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Multi-organ linkage pathophysiology and therapy for NAFLD and NASH

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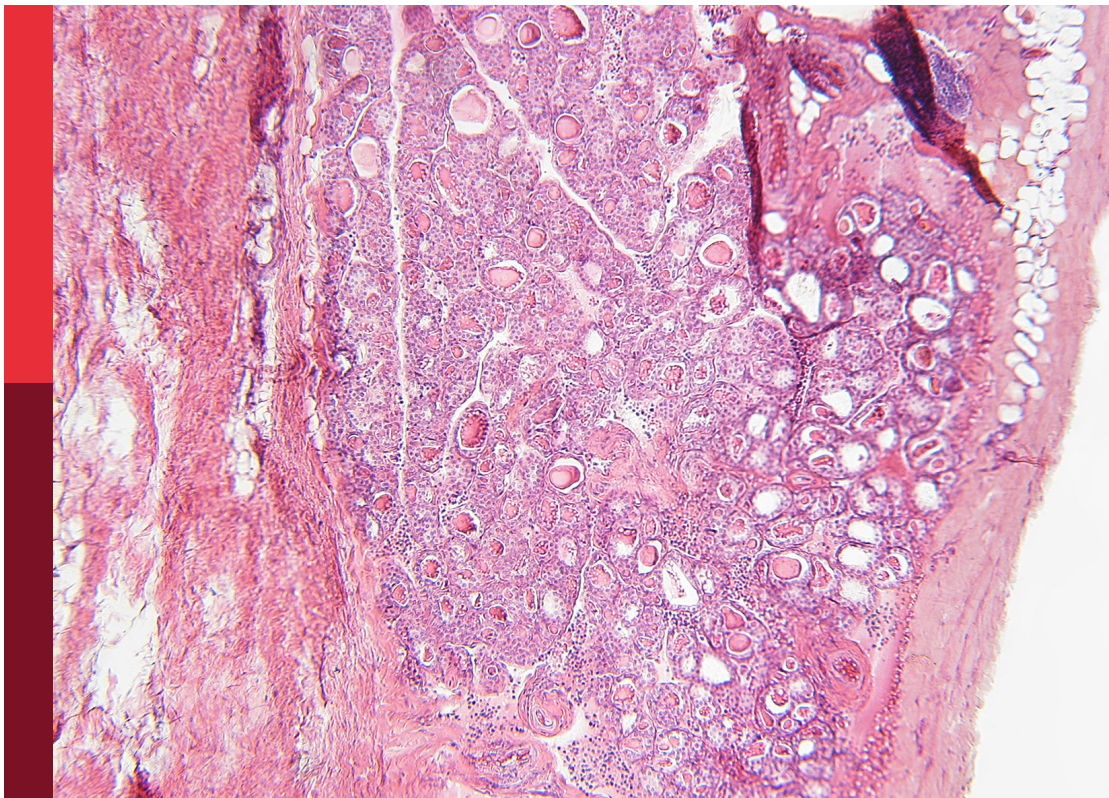

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Multi-organ linkage pathophysiology and therapy for NAFLD and NASH

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Editorial: Multi-organ linkage pathophysiology and therapy for NAFLD and NASH

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KEYWORDS

non alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), metabolic dysfunction-associated steatotic liver disease (MASLD), metabolic dysfunction-associated steatohepatitis (MASH), multi-organ linkage

Editorial on the Research Topic
Multi-organ linkage pathophysiology and therapy for NAFLD and NASH

Introduction

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), now referred to as metabolic dysfunction-associated steatotic liver disease (MASLD) and metabolic dysfunction-associated steatohepatitis (MASH), respectively, are major public health problems associated with obesity and diabetes (1, 2). Their complex pathophysiology involves multiple organs and metabolic pathways, challenging diagnosis, risk assessment, and treatment. This Research Topic, "*Multi-organ linkage pathophysiology and therapy for NAFLD and NASH*", provides new perspectives on organ linkages, pathogenesis, and therapeutic strategies for the management of NAFLD/MASLD and NASH/MASH.

Extrahepatic malignancy risk

Previous studies have examined extrahepatic malignancy risk in NAFLD (3, 4), but often overlooked the influence of obesity on cancer risk. To address this gap, Albhaisi et al. matched NAFLD patients with a non-NAFLD group to minimize confounding and found no increased extrahepatic cancer risk in NAFLD. However, given the robust negative impact of obesity on carcinogenesis and the strong association between NAFLD and obesity, carcinogenesis in NAFLD warrants some attention in general practice, as previous studies have shown.

Algorithm for assessing liver fibrosis risk in non-obese MAFLD

Asians, due to their genetic background, may encounter unique circumstances regarding the prognosis of non-obese (lean) NAFLD (5). Lee et al., researchers in Hong Kong, have proposed a distinctive sequential algorithm to assess liver fibrosis risk in non-diabetic overweight/obese individuals with metabolic dysfunction-related fatty liver disease (MAFLD). This algorithm integrates aspartate aminotransferase (AST) abnormalities and HOMA-IR≥2.5, alongside elastography, to stratify liver fibrosis risk in this population. Similar AST levels and liver fibrosis associations have been noted in other Asian cohorts, supporting these findings (6).

Predicting fibrosis stage undergoing bariatric surgery

Bariatric surgery is emerging as a beneficial treatment for NAFLD/NASH. Huang et al. studied 373 patients who underwent intraoperative liver biopsy during bariatric surgery in China. They aimed to predict fibrosis stage F2 or higher (9.1%) using noninvasive models. In multivariate analysis, age, diabetes, c-peptide, and AST were significant predictors. Models like APRI, FIB-4, and HFS showed predictive accuracies (AUC: 0.745–0.781). These predictive abilities are expected to improve combined with MRI/US elastography and previously reported markers (7, 8).

Comparative efficacy of GLP-1 receptor agonists in NAFLD

Novel therapeutics targeting G protein-coupled receptors are in development for obesity and diabetes (9). Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are increasingly used for managing obesity and diabetes mellitus, necessitating an understanding of how different GLP-1RA formulations impact outcomes. Yuan et al. conducted a network meta-analysis of 14 randomized controlled trials, finding that twice-daily exenatide was most effective in reducing liver fat content, while once-daily semaglutide showed superior efficacy in reducing AST and ALT levels.

Folate levels and NAFLD risk in adolescents

Folic acid deficiency heightens NAFLD risk in adults (10). Wen et al. used NHANES to study folate levels and NAFLD in adolescents (12–19 years). They found serum total folate or 5-methyl-tetrahydrofolate negatively correlated with CAP or liver stiffness. Mechanisms explored included inhibited lipid metabolism, impaired lipid transport, and folate-induced reductions in blood glucose and lipid concentrations.

The liver-brain axis

Mai and Mao conducted a study investigating the causal relationship between NAFLD and cortical structure. They used Mendelian randomization methodology, incorporating genetic predictors of NAFLD and liver adiposity, alongside summary statistics from the ENIGMA Consortium's genome-wide association study (GWAS). The findings revealed associations between NAFLD and liver adiposity with decreased surface area of the parahippocampal gyrus and increased thickness of the entorhinal cortex. These results suggest that NAFLD is linked to structural alterations in specific brain regions, emphasizing the potential influence of the hepatic-brain axis.

The liver-bone axis

In a review by Chondrogianni et al., the link between NAFLD and osteoporosis was explored through experimental and clinical evidence. Both diseases are prevalent globally, often coexisting. Emerging data suggest common molecular pathways like sarcopenia, the RANKL-OPG-RANK pathway, and the Wnt pathway (11). However, not all epidemiological studies confirm a direct association. Comprehensive understanding of the liver-bone axis requires large prospective cohort studies and intervention trials supported by robust basic research.

Closing remarks

This Research Topic incorporates a variety of research articles utilizing database studies, a valuable method for examining numerous cases and outcomes. However, we also stress the importance of cohort studies for the certainty of NAFLD diagnosis and detailed presentation of individual cases, supported by liver biopsy tissue diagnosis. By uncovering key mechanisms and identifying novel therapeutic targets, these studies will aid in developing personalized approaches for managing NAFLD/MASLD and NASH/MASH.

Author contributions

TK: Writing – review & editing, Writing – original draft. TY: Writing – review & editing. GE: Writing – review & editing.

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Conflict of interest

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

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Nonalcoholic fatty liver disease is specifically related to the risk of hepatocellular cancer but not extrahepatic malignancies

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Objective: We performed a matched cohort study among individuals with and without nonalcoholic fatty liver disease (NAFLD) to determine: 1) the incidence of cancers (extrahepatic and liver) and their spectrum and 2) if NAFLD increases the risk of extrahepatic cancers.

Methods: The NAFLD and non-NAFLD (control) cohorts were identified from electronic medical records *via* International Classification of Diseases (ICD) codes from a single center and followed from 2010 to 2019. Cohorts were matched 1:2 for age, sex, race, body mass index (BMI), and type 2 diabetes.

Results: A total of 1,412 subjects were included in the analyses. There were 477 individuals with NAFLD and 935 controls (median age, 52 years; women, 54%; white vs. black: 59% vs. 38%; median BMI, 30.4 kg/m^2; type 2 diabetes, 34%). The cancer incidence (per 100,000 person-years) was 535 vs. 1,513 (NAFLD vs. control). Liver cancer incidence (per 100,000 person-years) was 89 in the NAFLD group vs. 0 in the control group, whereas the incidence of malignancy was higher across other types of cancer in the control group vs. in the NAFLD group.

Conclusions: The overall extrahepatic cancer risk in NAFLD is not increased above and beyond the risk from background risk factors such as age, race, sex, BMI, and type 2 diabetes.

