneously act systemically and on the key insulin-sensitive tissues, attenuating inflammation thus facilitating better insulin sensitivity.

in parallel causes activation of sterol regulatory element binding protein 1c (SREBP1c). As demonstrated in transgenic rat hepatocytes, this is a consequence of Akt-stimulated mammalian target of rapamycin complex-1 (mTORC1) activity which regulates the transcription and stability of SREBP1c (36). Activated SREBP1c stimulates increased expression of genes encoding key enzymes in FA biosynthesis including those of the fatty acid elongase complex, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and ATP citrate lyase (37). A seeming paradox is observed inside the liver with developing obesity and progression towards T2D-associated NAFLD. Impairment of gluconeogenesis suppression occurs concomitant with *de novo* lipogenesis (DNL) and IR. This can be partially explained as a function of liver insulin signaling stimulating hepatic DNL whose biochemical pathway products predispose and drive the impairment of gluconeogenesis suppression. These biochemical pathway products and their concentrations, evidence suggests, determine the onset and rate of hepatic structural and cellular damage observed in the onset of NAFLD in mice (38). The question that remains to be better understood is, what is the point in hepatic insulin signaling where its effects on glucose and lipid metabolism diverge?

Some evidence suggests that mTORC1 may be one such point of divergence at the level of hepatic hyperinsulinemia and resistance. Studies in rodents have shown that the blockade of Akt and PI3K activity prevents insulin-mediated expression of genes of enzymes involved in gluconeogenesis while mTORC1 prevented insulin-dependent induction of SREBP1c without any effect on suppression of expression of gluconeogenetic genes (39). mTORC1 is a nutrient-sensing biochemical control point promoting its re-distribution to the lysosome (40–42). However,

as demonstrated in transgenic mouse models, mTORC1, on its own, is insufficient to cause *de novo* lipogenesis and NASH, at least in the absence of Akt2 (43). The nuance in these observations is best evaluated noting the role of tuberous sclerosis complex (TSC) proteins (44, 45). A number of mouse models have shown that Akt stimulation of mTORC1 is conditioned on TSC2 inhibition. Hepatic deletion of TSC1 results in an insulin-depended mTORC1 activation and protects from steatosis and *de novo* lipogenesis (44, 46). Additional studies in mice exhibiting hepatocyte-targeted inactivating genetic modifications of Akt, FoxO1, and TSC1, insulin-dependent co-ordinate activation of mTORC1 and FoxO1 inhibition were considered to be sufficient and possibly-necessary for insulin-dependent *de novo* lipogenesis (47, 48).

The stress response as one of the mechanisms involved in the evolution of hepatic IR

Co-incident with the onset of NAFLD, are a series of changes inside hepatocytes indicating an acute stress response; changes concomitant with intra-hepatic inflammation (49). Central to this stress response is the unfolded protein response (UPR) with its fulcrum point the endoplasmic reticulum (ER). Hepatic ER stress has been observed in NAFLD (50) and related to its progression, including its mechanistic relationship with hepatic insulin resistance (51). ER stress has been coupled to steatohepatitis-associated insulin resistance (52). Moreover, *de novo* lipogenesis in the liver has also been linked to hepatocyte ER stress (53). Pharmacologic suppression of Caspase-2 as well as Caspase-2 disruption, observed in hepatocyte ER stress-associated NASH prevented fibrosis and inflammation by preventing SREBP1 and SREBP2 activation. These observations suggested that ER stress could participate in the early onset of hepatic insulin resistance, *de novo* lipogenesis and the progression towards NAFLD.

Amino acids in the evolution of hepatic IR

It stands to reason that, especially under HFD conditions, lipids and FFAs are widely-viewed as the basis of IR, systemic or hepatic, however, other metabolites, especially in high fat "Western diets" have been implicated. Several amino acids (AA) have been shown to contribute to IR (15). In humans, AA elevation in plasma impairs insulin-stimulated glucose disposal in skeletal muscle. The mechanism appears to be through the mammalian target of rapamycin (mTOR)/S6 kinase pathway and phosphorylation of IRS-1 (54). Branched-chain (BC) AA are constituents of liver gluconeogenesis and their levels in the circulation have been found to be correlated with IR in humans (55). In skeletal muscle under hypersinulinemic conditions, BCAA impair glucose disposal and augment ATP synthesis without any effect on mitochondrial abundance of DNA (56, 57). In contrast, transient dietary reduction of BCAA reduces post-prandial insulin secretion and improves adipose metabolism (58).

Leukocytes, immunokines, and inflammation: cause or outcome of hepatic IR, in response to metabolic stress?

Macrophages are possibly the first leukocytes to accumulate inside the liver of obese individuals concomitant to IR onset (peripheral and/or hepatic) (59). These cells impair insulin signaling mainly *via* secreted immunokines (60). Liver-resident macrophages have been implicated in the onset and progression of hepatic IR and a number of overlapping mechanisms have been identified in their activation. While the following observations have been made mainly in skeletal muscle, and muscle-associated adipose, one can anticipate similar mechanisms to participate in hepatic IR: Accumulation of lipids inside myotubes in humans and rodent models, stimulates NF-κB nuclear translocation, attenuated mitochondrial respiration, fragmentation and mitophagy and elevated production of reactive oxygen species (ROS) (61). Systemic IR is widely-reported to co-incide with macrophage accumulation and activation inside adipose (62), however, adipose IR can manifest adipose macrophage accumulation and activation (63), suggesting that, at least in some instances, IR can precede an inflammatory state and may in fact represent a "danger" signal causing the eventual activation of Kupffer cells and liver macrophages. Potential mechanisms underlying an IR-first cause could involve local hyperinsulinemia-stimulated activation of these leukocytes and/or hyperinsulinemia-stimulated increase in microvascular blood flow, hyperoxygenation and hepatic cell stress. Hyperinsulinemia would then be a consequence of pancreatic β cell impairment. A number of known mechanisms of peripheral IR could cause beta cell impairment *via* stress induction, UPR, and failure to sense glucose/produce insulin (64, 65).

Overnutrition and obesity lead to a systemic low grade chronic inflammatory state referred to as meta-inflammation, characterized by adipocyte necrosis and altered secretory phenotype in adipocytes (66–68). This results in the recruitment and release of proinflammatory cells and cytokines, such as TNFα expressed by macrophages and monocytes infiltrating obese adipocytes. Adipose tissue contains predominantly M2 macrophages, with a phenotypic switch to M1 in obese persons. M1 macrophages produce chemokines such as MCP-1 which recruit circulating monocytes to the liver and adipose tissue where they can undergo maturation into the pro-inflammatory M1 phenotype. Adipocytes also produce low levels of TNFα, leading to MCP-1 production and macrophage infiltration in adipocytes, triggering release of pro-inflammatory cytokines, such as IL-6 and IL-1β (69). The level of pro inflammatory cytokines in subcutaneous abdominal adipose tissue, inversely correlates with hepatic and systemic insulin sensitivity. Obese individuals with NAFLD have shown a decrease in hepatocyte insulin signaling compared to obese individuals with

normal intrahepatic triglycerides (70). This low grade chronic inflammatory state in adipose tissue further contributes to IR *via* TNFα mediated serine/threonine phosphorylation of IRS-1, leading to enhanced lipolysis and increased exposure of hepatocytes to lipids (71, 72), fueling the progression of NASH.

More recent human and rodent studies, however, show that macrophages alone may not be sufficient to be involved in hepatic pathology concomitant to obesity-driven IR. Accumulation of neutrophils occurs very close to, or concurrent with that of macrophages (73). Indeed, more recent data demonstrate a prominent role of neutrophils over macrophages as being pivotal leukocytes that license and co-operate with macrophages in the onset of IR and T2D (74, 75). Neutrophil migration to sites of "danger" and their activation is a function of the balance of the CXCR2/CXCR4 chemokine receptor density on their surface (76). Neutrophil-attracting CXCR2 ligands are expressed in the pancreas, adipose and liver (77), suggesting that under potentially-stressful states, their secretion can be expected to recruit and activate neutrophils, which in turn would exacerbate and amplify a low grade inflammatory condition (78).