KEYWORDS

NAFLD, NASH, cancer, obesity, diabetes mellitus, metabolic syndrome

Introduction

Cancer is one of the leading causes of death in the United States and worldwide (1, 2). There is a large body of evidence that proves the association between malignancy and excess body weight (3–5). Most studies reported an increased incidence of gastrointestinal (GI) and hormone-related malignancies in individuals with obesity (4). Obesity has become a worldwide epidemic of the modern age (6, 7); therefore, the incidence of nonalcoholic fatty liver disease (NAFLD) has increased exponentially (8–10). NAFLD is closely associated with obesity and is seen in up to 80% of people with obesity (11). Less than 20% of patients with NAFLD have a normal body mass index (BMI) and no metabolic disorders (12). Numerous studies have established that malignancy is the second most frequent cause of death among patients with NAFLD (13, 14). Predictably, hepatocellular carcinoma (HCC) is the type of cancer that NAFLD is considered a major risk factor for, and this has been unanimously agreed upon by all relevant studies (15). Regarding extrahepatic malignancies, it is not known what specific types of cancer or the magnitude of risk that patients with NAFLD are at higher risk for compared to those without NAFLD. Furthermore, it remains unclear whether there are particular characteristics of malignancy risk among those with NAFLD that are distinct from those with obesity alone. A recent study by Allen et al. (16) has investigated the effect of NAFLD vs. obesity on incident cancers in a historical cohort of adults with NAFLD in Olmsted County, Minnesota, compared with age- and sex-matched controls. They reported that NAFLD and not obesity alone was associated with increased cancer risk, particularity of GI types (16). This study did not match cases and controls in BMI; instead, they used Poisson regression to examine the effect of NAFLD vs. obesity on malignancy risk. Another study by Kim et al. (17) reported that NAFLD is a risk factor for male colorectal carcinoma; however, it is important to note that they did not fully account for the interference of obesity on cancer risk (17). A study investigating the association between BMI and the development of GI cancers used BMI stratification and concluded that the NAFLD–GI cancer association was stronger in a population without obesity (18). NAFLD, like obesity, is not a localized disorder but rather a multisystem disease related to metabolism; therefore, it is highly essential to evaluate its role independently of obesity and metabolic dysregulation in certain diseases. Moreover, the question about the need for more accurate tools to characterize excess adiposity is being raised, such that BMI alone is an insufficient marker of obesity and may overlook other key contributors to disease outcomes. In order to support the importance of ruling out the effect of obesity when studying the role of NAFLD in extrahepatic malignancies, we aimed to determine the incidence and spectrum of the most common cancer types in the NAFLD population matched in age, sex, race, BMI, and type 2 diabetes with a non-NAFLD population.

Methods

Study population

We constructed a matched cohort study in a single center in the state of Virginia. The index dates for NAFLD cohort identification were between 2010 and 2012, and the study follow-up time was between 2010 and 2019. The two groups were identified from electronic medical records. The NAFLD cohort was composed of adults diagnosed with NAFLD. Each patient with NAFLD was individually matched to two individuals without NAFLD (control) at the time of index NAFLD diagnosis date who did not have a diagnosis of any known liver disease during the study inclusion period. Characteristics of the study population are summarized in Table 1. Individuals with NAFLD were identified by the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD 9-CM) codes for NAFLD, which included code numbers 571.5 (cirrhosis of the liver without mention of alcohol), 571.8 (other chronic nonalcoholic liver disease), and 571.9 (unspecified chronic liver disease without mention of alcohol) and ICD-10-CM codes K75.81 [nonalcoholic steatohepatitis (NASH)] and K76.0 (fatty liver, NOS) (Appendix Table 1), along with elevated alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) (defined as ALT and AST ≥30 for men; ≥20 for women), radiographic evidence of hepatic steatosis, and absence of other liver diseases within 3 years prior to NAFLD diagnosis index date (Appendix Table 1). The control cohort was defined by absence of any known liver disease, normal liver enzymes, and hepatic imaging without fatty liver within 3 years prior to the index visit date. Those with any prior history of cancer prior to the index date or BMI ¾15 or ≥60 kg/m^2 within 3 years prior to the NAFLD diagnosis index date were excluded for both groups. In addition, we excluded all study individuals with no healthcare visit/encounters after the index date or with less than 1 year of follow-up. The two groups were matched 1:2 for age, sex, race, BMI, and type 2 diabetes. One of the study investigators (SA) reviewed the complete medical records of a 10% random sample of individuals with NAFLD codes to confirm the validity of the code-identified study participants. In-depth chart review identified NAFLD diagnosis with a positive and negative predictive value of 86% and 87%, respectively.

Outcomes

Both groups were followed prospectively until death, last medical visit, or December 2019. Primary outcomes were incident cancers documented after the index NAFLD diagnosis. We looked into all cancers without limitation to certain classifications or subgroups. The cancer ascertainment

TABLE 1 Baseline demographic characteristics of the study population.

	Total (n=1,412)	NAFLD (n=477)	Control (n=935)	p-value*
Women, n (%)	765 (54%)	269 (56%)	496 (53%)	0.2555
Race, n (%)				0.4892
African American	532 (38%)	174 (36%)	358 (38%)	
White	829 (59%)	274 (57%)	555 (59%)	
Other	51 (3%)	29 (7%)	22 (2%)	
BMI group, n (%)				0.2128
1 (<25 kg/m^2)	276 (19%)	85 (18%)	191 (20%)	
2 (25–30 kg/m^2)	390 (28%)	122 (26%)	268 (28%)	
3 (30–35 kg/m^2)	349 (25%)	118 (25%)	231 (25%)	
4 (35–40 kg/m^2)	232 (16%)	88 (18%)	144 (15%)	
5 (≥40 kg/m^2)	165 (12%)	64 (13%)	101 (11%)	
BMI, median (IQR)	30.4 (26–36)	31 (27-36.6)	30.1 (26-35.6)	0.0656
Age, median (IQR)	52 (44-60)	51 (43-59)	52 (44-60)	0.2728
ALT, median (IQR)	21 (16-33)	51 (32-84)	18 (14-21)	<0.0001
AST, median (IQR)	21 (17-30)	41 (28-69)	19 (16-21)	<0.0001
Diabetes, n (%)	473 (34%)	173 (36%)	300 (32%)	0.1153

*p-value for statistical assessment of group difference between NAFLD and control. For continuous variables, Wilcoxon signed-rank test was considered. The chi-square test was used for categorical variables. IQR, interquartile range.

was done by identifying the cancer diagnoses in the medical records using the ICD-9 and ICD-10 codes documented at least once at separate dates. The cancers of interest were the most common cancers, which were classified into two groups: hepatic (liver) and extrahepatic cancers [gastrointestinal (colon, esophageal, gastric, and pancreatic), breast, uterine/endometrial, ovarian, prostate, lung, kidney/urinary tract, blood/bone marrow, and skin].

One of the study investigators (SA) reviewed the complete medical records of a 10% random sample of individuals with cancer codes to confirm the validity of the code-identified outcomes. In-depth chart review identified cancer diagnosis with a positive and negative predictive value of 87% and 88%, respectively. Comorbidities of interest included type 2 diabetes, hypertension, lipid disorders, and psoriasis. We did not have data about smoking status at the time of diagnosis or matching. Comorbidities were defined based on diagnostic ICD-9 and ICD-10 codes (Appendix Table 2). The study was approved by the Institutional Review Board as an institutional review board exemption under 45 CFR 46.101 (b).

Statistical analysis

In order to reduce the confounding effects, paired matching in age, sex, race, BMI, and diabetes status was performed using propensity score matching. Baseline demographic characteristics were compared between NAFLD and matched control group using Wilcoxon signed-rank test for continuous variables (due to skewed distribution of data) and chi-square test for categorical variables. The Kaplan–Meier curves were estimated for cancer survival, along with the log-rank test for difference in survival probabilities between the NAFLD and control groups. Cancer types were identified in the electronic medical records using the codes listed in Appendix Table 3, and cancer incidence was estimated for both groups. The incidence rates were calculated per 100,000 person-years. Statistical analyses were performed using SAS Version 9.4 (SAS Institute, Cary, NC, USA).

Results

A total of 1,412 subjects were included in the study (NAFLD vs. control: 477 vs. 935). The median age was 52 years, with 54% of the subjects being women. The majority of subjects were Caucasian (59%) with a median BMI of 30.4 kg/m^2. The proportion of those who have type 2 diabetes was 34%. The median follow-up was 5.7 vs. 5.2 years (NAFLD vs. control). Individuals with NAFLD had a higher proportion of obesity, i.e., BMI ≥30, compared with controls (56% vs. 51%). A total of 77 incident cancer cases (12 in the NAFLD group and 65 in the control group) were identified after matching during follow-up (total follow-up time in years: 2,244.2 for NAFLD and 4,293.8 for control). The overall cancer incidence (per 100,000 person-years) was 535 vs. 1,513 (NAFLD vs. control). More specifically, HCC incidence (per 100,000 person-years) was 89 in NAFLD vs. 0 in control, whereas the incidence of malignancy was higher across other types of cancer in control vs. NAFLD. The most common cancer in the matched control group was lung cancer as compared to breast cancer in the NAFLD group. There was no significant difference in cancer survival between NAFLD and control groups except for HCC, which was associated with

higher mortality in the NAFLD group as compared to that in the control group (p = 0.0489); this was expected given that no one in the control group developed HCC. The spectrum and incidence of cancers are shown in Table 2 and Figures 1, 2.

Discussion

Our study highlights the importance of ruling out the effect of obesity when studying the association between NAFLD and the risk of extrahepatic complications such as malignancy. In this study, we found that the risk for extrahepatic malignancies is not increased above and beyond the risk from background risk factors that include age, sex, race, BMI, and type 2 diabetes. This finding is not in agreement with previous studies that reported that NAFLD by itself can be a risk factor for extrahepatic malignancies (16–18). However, similar to previous studies, we found that NAFLD was associated with an increased risk of HCC (15). The contradiction between our findings and what has been reported previously is quite puzzling but should not be dismissed because this brings us back to the importance of understanding the NAFLD-obesity-metabolic comorbidities conundrum. Interestingly, studies involving extrahepatic cancer risk and NAFLD, including our study, have selected different methods and statistical approaches to answer this important research question. At times, that could be one of the reasons for the differences in findings across studies, but there are numerous factors implicated. Approaching cancer prevention and management in NAFLD from the perspective of multisystem disease in the context of obesity and metabolic dysregulation is likely to be more effective in improving clinical practice and patient care than from the perspective of a single system driving cancer risk. In fact, there is currently a global multi-stakeholder endorsement of the new metabolic dysfunction-associated fatty liver disease (MAFLD) definition as an overarching term that describes fatty liver diseases associated with metabolic dysregulation because it more accurately reflects the underlying pathogenesis of the disease than does the previously used term, NAFLD, and this designation will advance the science of fatty liver disease and improve patient care (19–21). Obesity is a key driver of carcinogenesis and the development of cancers (22). The underlying mechanisms for cancer development might be attributed to metabolic dysregulations related to obesity. Several previous relevant studies did not match the study populations in BMI. Instead, they performed subgroup analyses separately for subgroups with and without obesity. This methodology, while plausible, may remain inadequate in confidently removing the confounding effect of obesity on cancer risk in NAFLD individuals. Our study provides estimates of cancer types that commonly occur in NAFLD and control groups. After matching major risk factors that include BMI, we show that NAFLD is unlikely to solely be responsible for mediating cancer risk independently of preexisting metabolic risk factors. With or without NAFLD, obesity and metabolic dysregulation remain major drivers of cancer risk. Perhaps the contribution of metabolic dysregulation to cancer risk is bigger than that of obesity because there are many obesity phenotypes that do not include fatty liver due to genetic predisposition and are not linked to increased risk of cancer (23–25). Some, but not all phenotypes, may increase the risk of cancer. Other fat distribution patterns and patterns of ectopic lipid deposition reflecting metabolic dysregulation may be linked to an elevated risk of certain types of cancer—not just excess adiposity alone. These fat distribution patterns typically

TABLE 2 Spectrum of cancers in NAFLD and controls.

Cancer type	Cancer event count		Incidence per 100,000 person-years [with 95% confidence interval (CI)]	
	NAFLD	Control	NAFLD	Control
Gastrointestinal/liver cancers	2	7	89 (83, 95)	163 (155, 171)
-Liver	2	0	89 (83, 95)	–
-Colon	0	4	–	93 (87, 99)
-Small intestine	0	2	–	47 (42, 51)
-Esophagus	0	1	–	23 (20, 26)
Breast	4	8	178 (170, 186)	186 (178, 195)
Uterus	0	3	–	70 (65, 75)
Ovary	0	2	–	47 (42, 51)
Prostate	0	8	–	186 (178, 195)
Lung/bronchus	3	13	134 (126, 141)	303 (292, 313)
Kidney/Urinary Tract	0	10	–	233 (223, 242)
Blood/Bone Marrow	2	5	89 (83, 95)	116 (110, 123)
Skin	0	4	–	93 (87, 99)
Other	1	5	45 (40, 49)	116 (110, 123)

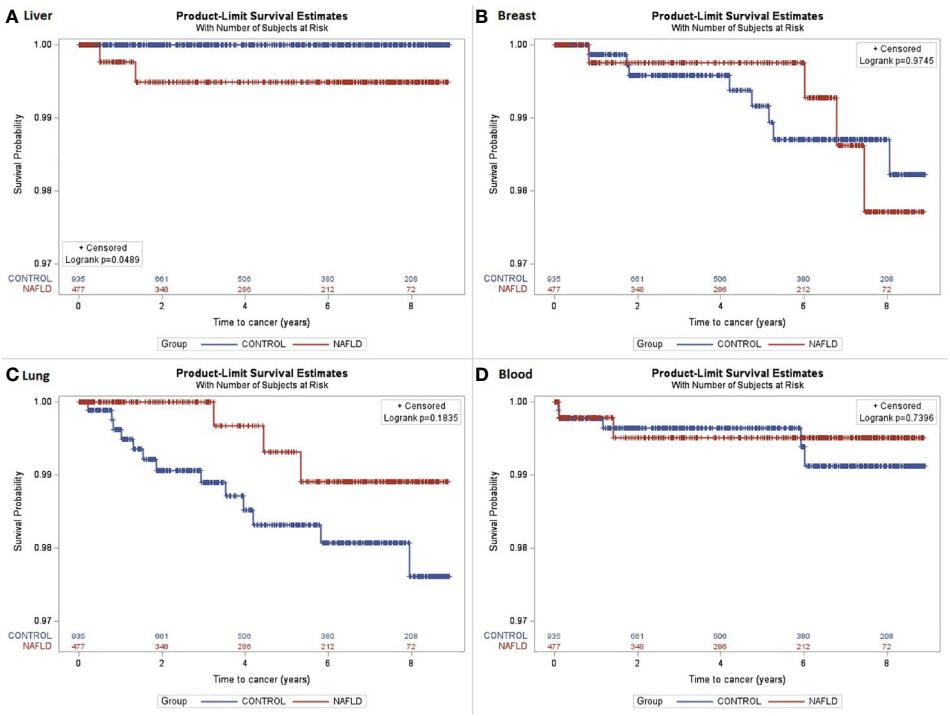

FIGURE 1
The Kaplan–Meier curves were estimated for cancer survival. **(A)** Liver cancer survival (log-rank p = 0.0489); **(B)** Breast cancer survival (log-rank p = 0.9745); **(C)** Lung cancer survival (log-rank p = 0.1835); **(D)** Blood/bone marrow cancer survival (log-rank p = 0.7396).

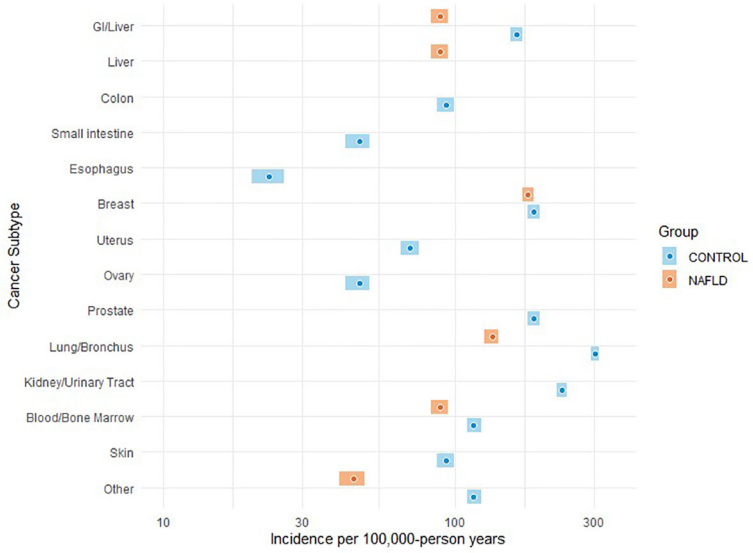

FIGURE 2
Plot chart showing the cancer incidence among individuals with NAFLD compared to age-, sex-, race-, BMI-, and diabetes-matched controls.

reflect insulin resistance and "metabolic" obesity that includes deposition of lipid in the visceral cavity, skeletal muscle, pancreas, and kidney—these can all occur in the absence of NAFLD (26–28). A recent study (16) suggested that NAFLD was associated with a higher risk of incident cancers, while obesity alone was not; however, ectopic fat deposition, which cannot be measured by BMI, seems to be the common underlying factor in the pathogenesis of metabolic disorders and metabolic cancers (29). Instead of considering NAFLD as a mediator of the obesity–cancer association, we suggest metabolic dysregulation to be a possible mediator of this association. Measuring metabolic dysregulation is a significant barrier for studying the NAFLD-obesity-cancer relationship. Whether NAFLD directly causes cancer is difficult to establish, and there could be another proximate cause for both fatty liver and cancer (29). It is known that weight loss of 5% reverses NAFLD. However, the Women Health Initiative study indicates that while an intentional 5% weight loss reduced the risk of endometrial cancer, it did not reduce the risk of other obesity-related cancers (colon, breast, pancreas, kidney, thyroid, or liver) (29, 30). The ultimate proof will probably come from long-term follow-up of patients specifically treated for NAFLD without other metabolic disturbances; this should clarify whether the ultimate cause lies in the liver or in the adipose tissue (29). Despite previous studies suggesting the higher risk of extrahepatic cancers in patients with NAFLD, this did not result in significant changes in clinical practice; however, there is a unanimous agreement on the importance of finding reliable and cost-effective noninvasive diagnostic markers of NAFLD to improve clinical care and facilitate NAFLD research. There is strong evidence to support the positive association between obesity and most common cancers (4, 31). Proposed mechanisms for this association include insulin and other hormones, insulin-like growth factor 1, adipokines, and systemic inflammation (32–34). Diabetes mellitus is another important confounding factor for the NAFLD–cancer association given that insulin resistance is a plausible mechanism linking cancer with NAFLD, which is why we matched the groups in type 2 diabetes as well. However, it cannot be assumed that matching diabetes appropriately accounts for differences in insulin resistance because type 2 diabetes may or may not account for varying degrees of insulin resistance. In the absence of liver biopsy in the general population and formally approved noninvasive diagnostic methods in addition to the unreliability of liver enzymes as biomarkers for NAFLD, it is difficult to ascertain possible distinct associations between the different stages of NAFLD (simple steatosis, NASH) and extrahepatic malignancies. For all of the abovementioned reasons, the remote effects of NAFLD leading to extrahepatic malignancies remain unclear. Our findings can be applied in clinical practice to guide counseling in individuals with obesity and to support larger studies investigating the effectiveness of cancer screening in obesity. This study has major limitations, which include the usual potential sources of bias seen with observational studies, sample size, duration of follow-up, being a single-center study, lack of reliable biomarkers for the diagnosis of NAFLD, unavailability of liver biopsy results, and unknown smoking status of both groups. The use of ICD codes to identify cases of NAFLD, which is likely to be a gross underestimate of the extent of the problem, is a major limitation of most NAFLD studies. Majority of individuals in the control group had missing lab values, so we could not estimate markers such as fibrosis-4 (FIB-4) score for comparison with the NAFLD group to evaluate for possible predictive ability of cancer risk. Furthermore, a proportion of the control group may have undiagnosed NAFLD, and we tried to address that by ensuring that they never had any ICD codes for NAFLD over the entire follow-up period; however, this method alone does not exclude possible undiagnosed or subclinical disease in the control group. Information about therapeutic interventions for obesity or NAFLD (e.g., dietary interventions, weight loss medications, etc.) and changes in BMI over time is missing in our study. The strengths of this study include the use of a cohort without NAFLD individually matched by age, sex, race, BMI, and type 2 diabetes with the NAFLD cohort. We conducted random in-depth chart review to confirm the diagnosis of cancer for both groups. We have randomly selected our study population, so it is difficult to explain why the patients were relatively young compared to previous studies, but this might be reflective of the characteristics of the patient population at our institution. This might partially explain the very low number of patients who developed HCC in addition to other factors such as underdiagnosing cancer and lack of data on regular cancer screenings. Nonetheless, the other demographic characteristics of the study population are roughly similar in general to other populations in the southern region of the United States, but any differences in the demographic distributions of other populations in other regions should be considered when attempting to generalize the results. There is no doubt that NAFLD is a multisystem disease with a vast range of complications, both intrahepatic and extrahepatic, but the more important and extremely dangerous player in driving carcinogenesis, regardless of NAFLD, is adiposity. Measures of obesity such as BMI are insufficient to accurately characterize excess adiposity given that BMI indicates neither the percentage of body fat mass nor the location of the fat (35) and would miss identification of visceral obesity even with a normal BMI can increase the risk of various extrahepatic complications in individuals with "lean NAFLD." Our findings highlight the importance of early management of obesity and provide a rationale for future larger studies on the effectiveness of cancer screening in obesity and larger studies of NAFLD after ruling out the effects of obesity and metabolic comorbidities such as diabetes. While the results of our study did not provide new aspects to the current understanding of NAFLD as an HCC cancer risk, they will potentially bring back the debate

regarding NAFLD being an independent risk factor for extrahepatic malignancies.