With the activation of leukocytes inside the liver, such as macrophages, growing intra-hepatic lipid deposition results in immunokine release [reviewed in (79) and (80)] which potentiates adipocyte lipolysis (81) concomitant to inhibition of hepatic insulin signaling (81, 82). Immunokines promote not only hepatic, but also systemic IR (83, 84), and cytokines like TNFα are detectable and upregulated in concentration inside the liver and adipose tissue of NASH patients (85), suggesting that upregulated TNFα in adipose might potentiate the progression of NAFLD in two ways: systemic IR and activation of a peripheral inflammation of insulin-responsive tissues (86). For example, adipose-produced IL-6 in liver stimulates hepatic SOCS3, suppressing insulin signaling, resulting in hepatic IR (87). Serum IL-6 concentrations are elevated in NAFLD and NASH (88).

Possible strategies to improve hepatic insulin sensitivity

The most obvious approaches to improving insulin sensitivity are diet changes and exercise that result in weight loss. However, work-life balance, in many instances, can impede commitment to defined diet and even low-level exercise activity. The distinct sex related disparities in the prevalence of NAFLD due to an interplay of sex hormones, age related hormonal changes as well as diseases such as polycystic ovarian syndrome and Turner's Syndrome may warrant exploration into sex-specific therapeutic strategies that have been presented and/or reviewed elsewhere (89–93).

An array of different medicinals has been developed specifically to lower glucose concentrations, improve insulin production and/or correct weight and attenuate inflammation. Table 1 presents the clinical studies where insulin sensitivity, and hepatic insulin sensitivity in particular, was one of the outcome measures. Other classes of drugs have been repurposed for these indications. Their effects on IR have been mild to variable. A single class of agent to improve insulin sensitivity together with prevention of IR-associated liver pathology remains to be discovered, although we have shown that a neutrophil-targeting CXCR2 antagonist could offer such a solution [see below, (105)].

Antihyperglycemic agents

Sulfonylureas lower blood sugar concentrations by stimulating insulin secretion independent of food intake, however, they are associated with hypoglycemia. While some studies demonstrated beneficial effects on IR, others could not (106, 107). Sulfonylurea use is slowly being replaced by newer agent classes to treat hyperglycemia.

Metformin remains a first-line glucose lowering agent. Although the underlying mechanism of action remains incompletely understood, it appears that it inhibits the hepatic glycerol-3-phosphate dehydrogenase activity, resulting in suppression of glycerol-induced gluconeogenesis and increased cytosolic redox state. Together, these actions lead to a reduction in lactate dehydrogenase and lactate-induced endogenous glucose production (108). Other possible mechanisms of action include the inhibition of complex I followed by increased AMP, activating AMP kinase and facilitating fatty acid oxidation in liver and reduced expression of genes encoding enzymes involved in gluconeogenesis. Additionally, AMP interferes with glucagon signaling and gluconeogenesis (108). In non-hepatic tissues, metformin increases insulin stimulated glucose utilization (108) and AMP kinase activity (109). A meta-analytic inspection of 11 randomized controlled trials (RCT) in obese and overweight adolescents, revealed that metformin reduced fasting plasma glucose (FPG) at less than 6 months, without impacting insulin sensitivity (110). Another meta-analysis of 31 RCT using metformin for more than 8 weeks in individuals at high risk for T2D revealed that it improved insulin sensitivity concurrent with a reduced incidence T2D (111). An additional meta-analysis in patients with NAFLD revealed benefit in insulin sensitivity without, however, any improvement in NAFLD liver histology (112).

Peroxisome proliferator-activated receptor agonists

PPAR agonists, particularly those for PPARγ, have shown promising efficacy in improving IR and liver histology in T2D-associated NAFLD. As a class, they also suppress the production of pro-inflammatory immunokines concurrent with stimulation of adiponectin production (113, 114). Pioglitazone treatment of T2D patients has resulted in beneficial outcomes in NAFLD (62) resulting in improved liver and peripheral insulin sensitivity (101). While its use has been somewhat questioned due to adverse event concerns (115), a more recently-developed agent, lobeglitazone, exhibits improved safety with improvements in insulin sensitivity and liver steatosis in T2D-associated NAFLD (116). Another PPARγ-sparing agent, MSDC-0602K, also achieves

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TABLE 1 Clinical trials assessing the 3-month (and greater) outcomes on insulin sensitivity in overweight/obese individuals with or without type 2 diabetes.

Study Agent	Study Design	Main Outcome(s)	Metabolic Outcomes	Reference
Lixisenatide vs Placebo	Randomized, Placebo-controlled	Decreased HbA1c	Decreased - FPG - BW - 2hr PPG Increased - HOMAβ	Ahren et al. (94)
Dulaglutide vs Liraglutide	Randomized, Parallel	Decreased HbA1c	Decreased - FPG - BW - PPG	Dungan et al. (95)
Exenatide vs Placebo	Randomized, Placebo-controlled	Decreased - HbA1c - Hepatic triglycerides - Epicardial adipose	Decreased - BW	Dutour et al. (96)
Dulaglutide vs Liraglutide vs Placebo	Randomized, Placebo-controlled	Decreased HbA1c	Decreased (both agents vs. placebo) - HbA1c - FPG Increased - HOMA-2 %β	Miyagawa et al. (97)
Empagliflozin vs Placebo	Randomized, Placebo-controlled	Decreased Hepatic Lipid Content	Decreased - FPG - BW - Uric acid	Kahl et al. (98)
SAR425899 vs Liraglutide vs Placebo	Randomized, Parallel	Decreased HbA1c (both agents vs. placebo) Increased - HOMA-2 %S		Schiavon et al. (99)
Saroglitazar vs Placebo	Randomized, Placebo-controlled	Increased - Glucose Metabolism (M) - Insulin Sensitivty (M/I) - HOMA-β	Decreased - HbA1c - FPG - Triglycerides Increased - HDL-C	Jain et al. (100)
Pioglitazone vs Placebo	Randomized, Placebo-controlled	Increased - Glucose Disposal Rate - Insulin-Stimulated Suppression of Endogenous Glucose Production	Decreased - HbA1c - FPG - Plasma TG - Visceral Fat - BW Increased - BW - Fat Mass - Subcutaneous Fat	Miyazaki et al. (101)
Semaglutide vs Empagliflozin	Randomized Active Control	Decreased - HbA1c	Decreased - FPG - Fasting Plasma Insulin - Fasting C-Peptide - BW - CRP	Rodbard et al. (102)
Canagliflozin vs Placebo		Decreased - Hepatic Triglycerides Increased - Insulin-Stimulated Suppression of Endogenous Glucose Production - Beta Cell Function	Decreased - HbA1c - FPG - Fasting Plasma Insulin - BW Increased - Insulin Clearance - FFA	Cusi et al. (103)

(Continued)

TABLE 1 Continued

Study Agent	Study Design	Main Outcome(s)	Metabolic Outcomes	Reference
Saroglitazar vs Pioglitazone	Randomized, Parallel	Decreased - HbA1c - FPG	Decreased - Triglycerides - VLDL-C - LDL-C - HDL-C	Krishnappa et al. (104)

insulin-sensitizing peripheral effects safely (117). More recently, CHS-131 demonstrated significant dual-target outcomes, improving fasting insulin levels and insulin sensitivity, total plasma cholesterol, triglycerides, liver enzymes, and increased plasma adiponectin levels. Most importantly, CHS-131 improved liver histology and markers of hepatic fibrosis (118). Fibrates, ligands of PPARα, reduce fasting plasma glucose, insulin, and improve insulin sensitivity (119) although some questions remain about their true efficacy (120). Seladelpar and GW501516 are PPARδ agonists shown to improve insulin sensitivity in obese individuals (120, 121) with mechanisms of action that include increased fatty acid oxidation in skeletal muscle and attenuation of macrophage pro-inflammatory state (122). Another PPAR agent is Elafibranor, a PPARα/δ agonist, which reduces inflammation and enhances both peripheral and liver insulin sensitivity under obese conditions (123, 124), although the latter findings remain to be validated (125). Saroglitazar is a dual PPARα/γ agonist with whole body insulin sensitivity improvement without adverse events noted with the use of other PPARα/γ agonists (100, 104). A pan-PPAR agonist, lanifibranor, is currently being tested in phase II studies, with enabling data showing improved insulin sensitivity in T2D and improved intra-hepatic lipid content in T2D-associated NAFLD (clinicaltrials.gov #NCT03459079).