Conclusion

The overall cancer risk in NAFLD is not increased above and beyond the risk from background risk factors such as age, race, BMI, and type 2 diabetes. The risk of HCC is specifically related to NAFLD in this study population. However, our conclusions are limited by major study limitations; therefore, future studies investigating the association between NAFLD and extrahepatic malignancy should account for metabolic dysregulation and its comorbidities and the possible interference of obesity in the cancer risk and should try to minimize the confounding effect of obesity. In addition, there are numerous other factors implicated in the obesity–cancer relationship, such as insulin resistance and gut microbiota, and should be considered in future studies. Our study is calling for rethinking the NAFLD–extrahepatic cancer association and for considering a holistic approach for understanding and managing metabolic comorbidities for cancer prevention.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board at Virginia Commonwealth University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization, SA and AJS; Data curation, SA, DM and TG; Formal analysis, SA, DM, and LK; Investigation, AJS; Methodology, SA, DM, LK and AJS; Project administration, SA, TG and AJS; Resources, AJS; Software, DM and LK; Supervision, AJS; Validation, SA, DM and LK; Visualization, DM and LK; Writing – original draft, SA; Writing – review & editing, SA, DM, LK, TG and AJS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AJS is President of Sanyal Biotechnology and has stock options in Genfit, Akarna, Tiziana, Indalo, Durect, Exhalenz and Hemoshear. He has served as a consultant to Astra Zeneca, Nitto Denko, Ardelyx, Conatus, Nimbus, Amarin, Salix, Tobira, Takeda, Fibrogen, Jannsen, Gilead, Lilly, Poxel, Artham, Cymabay, Boehringer Ingelhiem, Novo Nordisk, Birdrock, Novartis, Pfizer, Jannsen and Genfit. He has been an unpaid consultant to Intercept, Echosens, Immuron, Galectin, Fractyl, Syntlogic, Affimune, Chemomab, Nordic Bioscience and Bristol Myers Squibb. His institution has received grant support from Gilead, Salix, Tobira, Bristol Myers, Shire, Intercept, Merck, Astra Zeneca, Malinckrodt, Cumberland and Novartis. He receives royalties from Elsevier and UptoDate.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1037211/full#supplementary-material

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Sequential algorithm to stratify liver fibrosis risk in overweight/obese metabolic dysfunction-associated fatty liver disease

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Background: Non-diabetic overweight/obese metabolic dysfunction-associated fatty liver disease (MAFLD) represents the largest subgroup with heterogeneous liver fibrosis risk. Metabolic dysfunction promotes liver fibrosis. Here, we investigated whether incorporating additional metabolic risk factors into clinical evaluation improved liver fibrosis risk stratification among individuals with non-diabetic overweight/obese MAFLD.

Materials and methods: Comprehensive metabolic evaluation including 75-gram oral glucose tolerance test was performed in over 1000 participants from the New Hong Kong Cardiovascular Risk Factor Prevalence Study (HK-NCRISPS), a contemporary population-based study of HK Chinese. Hepatic steatosis and fibrosis were evaluated based on controlled attenuation parameter and liver stiffness (LS) measured using vibration-controlled transient elastography, respectively. Clinically significant liver fibrosis was defined as LS ≥8.0 kPa. Our findings were validated in an independent pooled cohort comprising individuals with obesity and/or polycystic ovarian syndrome.

Results: Of the 1020 recruited community-dwelling individuals, 312 (30.6%) had non-diabetic overweight/obese MAFLD. Among them, 6.4% had LS ≥8.0 kPa. In multivariable stepwise logistic regression analysis, abnormal serum aspartate aminotransferase (AST) (OR 7.95, p<0.001) and homeostasis model assessment of insulin resistance (HOMA-IR) ≥2.5 (OR 5.01, p=0.008) were independently associated with LS ≥8.0 kPa, in a model also consisting of other

metabolic risk factors including central adiposity, hypertension, dyslipidaemia and prediabetes. A sequential screening algorithm using abnormal AST, followed by elevated HOMA-IR, was developed to identify individuals with LS ≥8.0 kPa, and externally validated with satisfactory sensitivity (>80%) and negative predictive value (>90%).

Conclusion: A sequential algorithm incorporating AST and HOMA-IR levels improves fibrosis risk stratification among non-diabetic overweight/obese MAFLD individuals.

KEYWORDS

obesity, MAFLD (metabolic associated fatty liver disease), overweight, fatty liver disease, population based study

Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a new nomenclature defining fatty liver disease with metabolic dysfunction proposed by an international expert panel in 2020, and affects one-third of the global adult population (1–3). MAFLD can be classified into three subtypes, based on the presence of hepatic steatosis co-existing with any one of the following including (1) type 2 diabetes (T2D-MAFLD); (2) overweight or obesity (overweight/obese MAFLD), defined as body mass index (BMI) ≥23kg/m^2 in Asians and 25kg/m^2 in Caucasians; and (3) in lean/normal weight individuals, the presence of any two of the other evidence of metabolic dysfunction (Lean MAFLD), including central obesity, elevated blood pressure (BP), dyslipidaemia, prediabetes, increased homeostasis model assessment of insulin resistance (HOMA-IR), and elevated circulating high-sensitivity C-reactive protein (hsCRP) levels (1).

In fatty liver disease, liver fibrosis is the most important determinant of adverse long-term outcomes. Higher stage of liver fibrosis is associated with all-cause, as well as liver-related, mortality and morbidity (4, 5). Early identification of individuals with clinically significant liver fibrosis, who are at risk of compensated advanced chronic liver disease (cACLD) (6, 7), is important to facilitate more targeted follow-up and surveillance, especially among overweight and obese individuals in whom the prevalence of MAFLD is over 50% (8). Several recent studies have suggested that the prevalence and risk of liver fibrosis differ across the three MAFLD subtypes (9–11). However, while individuals with overweight/obese MAFLD constitute the largest subgroup within the MAFLD population, no report thus far has evaluated the optimal strategy for stratifying liver fibrosis risk in these individuals. Previous studies in non-alcoholic fatty liver disease (NAFLD) have shown that the presence of metabolic dysfunction was closely associated with the development of liver fibrosis (12). Hence, we investigated whether incorporating additional metabolic risk factors into clinical evaluation would improve liver fibrosis risk stratification among individuals with overweight/obese MAFLD, using a contemporary population-based study of Hong Kong (HK) Chinese with comprehensive metabolic assessment.

Materials and methods

Study participants

All participants were recruited from the New HK Cardiovascular Risk Factor Prevalence Study (NCRISPS), an ongoing population-based, cross-sectional study established since December 2019, to determine the updated sex and age-stratified prevalence of cardiovascular risk factors, cardiovascular diseases (CVD) and related disorders in HK Chinese. The protocol was similar to the previously published HK Cardiovascular Risk Factor Prevalence Study (CRISPS) (13). Individuals aged 25 – 74 years were recruited from the community through systematic sampling of representative replicates of living quarters in HK, obtained from the Census and Statistics Department of the HK Special Administrative Region. Pregnant women, individuals with physical or mental illness which precluded them from travelling to the study centre for health assessment, or from providing informed consent, were excluded. The protocol of NCRISPS was approved by the Ethics Committee of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB Ref: UW-18-610). Written informed consent was obtained from all participants before any study-related procedures.

Clinical assessments

All NCRISPS participants attended the study visit after an overnight fast of at least 8 hours. Each participant completed a detailed questionnaire which included demographics (age, sex, occupation, income, education level, smoking and alcohol intake), family history, medical (personal history of diabetes, hypertension, hyperlipidaemia, CVD, cancers, chronic liver diseases in particular viral hepatitis, Wilson's disease, alpha-1 antitrypsin deficiency, autoimmune hepatitis and primary biliary cholangitis), and drug histories (anti-diabetic, lipid lowering, anti-hypertensive, and steatogenic medications such as amiodarone, tamoxifen, methotrexate etc.). Bloods were drawn for complete blood count and serum creatinine levels. Estimated glomerular filtration rate (eGFR) of the participants was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation as described previously (14).

Metabolic assessments

Anthropometric parameters including body weight (BW), height (BH), BMI, waist and hip circumferences (WC and HC, respectively) and BP were measured as previously described (13). Fasting bloods were drawn for glycated haemoglobin (HbA1c) and lipid profile. All participants, except those on anti-diabetic medications, underwent a 75-gram oral glucose tolerance test (OGTT). Moreover, except for those receiving insulin therapy, fasting insulin level was measured to determine the HOMA-IR level (15).

Hepatic assessments

Liver biochemistry including serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured at the Pathology Department of Queen Mary Hospital, Hong Kong, a laboratory accredited by the College of American Pathologists. The definitions of abnormal ALT and AST levels in the laboratory were based on age- and sex-specific reference ranges established using data collected from a cohort of local healthy Chinese (13). Hepatitis B surface antigen (HBsAg) were measured in all participants, whereas antibody against hepatitis C virus (Anti-HCV) was measured in those with elevated ALT or AST levels above the upper normal range (ALT: 58U/L for men, and 36 U/L for women aged ≤50 years and 45 U/L for women aged >50 years; AST: 38 U/L for men, and 30 U/L for women aged ≤50 years and 37 U/L for women aged >50 years), and/or HBsAg-positivity. Two commonly used non-invasive conventional fibrosis scores including Fibrosis-4 index (FIB-4) and NAFLD fibrosis score (NFS) were determined using published formulae (16).

During the study visit, all participants underwent vibration controlled transient elastography (VCTE) assessments, performed by trained operators using Fibroscan (Echosens, Paris, France) as per protocol described previously (17). In all participants, M probe was used during assessments, unless prompted by the VCTE machine. Controlled attenuation parameter (CAP) and liver stiffness (LS), which assessed the severity of liver steatosis and fibrosis, respectively, were measured with values represented by the median of 10 reliable measurements, defined when the interquartile range was <30% and the success rate was >60%. Only CAP values with an inter-quartile range of 40 dB/m were used to ensure data validity.

Definition of outcomes and clinical variables

Hepatic steatosis was defined as CAP ≥248 dB/m. Liver fibrosis was graded by LS cut-offs: <8.0 kPa (low risk), 8.0 – 9.5 kPa (intermediate to high risk), ≥9.6 kPa (advanced fibrosis and cirrhosis) (18). In this study, clinically significant liver fibrosis was defined as LS ≥8.0 kPa (7).

In this study consisting of exclusively HK Chinese, overweight and obesity were defined as BMI ≥23kg/m^2 and ≥27.5kg/m^2, respectively (1, 19). Central obesity was defined as WC ≥90cm in men and ≥80cm in women (1, 20). Hypertension was defined as BP ≥140/90mmHg or on anti-hypertensive medications. Dyslipidaemia was defined as fasting triglycerides (TG) ≥1.7 mmol/L, high density lipoprotein-cholesterol (HDL-C) <1.3 mmol/L in women and <1.0 mmol/L in men, low density lipoprotein-cholesterol (LDL-C) ≥3.4 mmol/L, or on lipid-lowering medications. Normal glucose tolerance (NGT) was defined as FG <5.6 mmol/L and 2-hour blood glucose (2hG) <7.8 mmol/L. IFG was defined as FG ≥5.6 mmol/L and <7.0 mmol/L, whereas IGT was defined as 2hG ≥7.8 mmol/l and <11.1 mmol/L. Prediabetes included IFG, IGT or elevated HbA1c ≥5.7% and <6.5%. Type 2 diabetes was defined as the presence of any two of the following biochemical abnormalities: FG ≥7.0 mmol/L, or 2hG ≥11.1 mmol/L on OGTT, or HbA1c ≥6.5%, or on anti-diabetic medications (21). CKD was defined as eGFR <60ml/min/1.73m^2. CVD was defined as any self-reported or medical history of cardiovascular event, including myocardial infarction, stroke, transient ischaemic attack, peripheral vascular disease, heart failure, recorded based on diagnostic codes (402, 404, 410-414,425-447, and 518.4) from the HK Hospital Authority database. Excessive alcohol intake was defined as daily alcohol consumption of >3 drinks in men and >2 drinks in women (1).

Independent cohorts for external validation

Two independent cohorts consisting of individuals without type 2 diabetes and fulfilled the diagnostic criteria of overweight/

obese MAFLD, based on reliable M probe measurements with VCTE, were used to form a pooled external cohort for validating our findings. The first cohort comprised individuals from the Obesity Clinic of Queen Mary Hospital, HK (N=40), whereas the second cohort involved participants from a longitudinal follow-up study of polycystic ovarian syndrome (PCOS) at Queen Mary Hospital, HK (N=31) (22).

Statistical analysis

All data were analysed with IBM SPSS Statistics 26.0 (http://www.IBM.com/SPSS). Data normality was determined by the Kolmogorov-Smirnov test. Values were reported as mean ± standard deviation (SD), medians with interquartile range (IQR), or percentages, as appropriate. Continuous variables between two groups were compared using independent *t*-test or Mann-Whitney U test, whereas one-way analysis of variance (ANOVA) or Kruskal-Wallis test were used to compare among multiple groups. Categorical variables were compared using Chi-square or Fisher Exact test, as appropriate. Bonferroni correction was applied for multiple comparisons. Cochran-Armitage test was applied for evaluating trend for binary variables, whereas ANOVA linear test or Jonckheere-Terpstra test was used for evaluating continuous variables. Multiple quantile regression analysis was conducted to investigate the associations of clinical variables with LS, accounting for the potential heterogeneity in the association of differently explanatory variables across the different quantiles of LS. Multivariable stepwise logistic regression analysis was performed to evaluate the independent determinants of the presence of LS ≥8.0 kPa and develop a screening algorithm for identifying individuals with overweight/obese MAFLD who had LS ≥8.0 kPa. Sensitivity, specificity, positive and negative predictive values (PPV and NPV) and the area under the receiver operating characteristic curve (AUROC) of the screening algorithm were evaluated to determine its performance. In all statistical tests, a two-sided p-value of <0.05 was considered significant.

Results

Overweight/obese MAFLD constituted the largest MAFLD subgroup

Of 1020 NCRISPS participants, 445 (43.6%) of them had fatty liver disease as defined by CAP ≥248 dB/m. All participants had valid CAP and LS measurements on VCTE using M probe. Participants who had fatty liver disease were significantly older (56.1 vs. 54.1 years) (p=0.004), being men (53.7% vs 40.9%) (p<0.001) and ever-smoker (28.1% vs. 20.7%) (p=0.006), with higher BMI (26.4 kg/m^2 vs. 22.3 kg/m^2), HOMA-IR (2.31 vs. 1.27) and prevalence of hypertension (43.4% vs. 25.7%), diabetes (19.1% vs 7.0%), dyslipidaemia (74.4% vs. 47.3%) (all p<0.001) and CKD (2.9% vs. 0.9%) (p=0.014) than those who did not. Among these 445 participants, 427 fulfilled the diagnostic criteria of MAFLD, and the majority (73%) had overweight/obese MAFLD, followed by T2D-MAFLD (20%) and lean-MAFLD (7%). Notably, participants with T2D-MAFLD, as compared to the other two subgroups, had significantly higher NFS (p=0.002), CAP (p<0.001) and LS measurements (p<0.001) despite similar serum ALT levels (Table 1).

Associations of metabolic risk factors with liver fibrosis risk in overweight/obese MAFLD

Among the 312 participants who did not have diabetes and had overweight/obese MAFLD, the majority (93.6%) were at low risk of liver fibrosis with LS <8.0 kPa. (Table 2) Higher stages of liver fibrosis were significantly associated with higher serum ALT (p for trend <0.001) and AST levels (p for trend <0.001), CAP (p for trend = 0.002) and NFS (p for trend =0.002). Moreover, with regard to the metabolic risk factors considered in the MAFLD definition (1), participants with higher stages of liver fibrosis had significantly higher prevalence of prediabetes based on OGTT and/or HbA1c (p for trend = 0.01), and were more insulin resistant as indicated by HOMA-IR ≥2.5 (p for trend <0.001). An increasing number of these metabolic risk factors was significantly associated with higher LS values (p for trend <0.001) (Table 2).

In multiple quantile regression analysis, in the first quantile, only BMI ≥27.5 kg/m^2 (p=0.017) and central obesity (p=0.004) were significantly associated with LS, whereas in the second quantile, abnormal AST level became the only significant determinant (p <0.001). In the third quantile and the 90th percentile, both abnormal AST level (p<0.001) and HOMA-IR ≥2.5 (p<0.001) were significant independent determinants of LS, in a model also consisting of hypertension, abnormal serum ALT level, prediabetes based on OGTT and/or HbA1c, as well as high TG or on lipid lowering medications. Moreover, the effects of abnormal AST and HOMA-IR ≥2.5 on LS increased with higher LS quantiles (Table 3).

Sequential screening algorithm for identifying individuals with non-diabetic overweight/obese MAFLD who had clinically significant liver fibrosis (LS ≥8.0 kPa)

Among these 312 participants with overweight/obese MAFLD and without diabetes, 20 (6.4%) of them had LS ≥8.0 kPa. Participants with LS ≥8.0 kPa had significantly higher BMI (p<0.001), abnormal ALT (p=0.038) and AST levels (p<0.001),

TABLE 1 Clinical characteristics of participants with MAFLD stratified by subtypes (N=427).

	Overweight/obese MAFLD	T2D-MAFLD	Lean-MAFLD	p-value
N	312	85	30	–
Men	175 (56.1%)	42 (49.4%)	15 (50%)	0.49
Age, years	54.5 ± 11.2	61.8 ± 8.4	57.5 ± 8.40	**<0.001**
Ever smoker	94 (30.1%)	22 (25.9%)	6 (20%)	0.42
Current drinker	76 (24.6%)	12 (14.1%)	7 (25.9%)	0.11
Excessive alcohol intake	2	0	0	1.00
BMI, kg/m^2	26.9 ± 3.0	27.2 ± 4.1	21.6 ± 0.8***	**<0.001**
BMI ≥ 27.5 kg/m^2	111 (35.6%)	34 (40%)	0	**<0.001**
Central obesity	221 (70.8%)	57 (67.1%)	12 (40%)*	**0.003**
Prediabetes based on OGTT and/or HbA1c	205 (65.7%)	0	21 (70.0%)	**<0.001**
FG and/or HbA1c	179 (57.4%)	0	17 (56.7%)	**<0.001**
Hypertension	126 (40.4%)***	61 (71.8%)	13 (43.3%)*	**<0.001**
Dyslipidaemia	228 (73.1%)*	75 (88.2%)	24 (80%)	**0.01**
CKD	6 (1.9%)	6 (7.1%)	1 (3.7%)	0.05
CAD	9 (2.9%)*	8 (9.4%)	4 (13.3%)	**0.004**
Stroke	6 (60%)	3 (30%)	1 (3.3%)	0.41
Cancer	16 (5.1%)	5 (5.9%)	1 (3.3%)	0.93
Viral hepatitis B or C	17 (5.4%)	6 (7.1%)	2 (6.7%)	0.72
ALT, U/L	26 (19-37)	26 (19-33)	22 (13-33)	0.17
AST, U/L	25 (21-29)*	22 (20-27)	24 (20-30)	**0.03**
FIB4	1.04 (0.75-1.37)	1.12 (0.86-1.29)	1.13 (0.92-1.62)	0.23
NFS	-1.86 ± 1.25**	-1.37 ± 1.06	-2.00 ± 1.05*	**0.002**
CAP, dB/m	296 ± 34*	308 ± 37	276 ± 28[c]	**<0.001**
LS, kPa	5.0 (4.3-5.8)*	5.4 (4.4-6.7)	4.4 (4.0-4.8)*	**<0.001**
LS category				**0.002**
LS ≥ 8.0 kPa	20 (6.4%)**	14 (16.5%)	2 (6.7%)	**0.011**
LS ≥ 9.6 kPa	8 (2.6%)	6 (7.1%)	2 (6.7%)	0.33

Values expressed as mean ± standard deviation or median (25^{th} – 75^{th} percentile) or numbers (%). Bonferroni correction was applied for multiple comparisons; *, p<0.05; **, p<0.01; ***, p<0.001 vs. T2D-MAFLD as the reference group.
None of the participants had other chronic liver diseases or use of steatogenic medications.
MAFLD, metabolic dysfunction-associated fatty liver disease; T2D, type 2 diabetes; BMI, body mass index; OGTT, oral glucose tolerance test; HbA1c, glycated haemoglobin; FG, fasting glucose; CKD, chronic kidney disease; CAD, coronary artery disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIB-4, Fibrosis-4 index; NFS, non-alcoholic fatty liver disease fibrosis score; CAP, controlled attenuation parameter; LS, liver stiffness.
Bold values are those with statistical significance.

NFS ≥-1.5 (p=0.003), prevalence of prediabetes based on OGTT and/or HbA1c levels (p=0.031) and HOMA-IR ≥2.5 (p=0.001) than those without (Supplementary Table S1).

To derive a screening algorithm for identifying individuals with overweight/obese MAFLD who had LS ≥8.0 kPa, multivariable stepwise logistic regression analysis was conducted in a stepwise fashion, based on the availability of parameters during the routine clinical care for patients with MAFLD. In the first step which consisted of BMI ≥27.5kg/m^2, a cut-off used to define obesity among Asian individuals (19), as well as abnormal transaminase levels in the model, only abnormal AST level was independently associated with the presence of significant liver fibrosis (OR 8.96, 95%CI 3.3 – 24.3, p<0.001). In the next step when metabolic risk factors

TABLE 2 Associations of clinical characteristics with the severity of liver fibrosis among participants with overweight/obese MAFLD (N=312).