Fatty acid synthetases

A randomized single blinded phase 2a clinical trial evaluated the efficacy of a fatty acid synthetase inhibitor TVB-2640 on *de novo* lipogenesis in a population of NASH patients (126). Fatty acid synthetases convert metabolites of simple sugars to palmitate (126). The rationale behind this was to reduce *de novo* lipogenesis in patients with NASH. The outcome demonstrated decreased liver fat by 9.6% in a population with fatty liver and fibrosis that included subjects with diabetes.

Incretins

GLP-1 agonists like exenatide, liraglutide, semaglutide, and lixisenide can improve insulin sensitivity, although it is not clear if this effect is in the periphery or in the liver as well (94, 96, 102, 127, 128). Glucose-dependent insulinotropic polypeptide (GIP; tirzepatide) use also achieved some insulin sensitivity improvement in T2D, although again it is unknown if this acted at the level of the liver (127). Reduced hepatic inflammation and lipid deposition was demonstrated in T2D-associated liver pathology following a tri-pathway-targeting approach using HM1521, an agent that targets glucagon/GIP/GLP-1Ra in mice and in humans (117, 127).

α-Glucosidase inhibitors and sodium glucose co-transporter-inhibitors

While α-Glucosidase inhibitors (AGI) are not *a priori* thought of as agents that could affect IR, clinical studies have shown that they can, following establishment of a steady dose level (129, 130). These effects are expected to be extra-hepatic and a consequence of attenuation of hyperglycemia. In a similar manner, Sodium Glucose Co-transporter-2 Inhibitors (SGLT2I) have also demonstrated some insulin sensitivity enhancing effect (103, 131, 132) including a positive effect on liver IR (103) with neutral outcomes on non-hepatic IR (133).

Leukocyte and immunokine-targeting anti-inflammatory agents

It stands to reason that the accumulation of pro-inflammatory leukocytes and elevation of the concentration of their pro-inflammatory soluble mediators inside insulin-sensitive tissues is a high-priority target of therapy aimed to restore normal insulin-sensitivity in T2D as well as prevent any T2D-associated liver impairment that can be a consequence of, or drive hepatic IR. Salicylates were among the earliest agents tested for this objective and demonstrated mild improvement in peripheral glucose disposal (134, 135).

Inhibition of TNFα action with a variety of antibodies (etanercept, infliximab, adalimumab) improved insulin sensitivity in some patients, however, the heterogeneity of the study populations requires validation of those outcomes (135, 136). Targeting the IL-1β system (using IL-1 receptor antagonist protein, or antibodies like canakinumab and gevokizumab) improves glucoregulation overall, absent of any discernible effects on insulin resistance in T2D (135). In contrast, using the IL-6-targeting antibody tocilizumab, which aims to break the IL-6-mediated interference of insulin signaling, achieved statistically-relevant improvement of insulin sensitivity in obese patients (137).

Some excitement was generated when initial results from pre-clinical and early-clinical outcomes were reported showing improved hepatic function with the use of cenicriviroc, a dual CCR2/CCR5 chemokine receptor antagonist in hepatic pathology,

however these reactions were tempered when the agent was unable to improve insulin sensitivity in patients with NASH (138).

As neutrophil accumulation into areas characterized by molecular and microenvironmental structural anomaly is a function mainly of the balance of CXCR2 and CXCR4 ligands and the neutrophil cell surface ratio of CXCR2:CXCR4 chemokine receptors (76), modulation of signaling *via* these receptors was proposed to be potentially therapeutic for T2D progression, IR, and possibly NAFLD. CXCR2-deficient mice are resistant from high fat diet-induced IR and T2D and are characterized by reduced macrophage accumulation in adipose (139). We recently demonstrated that a selective CXCR2 antagonist, AZD5069 (140) treatment of high fat diet-fed mice, improved insulin sensitivity and insulin-induced suppression of hepatic glucose production, decreased hepatic lipid storage, and significantly-prevented the progression towards liver pathology reminiscent of NAFLD.

Myeloperoxidase (MPO) is a key enzyme in neutrophil respiratory burst, that generates reactive oxidation species. Studies have shown an increase in the prevalence of MPO-positive Kupffer cells and neutrophils in the liver during NASH. The free radicals produced by MPO could participate in liver damage, directly (on hepatocytes) and/or on the stroma. MPO-deficient mice fed a high fat diet were protected against NASH-related liver injury. Additionally, mice fed with an oral MPO inhibitor exhibited reduced transaminitis and fibrosis (141). Thus, this enzyme, targeted alone or together with CXCR2 inhibitors/antagonists could represent a novel therapeutic approach in liver IR-related NASH (142, 143).

Currently there are no FDA-approved single agent treatments for the concurrent management of insulin sensitivity and the prevention (or at least the attenuation of progression to) to NAFLD/NASH in individuals with metabolic syndrome and T2D. The closest drug to market is obeticholic acid which recently completed a phase 3 clinical trial, but has yet to be approved by the FDA due to safety concerns in long term adverse effects (144). Our outcomes with AZD5069, as a single agent, showing benefits in the prevention of progression of insulin resistance and liver pathology reminiscent of NASH/NAFLD, as well as clinical trials in humans showing that AZD5069 was very well-tolerated with few side effects (145), offer an opportunity for this and possibly other similar drugs (e.g. ladarixin (146),) to enter clinical consideration as adjunctive treatments to standard of care of obesity and T2D to prevent and/or attenuate insulin resistance and liver pathology.

AZD5069 and similar agents may be found to exert their overall effects in a wider-ranging manner. For example, by preventing CXCR2-stimulated inhibition of insulin-induced glucose transport in muscle cells (147). Additionally, by preventing the effects of IL-8 (produced by growing adipose) on insulin-induced Akt phosphorylation in adipocytes (148, 149). This furthers strengthens the rationale that these agents can be potentially helpful treatments in insulin resistance-incident obesity and T2D. Finally, ongoing studies in our laboratory will soon determine if neutrophil antagonism impacts macrophage accumulation and function and thus, in an indirect manner, AZD5069 and similar agents, such as ladarixin (146), could prevent accumulation and further activation of liver-resident macrophages.

Modulation of lipid and energy metabolism

Improvement in insulin sensitivity in obesity and T2D-associated NAFLD have been achieved using lipid metabolism-modifying agents like ketohexokinase inhibitor, a protein tyrosine phosphatase-1B inhibitor, or an ω3-fatty acid [reviewed in (117)]. Liver-targeted dinitriophenyl (DNP)-methyl ether (DNPME) and mitochondrial protonophore (CRMP) aiming to motivate hepatic fatty acid oxidation while reducing lipid accumulation improved systemic IR in rodent and non-human primate models of obesity-associated NAFLD (150). Another mitochondrion-acting agent, BAM15, also showed evidence of improving systemic IR and liver inflammation as well as pathology in mouse models of obesity (150). Precise targeting of sensitive points inside these pathways without systemic adverse events or toxicities remains a largely-unexplored area of T2D pharmaceutical research, especially for the objective of improving IR concurrent with delaying or obviating liver pathology.

Discussion

It is now evident that inflammation dependent pathways have a clear pathological role in the propagation of NAFLD. Initially, IR and hepatic lipid accumulation result in oxidative stress and activation of inflammatory pathways in the liver. In fact, inflammation plays a key role in IR as well. Overnutrition and increased caloric intake, set the stage for IR *via* multiple mechanisms. IR and hepatic lipid accumulation result in oxidative stress and activation of inflammatory pathways in the liver. Additionally, ER stress culminates in the UPR aimed at reducing ER burden while simultaneously increasing the translation of pro-apoptotic proteins. Finally, obesity-mediated adipocyte inflammation and necrosis results in a systemic meta-inflammation mediated by macrophages and cytokines such as TNFα and IL-8. IR contributes to hepatic steatosis through an increase in the circulating FFA, further leading to inflammation dependent liver injury resulting in NASH. This happens through liver macrophages in combination with, as emerging evidence indicates, the increased recruitment of neutrophils through CXCR2 signals. This recruitment of inflammatory cells to the liver plays a key role in the pathogenesis of NASH. Functionally, peripheral IR, especially in the liver further impairs systemic glucoregulation. The liver is a key site of gluconeogenesis, typically down regulated by insulin *via* the interference in transcription of gluconeogenic genes. Insulin physiologically favors lipogenesis and inhibits gluconeogenesis. Paradoxically, during IR states in the liver, there continues to be an increase in lipogenesis and gluconeogenesis referred to as selective IR. This culminates in NASH and systemic hyperglycemia, contributing to the diabetic phenotype.

With respect to therapeutics, a novel approach is to target IR and interfere with the natural disease progression of NASH. Bearing in mind that IR often precedes NASH and has an overlapping pathogenesis in the form of systemic meta-inflammation,

combination therapy targeting at least two distinct inflammation networks would have maximum synergistic value. CXCR2 antagonists are a novel approach that have demonstrated both an improvement in insulin sensitivity and interference in the natural disease progression of NASH, through an interference in recruitment of inflammatory cells. CXCR2 antagonists in combination with PPARγ agonists may have a synergistic role considering the latter's proven efficacy in improving insulin sensitivity and potential in NASH treatment. PPARγ agonists improve insulin sensitivity by increasing adiponectin and GLUT-4 translocation. Though limited by their side effects such as pulmonary edema in clinical practices new alternatives like CHS-131 show promise in this aspect, alone or in combination.

Author contributions

SN and BP wrote the original draft of the manuscript with significant intellectual guidance by NG. SN and NG edited all versions of the manuscript. NG assumes responsibility of the final submitted draft. All authors contributed to the article and approved the submitted version.

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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Association between the lean nonalcoholic fatty liver disease and risk of incident type 2 diabetes in a healthy population of Northwest China: a retrospective cohort study with a 2-year follow-up period

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Aims: We aimed to explore the metabolic features of lean nonalcoholic fatty liver disease (Lean-NAFLD) and its association with the risk of incident type 2 diabetes in young and middle-aged people.

Methods: We conducted a retrospective cohort study of 3001 participants who were enrolled in a health check-up program from January 2018 to December 2020 in the Health Management Center of Karamay People's Hospital. The age, sex, height, weight, body mass index (BMI), blood pressure, waist circumference (WC), fasting plasma glucose (FPG), lipid profiles, serum uric acid and alanine aminotransferase (ALT) of the subjects were collected. The cutoff point of BMI for lean nonalcoholic fatty liver disease is <25 kg/m^2. A COX proportional hazard regression model was used to analyze the risk ratio of lean nonalcoholic fatty liver disease to type 2 diabetes mellitus.

Results: Lean NAFLD participants had many metabolic abnormalities, such as overweight and obesity with nonalcoholic fatty liver disease. Compared with lean participants without nonalcoholic fatty liver disease, the fully adjusted hazard ratio (HR) for lean participants with nonalcoholic fatty liver disease was 3.83 (95% CI 2.02-7.24, p<0.01). In the normal waist circumference group (man<90cm, woman<80 cm), compared with lean participants without NAFLD, the adjusted hazard ratios (HRs) of incident type 2 diabetes for lean participants with NAFLD and overweight or obese participants with NAFLD were 1.93 (95% CI 0.70-5.35, p>0.05) and 4.20 (95% CI 1.44-12.22, p<0.05), respectively. For excess waist circumference (man≥90 cm, woman ≥80 cm) compared with lean participants without NAFLD, the adjusted hazard ratios (HRs) of incident type 2 diabetes for

lean participants with NAFLD and overweight or obese participants with NAFLD were 3.88 (95% CI 1.56-9.66, p<0.05) and 3.30 (95% CI 1.52-7.14, p<0.05), respectively.

Conclusion: Abdominal obesity is the strongest risk factor for type 2 diabetes in lean nonalcoholic fatty liver disease.

KEYWORDS

nonalcoholic fatty liver disease, risk factor, type 2 diabetes, visceral fat obesity, cohort study

1 Introduction

At present, NAFLD has become one of the most common liver diseases affecting the health condition of adults and children in the world (1, 2) and has brought a huge burden to the global health care system. Approximately 25% of the global population is affected by NAFLD, and Middle Eastern countries and South America have the highest incidence of NAFLD in the world (3, 4). NAFLD is characterized by the accumulation of more than 5% fat in hepatocytes (5), which includes hepatic steatosis, steatohepatitis and liver fibrosis, and further development of the lesions can lead to cirrhosis and hepatocellular carcinoma (6) NAFLD is a multisystem disease that increases the risk of type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), some types of extrahepatic malignancies, and chronic kidney disease (CKD), and the magnitude of this risk parallels the severity of NAFLD (especially the stage of liver fibrosis) (7, 8).

NAFLD is closely related to type 2 diabetes mellitus. NAFLD and T2DM often coexist and act synergistically, increasing the risk of hepatic and extrahepatic adverse clinical outcomes (1). T2DM is also one of the strongest risk factors for faster progression of NAFLD to nonalcoholic steatohepatitis, advanced fibrosis, or cirrhosis (T2DM plays an important role in disease progression to NASH, liver fibrosis, and cirrhosis). The global prevalence of NAFLD in patients with T2DM was 55.5% (95% CI: 47.3-63.7) (9) more than half of T2DM patients have been diagnosed with NAFLD, and there is a strong correlation between them. Obesity, physical inactivity and metabolic syndrome are common risk factors (1, 10–12).

Nonalcoholic fatty liver disease (NAFLD) can be classified into lean or nonoverweight obese (BMI < 25 kg/m^2) and overweight obese (BMI≥25 kg/m^2) according to BMI (2, 13). A systematic review and meta-analysis reported in 2020. The prevalence rates of lean NAFLD and nonobese NAFLD in the general population are 5.1% and 12.1%, respectively. In the NAFLD population, lean NAFLD and nonobese NAFLD accounted for 19.2% and 40.8%, respectively (14). Studies have shown that not only overweight and obese NAFLD may have liver and extrahepatic complications. “Lean” or “nonobese” patients with nonalcoholic fatty liver disease (NAFLD) also have hepatic and extrahepatic complications, suggesting that metabolic phenotype is more important than the clinical classification of body mass index in the prognostic assessment of NAFLD (15). However, to date, the characteristics of the lean NAFLD population are still unclear, and there are still few studies on the prevalence and outcome of lean nonalcoholic fatty liver disease based on race (16).

Studies have shown that there is a bidirectional interaction between NAFLD and type 2 diabetes (12, 17). However, the direct relationship between NAFLD and the incidence of type 2 diabetes is still less studied, and the causal relationship between the two is still unclear, especially the association between “lean” or “nonobese” nonalcoholic fatty liver disease (NAFLD) and the incidence of type 2 diabetes. Further studies are needed (2) Compared with overweight and obese NAFLD, the incidence of type 2 diabetes in people with “lean” or “nonobese” nonalcoholic fatty liver disease is also less studied worldwide, especially in China. This retrospective cohort study was conducted to investigate the association between lean nonalcoholic fatty liver disease (NAFLD) and the risk of type 2 diabetes mellitus (T2DM) in healthy people undergoing physical examination in Karamay, Northwest China.

2 Materials and methods

2.1 Subjects (study design and study participants)

In Karamay, Northwest China, the Xinjiang Oilfield Company organizes a medical health checkup program for employees and citizens every year. The medical examinations were carried out at the Medical Examination Centre of Karamay People’s Hospital. This study is a retrospective cohort study. Adults who underwent annual physical examination in the Health Management Center of Karamay People’s Hospital of Xinjiang from January 1, 2018, to December 31, 2020, were selected as the study population. The inclusion criteria were as follows: (1) Participants participated in the annual physical examination (baseline examination) at the Health Examination Center of Karamay People’s Hospital from January 2018 to December 2018. (2) Age ≥20 years, no history of diabetes. (3) Participation in annual employee health check-ups in 2019 and

2020. The exclusion criteria for subjects of study were as follows:(1) Queer alcohol intake (male>30 g/day, female>20 g/day); (2) Combined with viral hepatitis, drug-induced liver disease, hepatolenticular degeneration, autoimmune liver disease and other specific diseases that can lead to fatty liver; (3) Baseline examination, fasting blood glucose ≥ 6.1 mmol/L; (4) Loss of fasting blood glucose during baseline examination or physical examination follow-up (5); Loss of abdominal ultrasound and other parameter data during physical examination follow-up.