	LS <8.0 kPa	LS 8.0 – 9.5 kPa	LS ≥9.6 kPa	p for trend
N	292	12	8	–
Men	162 (55.5%)	9 (75.0%)	4 (50.0%)	0.69
Age, years	54.6 ± 11.2	52.6 ± 12.2	59.4 ± 11.9	0.49
Ever-smoker	89 (30.5%)	2 (16.7%)	3 (37.5%)	0.88
Current drinker	70 (24.0%)	4 (33.3%)	2 (25.0%)	0.67
Excessive alcohol intake	2	0	0	1.00
BMI, kg/m^2	26.8 ± 2.82	29.6 ± 5.17	29.7 ± 4.21	**<0.001**
BMI ≥27.5 kg/m^2	99 (33.9%)	6 (50.0%)	6 (75.0%)	**0.009**
HOMA-IR	2.15 (1.63-2.93)	3.20 (2.38-4.63)	4.40 (2.65-7.15)	**<0.001**
Metabolic risk factors				
Central obesity	203 (69.5%)	12 (100.0%)	6 (75.0%)	0.16
Hypertension	181 (62.0%)	9 (75.0%)	7 (87.5%)	0.09
Prediabetes based on OGTT and/or HbA1c	187 (64.0%)	10 (83.3%)	8 (100%)	**0.01**
FG and/or HbA1c	165 (56.5%)	9 (75.0%)	5 (62.5%)	0.36
Low HDL-C or on lipid lowering medications	109 (37.3%)	5 (41.7%)	2 (25.0%)	0.65
High TG or on lipid lowering medications	145 (49.7%)	7 (58.3%)	5 (62.5%)	0.37
HOMA-IR ≥ 2.5	110 (37.7%)	9 (75.0%)	7 (87.5%)	**<0.001**
Number of metabolic risk factors	3.09 ± 1.45	4.25 ± 1.54	4.25 ± 1.16	**0.001**
Viral hepatitis B or C	16	0	1	1.00
ALT, U/L	25 (19-34)	38 (27-70)	43 (36-45)	**<0.001**
AST, U/L	24 (21-29)	28 (23-31)	44 (32-56)	**<0.001**
FIB4	1.03 (0.75-1.36)	1.28 (0.86-1.33)	1.68 (1.19-2.52)	**0.01**
NFS	-1.91 ± 1.24	-1.61 ± 1.17	-0.48 ± 0.88	**0.002**
CAP, dB/m	294.9 ± 33.7	303.8 ± 30.8	321.3 ± 37.6	**0.022**
LS, kPa	4.9 (4.2-5.6)	8.3 (8.1-8.6)	11.6 (10.4-20.4)	**<0.001**

Values expressed as mean ± standard deviation or median (25th – 75th percentile) or numbers (%). Cochran-Armitage test was applied for evaluating trend for binary response, whereas ANOVA linear test or Jonckheere-Terpstra test was applied for evaluating trend for continuous responses.
MAFLD, metabolic dysfunction-associated fatty liver disease; BMI, body mass index; OGTT, oral glucose tolerance test; HbA1c, glycated haemoglobin; FG, fasting glucose; HDL-C, high density lipoprotein-cholesterol; TG, triglyceride; HOMA-IR, homeostasis model assessment of insulin resistance; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIB-4, Fibrosis-4 index; NFS, non-alcoholic fatty liver disease fibrosis score; CAP, controlled attenuation parameter; LS, liver stiffness.
Bold values are those with statistical significance.

including prediabetes (based on OGTT and/or HbA1c) and HOMA-IR ≥2.5 were also included in the model, both abnormal AST level (OR 7.95, 95%CI 2.83 – 22.4, p<0.001) and HOMA-IR ≥2.5 (OR 5.01, 95%CI 1.54 – 16.3, p=0.008) remained independently associated with LS ≥8.0 kPa. (Table 4) The results were similar when abnormal serum transaminase levels were replaced by elevated NFS (Supplementary Table S2), or when BMI ≥27.5kg/m^2 was replaced by a more generally used obesity cut-off of ≥30kg/m^2. Notably, among the metabolic risk factors (Supplementary Table S1), HOMA-IR ≥2.5 was the only independent determinant of significant liver fibrosis (OR 4.08, 95%CI 1.54 – 16.0) in multivariable logistic regression analysis.

Performance of sequential screening algorithm in derivation and validation cohorts

In NCRISPS, the sensitivity and specificity of this sequential screening algorithm (Figure 1) to identify individuals with non-

TABLE 3 Multiple quantile regression analysis showing the independent determinants of higher liver stiffness in participants with overweight/obese MAFLD (N=312).

	25th percentile		50th percentile		75th percentile		90th percentile	
	Beta (95% CI)	p-value	Beta (95% CI)	p-value	Beta (95% CI)	p-value	Beta (95% CI)	p-value
BMI ≥ 27.5 kg/m^2	0.40 (0.07 - 0.73)	**0.017**	0.30 (-0.04 - 0.64)	0.080	0.35 (-0.12 - 0.82)	0.146	0.38 (-0.56 - 1.31)	0.430
Central obesity	0.50 (0.16 - 0.84)	**0.004**	0.20 (-0.15 - 0.55)	0.263	0.45 (-0.04 - 0.94)	0.074	0.66 (-0.31- 1.64)	0.182
Abnormal AST level	0.40 (-0.26 - 1.06)	0.230	1.60 (0.93 - 2.27)	**<0.001**	1.53 (0.61 - 2.45)	**0.001**	3.92 (2.06 - 5.78)	**<0.001**
HOMA-IR ≥ 2.5	0.30 (-0.02 - 0.62)	0.066	0.30 (-0.03 - 0.63)	0.071	0.65 (0.20 - 1.10)	**0.005**	1.78 (0.87 - 2.69)	**<0.001**

The 25th, 50th, 75th and 90th quantiles corresponded to liver stiffness 4.3, 5.0, 5.8 and 7.0 kPa, respectively.
Model also included BMI, central obesity, hypertension, abnormal ALT level, prediabetes based on oral glucose tolerance test and/or HbA1c, high TG or on lipid-lowering medications.
MAFLD, metabolic dysfunction-associated fatty liver disease; 95%CI, 95% confidence interval; AST, aspartate aminotransferase; HOMA-IR, homeostasis model assessment of insulin resistance; BMI, body mass index; ALT, alanine aminotransferase; glycated haemoglobin; TG, triglyceride.
Bold values are those with statistical significance.

diabetic overweight/obese MAFLD who were at risk of clinically significant liver fibrosis was 90% and 58.6%, respectively. Importantly, the NPV was 98.8% with a PPV of 12.9%. (Table 5) The AUROC was 0.80 (95%CI 0.71 – 0.90). The performance was similar between men and women (Supplementary Table S3). In the pooled validation cohort, with baseline characteristics of the participants shown in Supplementary Table S3, the AUROC was 0.68 (95%CI 0.50 – 0.87). The sensitivity was 81.8% with an NPV of 91.3% (Table 5).

Discussion

In this contemporary population-based study of HK Chinese with comprehensive metabolic evaluation, we demonstrated that 1 in 3 of our local community-dwelling individuals had overweight/obese MAFLD. However, despite this high prevalence, only <3% and 6.4% of them had advanced and clinically significant liver fibrosis, respectively. Therefore, we have developed a simple, sequential screening algorithm based on abnormal AST, followed by elevated HOMA-IR levels. We demonstrated, with external validation, that the clinical performance of this algorithm was satisfactory with sensitivity over 80% and NPV over 90%. Although the specificity and PPV were relatively low, the high sensitivity and NPV were particularly important for a screening algorithm, which allowed us to optimally identify, among this large group of individuals with non-diabetic overweight/obese MAFLD, those who were at risk of cACLD and would require referral to hepatologists for VCTE and/or further hepatic evaluation.

Since the proposal of the new diagnostic entity of MAFLD, only a few studies have directly compared the three different MAFLD subgroups (9–11). The Rotterdam Study showed that the prevalence of hepatic fibrosis, defined as LS ≥8.0 kPa,

TABLE 4 Multivariable stepwise logistic regression showing the associations of clinical variables with LS ≥8.0 kPa in participants with overweight/obese MAFLD (N=312).

	OR (95% CI)	p-value
Step 1: BMI		
BMI ≥ 27.5 kg/m^2	2.92 (1.16-7.39)	**0.023**
Step 2: BMI, plus abnormal serum transaminase levels		
BMI ≥ 27.5 kg/m^2	2.43 (0.92-6.41)	0.07
Abnormal AST level	8.96 (3.30-24.3)	**<0.001**
Step 3: BMI, abnormal serum transaminase levels, plus Metabolic risk factors		
BMI ≥ 27.5 kg/m^2	1.66 (0.60-4.60)	0.33
Abnormal AST level	7.95 (2.83-22.4)	**<0.001**
HOMA-IR ≥ 2.5	5.01 (1.54-16.3)	**0.008**

Abnormal serum transaminase levels included abnormal ALT and AST levels; Metabolic risk factors included presence of prediabetes based on oral glucose tolerance test and/or HbA1c, and HOMA-IR levels.
MAFLD, metabolic dysfunction-associated fatty liver disease; LS, liver stiffness; OR, odds ratio; 95%CI, 95% confidence interval; BMI, body mass index; AST, aspartate aminotransferase; HOMA-IR, homeostatic model assessment of insulin resistance; ALT, alanine aminotransferase; OGTT, oral glucose tolerance test; HbA1c, glycated haemoglobin.
Bold values are those with statistical significance.