A total of 4085 people participated in the baseline examination at the Health Examination Center of Karamay People's Hospital in 2018 and the annual employee health examination follow-up in 2019 and 2020. Based on the inclusion and exclusion criteria, a total of 3001 participants were included in the cohort analysis (see Figure 1). This research project follows the Helsinki Declaration and China's clinical research management norms and regulations. The research plan was approved by the Medical Ethics Committee of Karamay People's Hospital. Informed consent was obtained from all participants.

2.2 Baseline data collection and measurement

Sex, age, ethnicity, height, weight, BMI, blood pressure, waist circumference and past medical history were collected by the investigators. The subjects' height and weight were measured in an overnight-fasted state, shoes were removed, light clothes were worn, and the readings were accurate to 0.5 kg and 0.5 cm, respectively. Body mass index (BMI) was calculated as body weight (kg) divided by squared height (m^2) (kg/m^2). Waist circumference (WC) was taken as the circumference of the midpoint line between the lowest point of the rib and the upper edge of the iliac crest under normal breathing conditions. Fasting blood glucose (FPG), alanine aminotransferase (ALT), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-$_C$), low-density lipoprotein cholesterol (LDL-$_C$) and blood uric acid (BUA) were collected. The triglycerides and glucose index (TyG) were calculated as ln (fasting TG (mg/dL) ×FPG (mg/dL)/2) (18).

2.3 Ultrasound examination and diagnosis of NAFLD

Abdominal ultrasound examination was performed on subjects using a color Doppler ultrasound diagnostic instrument E9 (GE Company, USA) with a transduce of 3.5 MHz. All subjects were diagnosed with fatty liver according to the results of ultrasound examination. Inspectors of the clinical information of the subjects, according to the subjects of liver tissue echoes, the differences between the liver and right kidney and blood vessels of the structure of the visibility diagnosis, ultrasonic tip liver frontcourt echogenicity ("bright liver "), the far field echo attenuation, and the display are not clear, such as structural characteristics of the intrahepatic duct in the exclusion of alcohol, virus, autoimmune, drugs and other causes of fatty liver. The by experienced sonographers (19, 20).

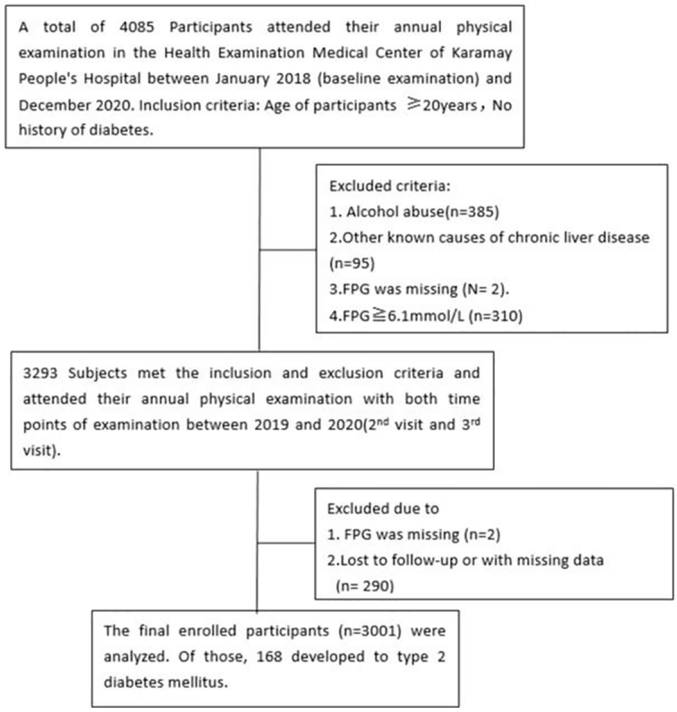

FIGURE 1
Flowchart of the present observational cohort study.

2.4 Endpoint and diagnosis of type 2 diabetes

The outcome event (study endpoint) was the onset of type 2 diabetes mellitus during the annual health check-up from 2019 to 2020. Survival was defined as the time from January 2019 to the date of diagnosis of type 2 diabetes at physical examination and was censored at the last follow-up physical examination in 2020 or at the last follow-up physical examination in 2020 without diabetes. Diabetes was diagnosed according to the 1999 World Health Organization (WHO) criteria: diabetes mellitus, fasting blood glucose ≥7.0 mmol/L, oral glucose tolerance test (OGTT) 2-hour postprandial blood glucose (2hPG) ≥11.1 mmol/L, or self-reported use of hypoglycemic drugs. Prediabetes: 6.1 mmol/L ≤ fasting glucose ≤7.0 mmol/L is impaired fasting glucose (IFG), and 7.8 mmol/L ≤2 hPPG ≤ 11.1 mmol/L is impaired glucose tolerance (IGT). Normal blood glucose: fasting blood glucose ≤6.1 mmol/L and OGTT 2hPPG ≤ 7.8 mmol/L (21).

2.5 the category used to define BMI and WC groups of NAFLD

The 3,001 participants were divided into four groups based on whether they were overweight/obese and NAFLD. The four groups were non-overweight/obese group without NAFLD (n = 1398), non-overweight/obese group with NAFLD (n = 160), overweight or obese group without NAFLD (n = 758), overweight or obese group with NAFLD (n = 685). BMI ≥ 25kg/m2 was defined as overweight/obese. WC≧ 90 cm in men,and WC≧≥ 80 cm in women was defined as abdominal obesity (22).

2.6 Statistical analysis

Excel 2007 was used to establish the database and manage the data, double input the data and correct the errors. SPSS 22.0 statistical package (IBM, Armonk, New York) was used for data processing for all statistical analyses. A normality test was performed on continuous variables of measurement data. Measurement data with a normal distribution are expressed as the mean ± standard deviation ($\bar{x}\pm s$), and continuous data with a skewed distribution are expressed as the median and interquartile range (IQR). The Kruskal–Wallis H test or Mann–Whitney U test was used for comparisons among groups. Categorical variables are expressed as percentages. The chi-square test was used to compare categorical variables. The 3001 participants were divided into four groups based on the presence or absence of overweight and NAFLD. Taking lean subjects without NAFLD as the reference group (compared with lean subjects without NAFLD), a COX proportional hazards regression model was used to analyze overall overweight (or obesity) without NAFLD, lean with NAFLD and overweight (or obesity) with NAFLD, and abdominal obesity (WC ≥ 90 cm in men and ≥ 80 cm in women) and nonabdominal obesity subgroups were associated with the risk of type 2 diabetes, and their hazard ratios and 95% confidence intervals were calculated. Hazard ratios (HRs) with 95% confidence intervals (CIs) for the incidence of diabetes were calculated for each study phenotype using Cox proportional-hazard regression models, with lean subjects without NAFLD as the reference group. The Kaplan–Meier method was used for survival analysis to draw the risk function curves of the above four categories of type 2 diabetes, and the log-rank test was performed to compare whether there was a difference in the risk of type 2 diabetes among the four groups. The Stata 17.0 was used to plot the figure of cumulative hazard estimates. The difference was statistically significant with a P value of <0.05 (two-tailed).

3 Results

3.1 Baseline clinical characteristics of subjects

A total of 3001 subjects were enrolled in the study. The average age of these people was 43 (34-49) years. The BMI was 24.84(22.55-27)kg/m^2, and there were 2255 men (75.1%) and 746 women. Of these, 845 had nonalcoholic fatty liver disease, while 2156 subjects had no NAFLD. A total of 81.1% of those with nonalcoholic fatty liver disease were overweight or obese, and 35.2% of those without NAFLD were overweight or obese. The number of subjects with lean nonalcoholic liver was 160, and the average BMI of these subjects was 23.86(23.05-24.48)kg/m^2. The number of subjects with overweight or obesity with nonalcoholic liver was 685, and the average BMI of these subjects was 28.65 (26.96-30.88)kg/m^2. In both the overweight (or obesity) with NAFLD group and the lean with NAFLD group, the baseline levels of fasting blood glucose, triglycerides, total cholesterol, low-density lipoprotein cholesterol, alanine aminotransferase, blood uric acid and TyG index were higher than those of any group without NAFLD, while the high-density lipoprotein cholesterol was lower than that of any group in the without NAFLD group (Table 1).

3.2 Incidence of type 2 diabetes in subjects with or without NAFLD

The follow-up period was 104 weeks (2.0 years). The results are shown in Table 2. The incidence rate of T2DM was 1.72% (24/1398) in the nonoverweight without NAFLD group, 11.88% (19/160) in the nonoverweight with NAFLD group, 5.01% (38/758) in the overweight without NAFLD group and 12.70% (87/658) in the overweight with NAFLD group. The number of participants with incident T2DM was larger in the NAFLD group than in the non-NAFLD group. The risk rate of type 2 diabetes was higher in the nonalcoholic fatty liver group than in the non-NAFLD group. In the case of unadjusted age, sex and other risk factors, the subjects in the lean with NAFLD group and overweight or obese with NAFLD group had HRs of 7.23 (95% confidence interval (CI) 3.96–13.20) and 7.77 (95% confidence interval (CI) 4.95–12.21), respectively, for

TABLE 1 Comparison of baseline characteristics of four groups from the subjects with NAFLD and the subjects without NAFLD.

Parameters	Total	Lean without NAFLD	Over- weight/Obesity without NAFLD	Lean with NAFLD	Over- weight/Obesity with NAFLD	H/X^2	P value
Number of subjects	3001	**1398**	**758**	160	685		
Age(year)	43(34-49)	42(33-48)	45(36-51)	45(36-50)	41(34-49)	45.477	<0.001
Male, N (%)	2255(75.1%)	875(62.6%)	626(82.6)	136(85.0%)	618(90.2%)	232.1	<0.001
BMI(kg/m^2)	24.84(22.55-27.46)	22.43(20.81-23.70)	26.84(25.81-28.40)	23.86(23.05-24.48)	28.65(26.96-30.88)	2296.922	<0.001
Waist circumference (cm)	89(81-96)	80.00(74.00-87.00)	94.00(89.00-99.00)	88.00(84.00-92.00)	99.00(93.00-104.00)	1641.133	<0.001
SBP (mmHg)	124.0(114.0-136.0)	120.00(109.00-129.00)	127.00(117.00-137.00)	128.50(116.00-137.00)	132.00(121.00-143.50)	331.834	<0.001
DBP (mmHg)	77.0(69.3-87.0)	73.00(66.00-81.00)	80.00(72.00-88.00)	78.50(72.75-88.00)	84.00(75.00-92.00)	325.173	<0.001
FBG (mmol/L)	5.37(5.14-5.73)	5.32(5.10-5.60)	5.38(5.18-5.74)	5.49(5.16-5.90)	5.47(5.18-5.92)	79.428	<0.001
TC (mmol/L)	4.57(3.92-5.19)	4.44(3.83-5.03)	4.56(3.99-5.20)	4.79(4.00-5.42)	4.83(4.16-5.45)	64.978	<0.001
HDL-C (mmol/L)	1.28(1.08-1.54)	1.45(1.22-1.73)	1.23(1.05-1.44)	1.19(1.03-1.45)	1.10(0.94-1.25)	561.377	<0.001
LDL-C(mmol/L)	3.02(2.50-3.58)	2.86(2.40-3.45)	3.05(2.56-3.61)	3.185(2.68-3.76)	3.25(2.70-3.80)	93.608	<0.001
TG (mmol/L)	1.42(0.97-2.15)	1.10(0.81-1.58)	1.50(1.10-2.17)	1.83(1.37-2.51)	2.12(1.51-3.01)	614.575	<0.001
ALT (U/L)	23.0(16.0-33.0)	18.0(13.0-15.0)	23.0(17.0-32.0)	30.50(22.0-41.75)	35.0(25.0-53.0)	705.754	<0.001
BUA (μmol/L)	334.0(276.0-394.0)	298.0(248.0-354.0)	342.0(291.0-397.0)	363.5(313.25-419.0)	387.09(335.5-444.0)	519.812	<0.001
TyG index	5.59(5.20-6.05)	5.33(5.01-5.690)	5.67(5.31-6.08)	5.90(5.55-6.26)	6.04(5.66-6.42)	649.744	<0.001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TGs, triglycerides; TC, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; ALT, alanine aminotransferase; BUA, blood uric acid; TyG index, a product of triglycerides and fasting glucose. The continuous data were expressed as median and interquartile range (IQR). Kruskal-Wallis H test or Mann-Whitney U test was used for comparison between groups. The categorical variables are expressed as percentages. Chi-square test was used to compare categorical variables.

TABLE 2 Subgroup analysis of the Cox proportional hazard model for the incidence of type 2 diabetes from the 3001 subjects with NAFLD and the subjects without NAFLD.

Grouping of subjects	No of subjects	No of subjects Who developed diabetes (%)	Hazard Ratio (95% CI)	
			Model 1	Model 2
Lean without NAFLD	1398	24(1.72)	1	1
Over weight (or obesity) without NAFLD	758	38(5.01)	2.97(1.78-4.95) **	1.80 (1.05–3.08) *
Lean with NAFLD	160	19(11.88)	7.23(3.96–13.20) **	3.83(2.02-7.24) **
Over weight (or obesity) with NAFLD	685	87(12.70)	7.77(4.95–12.21) **	3.84(2.28-6.47) **

Model 1 Risk factors were unadjusted; Model 2 adjusted for age, sex, TC, LDL-C, HDL-C, SBP, DBP, ALT, BUA, and TyG index.
NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; TC, total cholesterol; LDL-C. low density lipoprotein cholesterol: HDL-C. High-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; BUA, blood uric acid; TyG index, a product of triglyceride and fasting glucose; *p<0.05; **p<0.001, by chi-square test.

the development of diabetes compared with those in the lean without NAFLD group. After adjusting for the above risk factors, the subjects in the lean with NAFLD group and overweight or obesity with NAFLD group had HRs of 3.83 (95% confidence interval (CI) 2.02-7.24) and 3.84 (95% confidence interval (CI) 2.28-6.47), respectively, for the development of diabetes compared with those in the lean without NAFLD group (p<0.001). The results suggested that the risk of developing type 2 diabetes was similar in the two groups.

3.3 The cumulative hazard ratios of incident T2DM and the results of Kaplan–Meier survival analysis

The cumulative hazard ratios of incident T2DM are indicated in Figure 2. Univariate COX regression results according to the presence or absence of nonalcoholic fatty liver disease and overweight or obesity groups showed that both NAFLD and overweight/obesity were significantly associated with an increased risk of incident T2DM. Lean subjects without NAFLD were taken as the reference group, and the Logran method was used to compare the differences in the distribution of "survival" (pairwise comparisons of differences in the incidence of type 2 diabetes) among the four groups. The incidence of type 2 diabetes was the same in the lean with NAFLD group and the overweight/obesity with NAFLD group (3.83 vs 3.84, p=0.778). The incidence of type 2 diabetes was significantly different among the other groups, p<0.01 (Table 3).

3.4 Results of COX regression subgroup analysis of lean nonalcoholic fatty liver disease and risk of T2DM

According to the level of waist circumference, the study population was divided into a normal waist circumference group (man<90 cm, woman<80 cm) and an excessive waist circumference group (man≥90 cm, woman≥80 cm). In the normal waist circumference group, the lean without NAFLD group was used as the reference group. After adjusting for risk factors such as age, sex and blood pressure, the overweight or obesity without NAFLD group and lean with NAFLD group had a risk of type 2 diabetes of 0.60 (0.14-2.66) and 1.93 (0.70-5.35), respectively (p>0.05), and the overweight or obesity with NAFLD group had a risk of type 2 diabetes of 4.20 (1.44-12.22), p<0.01, while in the excessive waist circumference group. After adjusting for risk factors such as age, sex and blood pressure, the lean without NAFLD group was taken as the reference group. The lean with NAFLD group and overweight (or obesity) with NAFLD group had a risk of type 2 diabetes of 3.88 (1.56-9.66) and 3.3 (1.52-7.14), respectively (p<0.01), while the overweight or obesity without NAFLD group had a risk of type 2 diabetes of 1.80 (0.82-3.93), p>0.05 (Table 4).