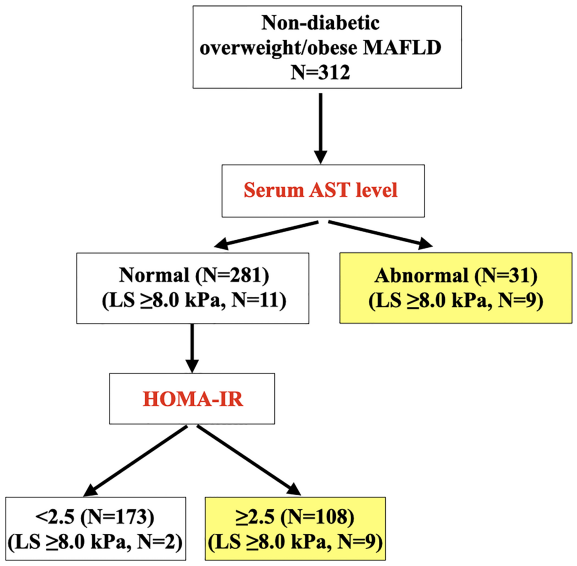

FIGURE 1
Sequential clinical algorithm for identifying overweight/obese participants with MAFLD at risk of clinically significant liver fibrosis (i.e. LS ≥8.0 kPa) (N=312). MAFLD, metabolic dysfunction-associated fatty liver disease; cACLD, compensated advanced chronic liver disease; LS, liver stiffness; AST, aspartate aminotransferase; HOMA-IR, homeostasis model assessment of insulin resistance.

increased significantly when individuals fulfilled all three diagnostic criteria of MAFLD which included type 2 diabetes, overweight/obesity or having two or more metabolic abnormalities, as compared to those satisfying only one or two inclusion criteria (23). It is also well established that type 2 diabetes is an important risk factor of fibrosis progression in fatty liver disease (24). A recent meta-analysis reported that 1 in 5 patients with type 2 diabetes had elevated LS (25). Consistently, in our study, individuals with T2D-MAFLD had significantly higher LS and prevalence of clinically significant liver fibrosis than the other two MAFLD subgroups. Indeed, several non-invasive fibrosis scores and novel biomarkers have been investigated over the years for their performance to stratify liver fibrosis risk specifically among individuals with T2D-MAFLD (26–28). (29) On the other hand, as shown by us and others, non-diabetic overweight/obese MAFLD constitutes the largest MAFLD population within the community (9–11). Although the studies were not directly comparable, our 73% prevalence of non-diabetic overweight/obese MAFLD was overall similar to the 77.5% reported in a community-based survey in Beijing, and lower than the 95.2% in a Korean study (9, 10). However, we found that their overall risk of significant liver fibrosis was much lower than that of T2D-MAFLD and correlated significantly with the presence of additional metabolic comorbidities.

Hence, in this study, we evaluated whether taking into consideration the presence of additional metabolic risk factors would improve the identification of clinically significant liver fibrosis specifically among individuals with overweight/obese MAFLD. The contemporary study population, together with the comprehensive metabolic assessments, which included OGTT in all our participants (except for those taking anti-diabetic medications), are two major strengths of our study. Indeed, we found that 41.7% of our study participants had MAFLD, a prevalence rate that was considerably higher than the 25.9% reported in a local population study using the HK census database performed over a decade ago (30). Although the two studies differed in the imaging modality employed for the detection of hepatic steatosis, our updated local MAFLD prevalence was overall in keeping with that reported globally in a recent meta-analysis (2). This probably reflected the soaring prevalence of obesity and related metabolic diseases such as prediabetes both locally and globally (31, 32), and the additional use of OGTT for evaluating glycaemic status in our study.

We found that, of all the metabolic risk factors including obesity, central adiposity, hypertension, dyslipidaemia and prediabetes diagnosed based on OGTT and/or HbA1c, elevated HOMA-IR was the only independent determinant of LS >8.0 kPa. These findings, which were derived from non-diabetic overweight/obese MAFLD participants, concurred with those reported in a previous study of obese individuals with NAFLD that HOMA-IR was an independent predictor of worsening histological fibrosis (33). Indeed, amongst the multiple hits in the pathogenesis of fatty liver disease, insulin resistance is a key driver of its progression (34). With increased

TABLE 5 Performance of the sequential screening algorithm in NCRISPS and the pooled validation cohort for identifying non-diabetic overweight/obese participants with MAFLD at risk of at risk of clinically significant liver fibrosis (i.e. LS ≥8.0 kPa).

	NCRISPS (Derivation cohort)	Pooled validation cohort
Sensitivity	18/20 (90.0%)	9/11 (81.8%)
Specificity	171/292 (58.6%)	20/60 (35.0%)
PPV	18/139 (12.9%)	9/48 (18.8%)
NPV	171/173 (98.8%)	21/23 (91.3%)

NCRISPS, New cardiovascular risk factor prevalence study; MAFLD, metabolic dysfunction-associated fatty liver disease; LS, liver stiffness; PPV, positive predictive value; NPV, negative predictive value.

lipolysis in the adipocytes and *de novo* lipogenesis in the liver, free fatty acid accumulates and lipo-toxicity ensues. Hepatocyte injury causes inflammation with increased cytokines production by the Kupffer cells, vascular remodeling, and activation of regenerative processes. Repetitive unsuccessful regenerative responses lead to progressive scarring, advanced fibrosis and cirrhosis (35). Furthermore, hyperinsulinaemia, which occurs secondary to insulin resistance, also promotes hepatic fibrosis through stimulating the proliferation of hepatic stellate cells, collagen synthesis, and up-regulation of the hepatic expression of connective tissue growth factors (36, 37).

In this study, with the inclusion of OGTT for metabolic evaluation in our study, we found that both HOMA-IR≥2.5 and prediabetes based on either OGTT or HbA1c were important metabolic risk factors of liver fibrosis among individuals with non-diabetic overweight/obese MAFLD. However, our findings in multivariable analyses showed that elevated HOMR-IR outperformed prediabetes, which included also individuals with normal HbA1c but abnormal OGTT, a cumbersome test to perform. This led to the development of the current screening algorithm as a simpler strategy to use clinically, based on parameters that can be conveniently measured during the routine care for patients with MAFLD. On the other hand, we found that FIB-4, a commonly used non-invasive fibrosis score, was not significantly associated with LS ≥8.0 kPa, which was likely due to the low prevalence of liver fibrosis in this community-based cohort. Moreover, although we demonstrated that replacing abnormal AST level with NFS resulted in similar conclusions, it is noteworthy that NFS is a composite score that requires several other parameters including platelet count, serum albumin and ALT levels. Certainly, the relatively small sample size of the two external cohorts to validate our findings was a major limitation of the study. Nonetheless, we found that the clinical performance of this screening algorithm remained satisfactory in the pooled validation cohort with reasonable sensitivity and high NPV of over 90%, indicating that this algorithm should be also applicable to individuals who are relatively insulin resistant either due to PCOS or more severe obesity.

Our study had several other limitations. First, the cross-sectional study design precluded the evaluation of a causal relationship between metabolic dysfunction and the development of liver fibrosis, or cACLD, in patients with overweight/obese MAFLD. Secondly, the sample size of both derivation and validation cohorts were relatively small, and all our participants were HK Chinese. Further studies in other populations with a larger sample size are required to confirm these findings and validate our proposed clinical algorithm. Moreover, serum hsCRP level was not measured and liver biopsy was not performed in our study participants. However, it was not feasible and ethically not justified to perform liver biopsy in these asymptomatic community-dwelling individuals. Nonetheless, the prevalence of 6.4% with LS ≥8.0 kPa on VCTE in our study, which involved community-dwelling individuals aged ≥25 years, was overall in line with those reported in two recent Korean studies based on magnetic resonance elastography (9, 10). In these two studies conducted among individuals attending health check-up aged ≥18 years and ≥40 years, the prevalence of significant liver fibrosis was found to be 4.2% and 9.9%, respectively (9, 10). Lastly, although the recruitment process from the community was through random sampling, participation in this population-based study was entirely voluntary. Therefore, it is possible that the participants were overall relatively more health conscious, which could have also explained the small number of individuals with excessive alcohol intake that is associated with increased liver fibrosis development in MAFLD (38). Moreover, the relatively small sample size of individuals with viral hepatitis or the significant alcohol intake also rendered it difficult for further subgroup analysis based on MAFLD with single or dual etiologies.

Conclusion

It is conceivable that a MAFLD pandemic, in particular overweight/obese MAFLD, will soon follow alongside the rising global prevalence of obesity (2, 39). From a clinical perspective, our findings suggest the recommendations of measuring serum AST level in all individuals with non-diabetic overweight/obese MAFLD detected on imaging techniques such as ultrasound or blood biomarkers, and have serum fasting insulin level measured to determine the HOMA-IR if their serum AST levels are normal. Individuals who have elevated serum AST level and/or HOMR-IR ≥2.5 should be referred for VCTE and/or hepatologist assessment for the presence of clinically significant liver fibrosis. Since MAFLD research has only started since 2020, future prospective studies should focus on the role of metabolic dysfunction in stratifying the long-term risks of incident adverse hepatic outcomes including liver-related mortality in this largest subgroup within the MAFLD population.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB Ref: UW-18-

610). The patients/participants provided their written informed consent to participate in this study.

Author contributions

C-HL researched the data and wrote the manuscript. DL, MY, RL, LM and Y-CW researched the data. CF, AL and JC performed statistical analyses. T-HL, JW, AX, H-FT, KT, BC, M-FY and KL critically reviewed and edited the manuscript. KL initiated and supervised the study, had full access to all the data and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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Prediction of MAFLD and NAFLD using different screening indexes: A cross-sectional study in U.S. adults

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Introduction: Metabolic dysfunction-associated fatty liver disease (MAFLD), formerly known as non-alcoholic fatty liver disease (NAFLD), has become the most common chronic liver disease worldwide. We aimed to explore the gender-related association between nine indexes (BMI/WC/VAI/LAP/WHtR/TyG/TyG-BMI/TyG-WC/TyG-WHtR) and MAFLD/NAFLD and examine their diagnostic utility for these conditions.

Methods: Eligible participants were screened from the 2017-2018 cycle data of National Health and Nutrition Examination Survey (NHANES). Logistic regression and receiver operating characteristic (ROC) curve were used to assess the predictive performance of 9 indexes for MAFLD/NAFLD.

Results: Among the 809 eligible individuals, 478 had MAFLD and 499 had NAFLD. After adjusting for gender, age, ethnicity, FIPR and education level, positive associations with the risk of MAFLD/NAFLD were found for all the nine indexes. For female, TyG-WHtR presented the best performance in identifying MAFLD/NAFLD, with AUC of 0.845 (95% CI = 0.806-0.879) and 0.831 (95% CI = 0.791-0.867) respectively. For male, TyG-WC presented the best performance in identifying MAFLD/NAFLD, with AUC of 0.900 (95% CI = 0.867-0.927) and 0.855 (95% CI = 0.817-0.888) respectively.

Conclusion: BMI/WC/VAI/LAP/WHtR/TyG/TyG-BMI/TyG-WC/TyG-WHtR are important indexes to identify the risk of MAFLD and NAFLD.

KEYWORDS

metabolic dysfunction-associated fatty liver disease, waist circumference, lipid accumulation product, triglycerideglucose index, BMI, TyG-WC, WHtR, TyG-WHtR

1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a syndrome including non-alcoholic fatty liver, non-alcoholic steatohepatitis, associated cirrhosis, liver cancer, and other diseases. It is defined by excessive fat accumulation in hepatocytes that is not caused by alcohol or other clear liver injury. NAFLD is the most common chronic liver disease in the world today, affecting the health of 25.24% adults (1). Previous studies have confirmed that NAFLD is closely associated with several metabolic diseases, such as hyperlipidemia, diabetes (2), and hypertension (3).NAFLD has been commonly linked to the metabolic syndrome (MetS). The 2020 International Expert Consensus recommended renaming NAFLD as metabolic dysfunction-associated fatty liver disease (MAFLD) to better meet clinical and research needs due to the rising prevalence of NAFLD, the improved understanding of its pathogenesis, and the drawbacks and shortcomings of previous exclusionary diagnoses (4). Although some existing studies suggest that MAFLD may be more advantageous in identifying advanced fibrosis and metabolic abnormalities, there still is limited evidence and not much research on MAFLD (5).