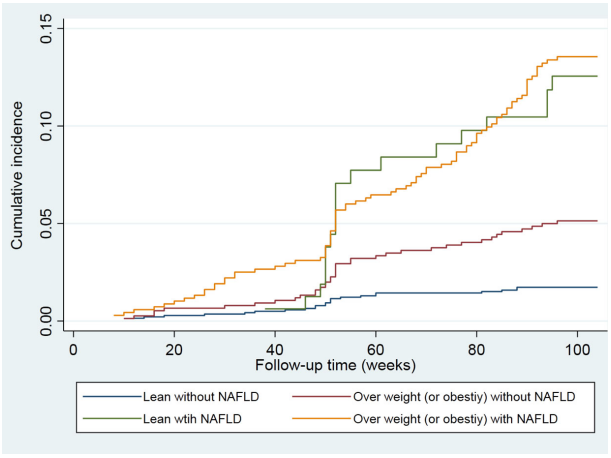

FIGURE 2
The cumulative hazard ratios of incident T2DM in different groups.

4 Discussion

In our present study, among 3001 eligible participants, 28.16% had NAFLD, and 5.33% had lean NAFLD. In the NAFLD population, 18.93% had lean NAFLD. The detection rate of NAFLD was 10.27% in those with BMI<25 kg/m^2 and 45.6% in those with BMI ≥ 25 kg/m^2. Our results are similar to those of previous studies. In a recent study from the United States, Zou B et al. found that the overall prevalence of NAFLD was 32.3%. Among patients with NAFLD, 29.7% were nonobese, and 13.6% had lean NAFLD (23). A large meta-analysis covering 84 studies worldwide showed that 19.2% of the subjects in the NAFLD population were lean, 40.8% were nonobese, and the prevalence of nonobese NAFLD and lean NAFLD was 12.1% and 5.1%, respectively (14). ShiY et al. reported a meta-analysis of 55,936 lean/nonobese subjects, and the total prevalence of NAFLD in lean and nonobese subjects was 10.2% and 15.7%, respectively (24). Zou ZY et al. reported an overall prevalence of NAFLD of 14.5% in a meta-analysis that included 155,846 nonobese participants (25). The prevalence of lean NAFLD increased between 1988 and 2017. Results of a meta-analysis of 33 observational studies involving 205,307 individuals from 14 countries. The global prevalence of lean NAFLD was 4.1% (95% CI: 3.4-4.8%). Among lean subjects, the prevalence of NAFLD was 9.7% (95% CI: 7.7-11.8%), and Asians had the highest prevalence of lean NAFLD (4.8%, 95% CI: 4.0-5.6%) (26). Table 1 shows that both overweight and obese NAFLD and lean NAFLD have much higher metabolic characteristics than those without NAFLD, and the risk of metabolic diseases is correspondingly increased

Among the 4 groups in the present study, the lean with NAFLD group had the oldest average age and male predominance (85.0%). BMI and waist circumference were higher than those in the lean without NAFLD group, and fasting blood glucose was the highest. Systolic blood pressure, serum total cholesterol, low-density lipoprotein, triglyceride and blood uric acid levels were also higher than those in the non-NAFLD group, and high-density lipoprotein was lower than those in the non-NAFLD group. The results of this study showed that the metabolic index value level of the lean with NAFLD group was basically the same as that of the

TABLE 3 Log rank (Mantel–Cox) test results of paired comparisons.

Grouping of subjects	N1		N2		N3		N4	
	X^2-value	P-value	X^2-value	P-value	X^2-value	P-value	X^2-value	P -value
N1			19.097	.000	56.323	.000	111.138	.000
N2	19.097	.000			10.699	.001	26.583	.000
N3	56.323	.000	10.699	.001			.079	.778
N4	111.138	.000	26.583	.000	.079	.778		

N1, lean without NAFLD; N2, overweight (or obesity) without NAFLD; N3, lean with NAFLD; N4, overweight (or obesity) with NAFL.

TABLE 4 Rates of incident and hazard ratio of type 2 diabetes based on waist circumference of the 3001 subjects with NAFLD and the subjects without NAFLD.

By waist circumference,	No of subjects	No of subjects who developed diabetes (%)	Hazard Ratio (95% CI)	
			Model 1	Model 2
Normal waist circumference (man<90 cm, woman<80 cm)				
Lean without NAFLD	1099	16(1.46)	1	1
Over weight (or obesity) without NAFLD	139	2(1.44)	0.99(0.23-4.30) #	0.60 (0.14-2.66) #
Lean with NAFLD	78	7(8.97)	6.35(2.61-15.44) **	1.93(0.70-5.35) #
Over weight (or obesity) with NAFLD	44	5(11.36)	8.08(2.96-22.07) **	4.20(1.44-12.22) *
Excess waist circumference (man≥90 cm, woman≥80 cm)				
Lean without NAFLD	298	8(2.68)	1	1
Over weight (or obesity) without NAFLD	619	36(5.82)	2.21(1.03-4.74) *	1.80(0.82-3.93) #
Lean with NAFLD	82	12(14.63)	5.70(2.33-13.95) **	3.88(1.56-9.66) *
Over weight (or obesity) with NAFLD	641	82(12.79)	5.0(2.42-12.34) **	3.30(1.52-7.14) *

Model 1 Risk factors were not adjusted; Model 2 adjusted for age, sex, TC, LDL-C, HDL-C, SBP, DBP, ALT, BUA, and TyG index.
NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; TC, total cholesterol; LDL-C. Low-density lipoprotein cholesterol: HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure DBP, diastolic blood pressure; ALT, alanine aminotransferase; BUA, blood uric acid; TyG index, a product of triglyceride and glucose index; #p>0.05; *p<0.01; **p<0.001, by chi-square test.

overweight (or obesity) with NAFLD group, which was the same as the results of other similar studies (13, 27).

Insulin resistance (IR) is not only the main pathogenesis of obese nonalcoholic fatty liver disease but also plays a key role in the pathogenesis of lean NAFLD (28). Studies have shown that the triglycerides and glucose index (TyG index) could be a reliable surrogate index for IR (18, 29), and the results of this study suggested that the insulin resistance level of people with lean NAFLD was higher than that of lean without NAFLD and overweight (or obesity) without NAFLD and was slightly lower than that of those with overweight (or obesity) with NAFLD. Our study suggests that insulin resistance plays an important role in the pathogenesis of lean fatty liver disease and type 2 diabetes mellitus.

To date, there are few studies on the association between lean fatty liver and the risk of type 2 diabetes, and the definition of lean fatty liver is based on BMI<25.0 kg/m^2 or <23.0 kg/m^2 without waist circumference stratification. Fukuda T and his colleagues had shown that 'A cutoff point of BMI 23 kg/m^2 was used to define overweight (≥23.0 kg/m^2) or nonoverweight (<23.0 kg/m^2). This was a population-based retrospective cohort study of 4629 participants who were enrolled in a health check-up program for a mean follow-up of 12.8 years. The adjusted hazard ratios for incident T2DM compared with the nonoverweight without NAFLD group were as follows: 3.59 (95% CI: 2.14–5.76) in the nonoverweight with NAFLD group, 1.99 (95% CI: 1.47–2.69) in the overweight without NAFLD group and 6.77 (95% CI: 5.17–8.91) in the overweight with NAFLD group. The adjusted hazard ratio in the nonoverweight with NAFLD group was significantly higher than that in the overweight without NAFLD group or that in the nonoverweight without NAFLD group (30). Another cohort study from the Japanese Physical Examination Population Database (JPEPD) with an average follow-up of 6 years showed that after adjusting for confounding factors, the fully adjusted HR (95% CI) for incident diabetes in lean NAFLD vs lean without NAFLD patients was 2.58 (95% CI: 1.68 -3.97) in the study population as a whole or in subgroups stratified by sex, and the risk of type 2 diabetes was the same for lean and overweight or obese with NAFLD (31).

In our study, lean nonalcoholic fatty liver was defined as a BMI of less than 25.0 kg/m^2. If waist circumference was not stratified,

after adjusting for related risk factors, compared with nonalcoholic fatty liver with BMI<25.0 kg/m^2. The adjusted hazard ratios for incident T2DM were 3.83 (2.02-7.24) in the lean with NAFLD group, 1.80 (1.05–3.08) in the overweight or obesity without NAFLD group and 3.84 (2.28-6.47) in the overweight or obesity with NAFLD group. Lean nonalcoholic fatty liver group than in the study of the risk of type 2 diabetes in the Japanese population is higher, the reason is that we study the lean nonalcoholic fatty liver disease in cutting point than their high, but lean nonalcoholic fatty liver disease group and overweight fuelling nonalcoholic fatty liver disease is the same risk for type 2 diabetes (3.83 vs3.84). After stratification by waist circumference, this study found that in the normal waist circumference group, compared with the BMI<25.0 kg/m^2 and nonalcoholic fatty liver groups, the hazard ratio of type 2 diabetes in the lean nonalcoholic fatty liver group was 1.93 (0.70-5.35, P>0.05) after adjusting for related risk factors. In the overweight waist circumference group (abdominal obesity group), the risk ratio of type 2 diabetes in the lean nonalcoholic fatty liver group was 3.88 (1.56-9.66), P<0.05. The results of this study showed that overweight nonalcoholic fatty liver disease is an independent risk factor for type 2 diabetes in the presence of a normal waist circumference, while lean nonalcoholic fatty liver disease is not an independent risk factor for type 2 diabetes. The high incidence of type 2 diabetes in people with lean nonalcoholic fatty liver disease may be due to the higher level of insulin resistance, higher blood lipid levels, and different degrees of steatohepatitis in this group.

In the presence of excess waist circumference (abdominal obesity), lean nonalcoholic fatty liver disease was an independent risk factor for type 2 diabetes, and the risk of type 2 diabetes was slightly higher than that of overweight and obese nonalcoholic fatty liver disease (3.88 vs 3.30). In abdominal obesity, there is an increase in visceral fat, which is a major source of free fatty acids and inflammatory cytokines. Increased levels of visceral fat can lead to insulin resistance and type 2 diabetes. Feng RN et al. found that abdominal obesity was closely related to type 2 diabetes in Chinese adults (32). NAFLD is strongly associated with the pathogenesis of type 2 diabetes mellitus. NAFLD is a multisystem disease characterized by "ectopic fat accumulation" in the liver, leading to a series of pathophysiological manifestations. When hepatic fat accumulation occurs, long-chain fatty acids (LCFAs) and triglycerides 3-phosphate (derived from glycolysis) form monoacylglycerol, diacylglycerol (DAG), and triacylglycerol (TAG) within hepatocytes. Lipid synthesis can increase the production of intermediates, such as diacylglycerol DAG, dipalmitoyl phosphate (Di-P PA), and other lipid products, such as ceramides. Increased production of these lipid products, especially DAG, leads to "resistance" within the hepatic insulin signaling pathway, and ceramide inhibits distal insulin signaling, which is also a mediator of inflammation and oxidative stress. In addition, liver fat accumulation can secrete hepatokines, which affect the insulin signaling pathway and subclinical inflammation, cause hepatic/peripheral insulin resistance and promote liver inflammation (33, 34). NAFLD often coexists with metabolic syndrome (MS) or predisposes patients to metabolic diseases. Therefore, the 2020 International Panel of Experts recommended that NAFLD be renamed metabolically associated fatty liver disease (MAFLD). The diagnostic criteria of MAFLD are based on histological (liver biopsy), abdominal imaging, and blood biomarker evidence of hepatic fat accumulation (hepatocellular steatosis). Combined with one of the following three conditions: overweight/obesity, type 2 diabetes mellitus and at least two risk factors for metabolic abnormalities (35), MAFLD is prone to develop into type 2 diabetes mellitus. According to the diagnostic criteria of MAFLD, overweight/obese fatty liver can be diagnosed as MAFLD, and lean fatty liver combined with risk factors for metabolic abnormalities also belongs to MAFLD. Ye JZ et al. conducted cohort cluster analysis on the MAFLD population and found that the high abdominal circumference cluster has a higher risk of T2DM and CVD after long-term follow-up, and its pathogenesis is related to the high waist circumference population often accompanied by hyperfree lipasemia and hyperinsulinemia (36).

This study also confirmed that the risk of type 2 diabetes in NAFLD patients with abdominal obesity was significantly higher than that in NAFLD patients with normal waist circumference, regardless of BMI. According to previous studies and the results of this study, regardless of whether it is MAFLD or not, the risk of type 2 diabetes in lean nonalcoholic fatty liver disease is similar. If NAFLD is MAFLD, whether it is lean nonalcoholic fatty liver disease or nonlean nonalcoholic fatty liver disease, it has a high risk of type 2 diabetes. BMI-driven approaches for NAFLD should be replaced by better diagnostic tools emphasizing the assessment of metabolic disorders and advanced liver fibrosis (16). It should be explored which clinical manifestations and outcomes of lean NAFLD meet the criteria for MAFLD and which do not.

There are several limitations of our study. As this study subjects were from healthy people undergoing physical examination, most of them were young and middle-aged people under 60 years old, so the correlation between lean elderly nonalcoholic fatty liver disease and the risk of type 2 diabetes was not covered in this study. Second. Due to the small number of women in the study population, this study was not conducted according to gender classification. Third. It is a single-center retrospective cohort study with a short follow-up period for the study population. In the future, a prospective multicenter cohort study with a longer follow-up period and a larger sample size is needed to strengthen the verification of the results. Fourth. For the reason of health examination, the glucose tolerance test was not used to exclude patients with type 2 diabetes during the baseline survey in this study. Therefore, a very small number of patients with impaired glucose tolerance with fasting blood glucose <6.1 mmol/L may participate in the follow-up study, but we believe that this has no significant impact on the study results. Fifth. Considering the short follow-up time of the present study, smoking, a small amount of alcohol consumption and exercise have little influence on the risk of type 2 diabetes, so the lifestyle data of smoking, alcohol consumption and exercise were not collected when the baseline data were collected.

5 Conclusion

The results of this study showed that abdominal obesity was a stronger risk factor for type 2 diabetes than overweight/obesity, with BMI ≥ 25 kg/m^2 as the cutoff point in nonalcoholic fatty liver disease. In waist circumference normal young and middle-aged

people, lean nonalcoholic fatty liver disease (BMI < 25.0 kg/m^2) is not an independent risk factor for type 2 diabetes. In the abdominal obesity population, lean nonalcoholic fatty liver disease is an independent risk factor for type 2 diabetes and causes the risk of type 2 diabetes and overweight fueling nonalcoholic fatty liver disease to be the same. The risk of lean nonalcoholic fatty liver disease in type 2 diabetes mellitus is affected by the number of risk factors for metabolic abnormalities; among them, the most important factor is abdominal obesity. It is of great significance to classify nonalcoholic fatty liver disease into metabolic fatty liver disease and classify its management and treatment according to the risk factors for type 2 diabetes mellitus, cardiovascular and cerebrovascular diseases and malignancy, as well as cluster risk factors for the prevention and treatment of nonalcoholic fatty liver disease complications.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Medical Research Ethics Committee of Karamay Hospital of Integrated Chinese and Western Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

NL designed the study and wrote the manuscript. HT contributed to data interpretation and reviewed the manuscript. NL and HT and WX contributed to data Statistical analysis. WX, SW, DL, MC, CX, MZ were involved in data collection and data cleaning. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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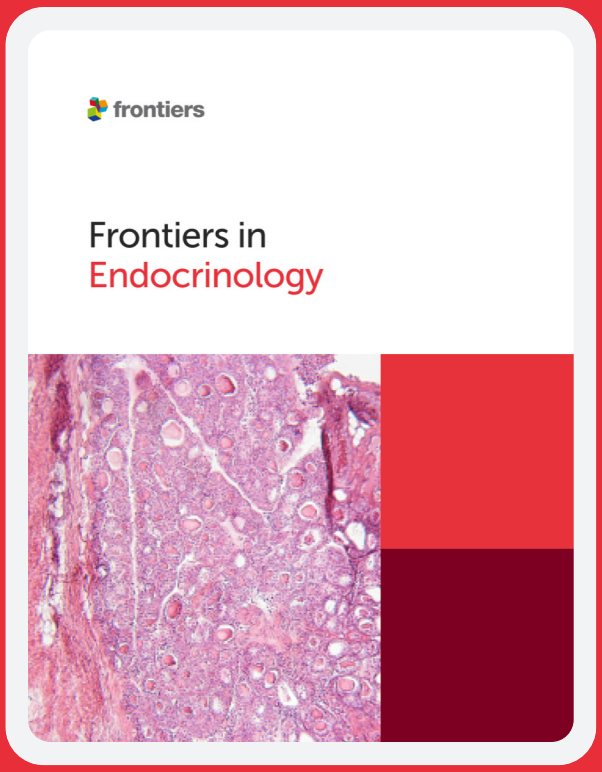

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