Patients with fatty livers have the risk of not only developing in cirrhosis and liver cancer, but also developing diabetes, cardiovascular disease, and kidney disease (6), which seriously affect their life quality and health. According to Younossi et al., NAFLD is anticipated to affect over 64 million people in the United States, with direct medical costs of about $103 billion annually ($1,613 for each patient) (7). Additionally, the prevalence of adult obesity, diabetes, and aging will all contribute to an increase in NAFLD-related liver disease and mortality. More than 800,000 liver deaths are projected between 2015 and 2030 (8). Therefore, early diagnosis and identification of fatty liver disease is critical in safeguarding the health of the population and reducing the financial burden of national health. Pathological biopsy, a gold standard for diagnosing fatty liver disease, is expensive, invasive, and accompanied by postoperative complications (9). Exploring easy-to-use, practical, and reliable predictors of fatty liver disease is clinically significant and valuable.

Obesity is one of the common causes of hepatic steatosis and is closely linked to insulin resistance (IR). However, increasing studies suggest that adipose tissue has a variety of functions and some adipose tissue is harmless to the body, such as brown fat (10). In addition, the distribution site of adipose tissue is also closely related to health status (11). We cannot simply assume that high body weight and excessive fat accumulation indicate poor health status since there are phenotypes of metabolically healthy obese (MHO) and metabolically unhealthy non-obese (MUNO) (12, 13). Visceral adiposity and lipid accumulation may, to some extent, assess more accurately the role of adipose tissue in the physiopathological processes as well as its value in predicting disease risk. Visceral adiposity index (VAI), proposed by Amato et al. (14), is a novel body fat index integrating waist circumference, body mass index (BMI), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) and is considered a reliable predictor of visceral adiposity. The relationship between VAI and metabolism-associated diseases is also widely investigated recently. A 4-year prospective cohort study suggested that VAI level was an independent risk factor for NAFLD, and there was a dose-response relationship between them (15). Lipid accumulation product (LAP), an easily accessible index consisting of waist circumference and triglycerides, may better reflect the extent of lipid accumulation compared to central obesity alone (16). Dai et al. confirmed that LAP was highly linked to the incidence and severity of NAFLD and a reliable predictor of NAFLD risk in Chinese adults (17). Triglyceride-glucose (TyG) index, a reliable surrogate for IR assessment, is closely associated with cardiovascular disease (18), diabetes mellitus (19), diabetic nephropathy (20) and various diseases. TyGis found to be important in identifying individuals at risk for NAFLD and assessing the progression of liver fibrosis (21, 22). More studies suggest that TyG-BMI, TyG-WC and TyG-WHtR are reliable indicators for NAFLD (23–25). BMI is widely used for obesity measurement, while waist circumference (WC) and waist-to-height ratio (WHtR) are important indicators for central obesity assessment. However, there is little research on the differences among BMI, WC, VAI, LAP, WHtR, TyG, TyG-BMI, TyG-WC and TyG-WHtR in predicting the risk of MAFLD/NAFLD.

This study intends to explore the differences among those indexes in predicting the risk of MAFLD/NAFLD based on the data of US adults in the 2017-2018 cycle from National Health and Nutrition Examination Survey (NHANES), aiming to provide a reliable reference for early detecting and identifying indicators of MAFLD/NAFLD.

2 Materials and methods

2.1 Study design and participants

All individuals aged ≥ 20 years from the cycle 2017 to 2018 of the NHANES in the United States were screened in this study. Profiles of the NHANES were described in previous study (26). The NHANES gathered a representative sample from the non-institutionalized U.S. population using a complicated, multi-stage, and probability sampling strategy. All data were collected with household interviews, mobile physical examinations, and laboratory tests. The participant screening flow chart was displayed in Figure 1. From all 9,254 individuals, we excluded participants aged< 20 years (n =3,685), drinking heavily (n=1,589), positive serology for hepatitis B, C and D (n=738), missing data of liver ultrasound transient elastography (FibroScan®) (n= 691), taking lipid-lowering drugs (n=650), and missing important data to calculate 9 indicators (n = 1,092). Finally, 809 participants were included for analysis. The Research Ethics Review Board of the National Center for Health Statistics examined and approved the NHANES protocol. Each participant completed a written statement of informed consent.

2.2 Definition of MAFLD and NAFLD

MAFLD was defined by presence of hepatic steatosis (HS) on ultrasound and meeting at least one of three conditions: overweight/obesity, presence of T2DM, or presence of metabolic disorder (27). Lean/normal-weight individuals with HS but no T2DM were considered to have a metabolic disorder if two or more of the following metabolic risk abnormalities were present: 1) WC ≥102cm

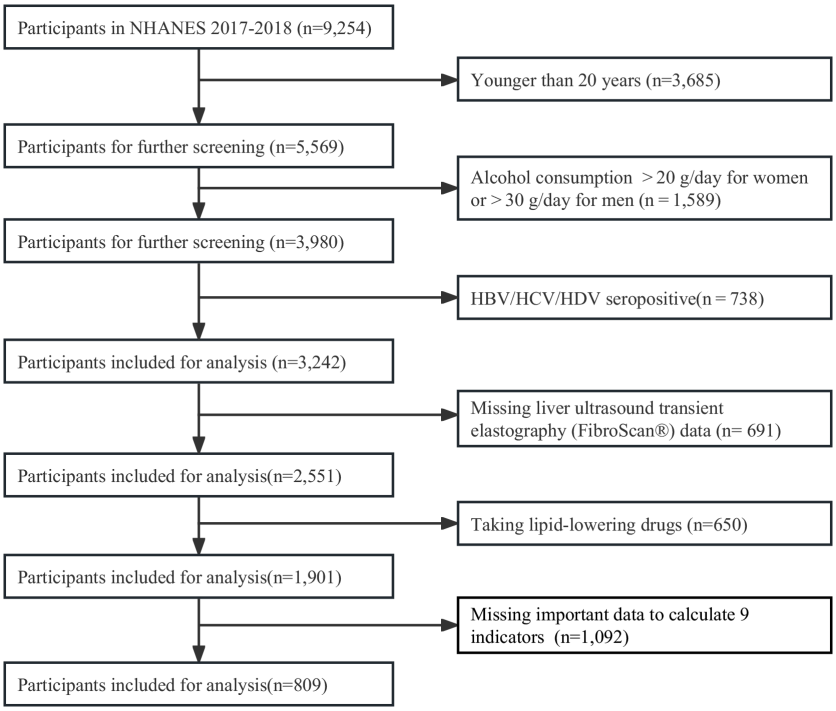

FIGURE 1
Flow chart for the selection of participants in the cross-sectional study.

in males or 88 cm in females, 2) blood pressure ≥130/85mmHg or specific medications, 3) serum TG ≥1.70mmol/L or specific medications, 4) HDL-C< 1.0mmol/L for males and < 1.3mmol/L for females, 5) prediabetes (a fasting glucose level between 5.6 and 6.9mmol/L, or a 2-hour post-load glucose level between 7.8 and 11.0mmol/L or an hemoglobin A1c (HbA1c) level between 5.7% and 6.4%), 6) a HOMA-IR score ≥ 2.5 and 7) a plasma C-reactive protein level > 2mg/L.

NAFLD was defined by presence of HS on ultrasound, excluding heavy drinking individuals (those consuming alcohol > 20 g/day for females or > 30 g/day for males) and other competing etiology for HS (those with hepatitis B/C/D positive serology). Considering that transient elastography (FibroScan®, TE) with controlled attenuation parameters (CAP) presented good accuracy in determining the level of hepatic steatosis (28), HS was diagnosed by FibroScan with CAP values ≥238 dB/m (29).

2.3 Nine indirect indexes and laboratory measurement

All participants were interviewed at home and physically examined at a mobile examination center (MEC). They were also required to fast at least nine hours before blood sampling. Height and weight were measured at the MEC following protocol and then used to calculate BMI, rounding to one decimal place. At the end of a normal exhale and while standing naturally with the legs spread out approximately 25-30 cm apart, WC was measured using an inelastic ruler with a minimum scale of one millimeter. The ruler was placed at the midpoint of the connecting line between the upper edge of the top of the iliac crest and the lower edge of the 12th rib (the narrowest part of the waist) and circled horizontally the abdomen, and readings were rounded to 0.1cm (30). After resting for at least 5 minutes, participants were measured with blood pressure using a standardized mercury sphygmomanometer in a sitting position.

Laboratory methods for measuring lipid profile, HbA1c, glucose, insulin, and plasma C-reactive protein level were described by CDC (31).

Alcohol consumption was calculated with self-reported information on drinking status within the last year. The consumed alcohol was reported in standard drinks and converted to grams using a multiplication factor of 14.

Indexes for assessment were calculated by using the following formulas (14, 16, 32):

$$BMI = \text{weight (kg)}/\text{height}^2\ \text{(m)};$$

$$VAI = \text{WC (cm)}/(39.68 + 1.88 \times \text{BMI (kg/m}^2)) \times \text{TG (mmol/L)}/1.03 \times 1.31/\text{HDL-C (mmol/L)} \text{ for males}$$

$$VAI = \text{WC (cm)}/(36.58 + 1.89 \times \text{BMI (kg/m}^2)) \times \text{TG (mmol/L)}/0.81 \times 1.52/\text{HDL-C (mmol/L)} \text{ for females}$$

$$LAP = [\text{WC (cm)} - 65] \times \text{TG (mmol/L)} \text{ for males}$$

$$LAP = [\text{WC (cm)} - 58] \times \text{TG (mmol/L)} \text{ for females}$$

$$WHtR = \text{WC (cm)}/\text{height (cm)}$$

$$TyG = \text{Ln}^{[\text{TG (mg/dL)} \times \text{FPG (mg/dL)}/2]}$$

$$TyG\text{-}BMI = TyG \times BMI$$

$$TyG\text{-}WC = TyG \times \text{WC (cm)}$$

$$TyG\text{-}WHtR = TyG \times WHtR$$

2.4 Covariates

Age, gender, ethnicity (Mexican American, non-Hispanic white, non-Hispanic black and others), family income-poverty ratio (FIPR) level, education level (less than high school, high school or equivalent, and college or above) and other demographic and lifestyle characteristics extracted from household questionnaires and used as covariates. Histories of hypertension, high cholesterol and diabetes referred to self-reported diagnosis of a particular disease. More details of the aforementioned characteristics are publicly available on the NHANES website.

2.5 Statistical analysis

The statistical analysis was performed in accordance with the CDC guidelines (https://www.cdc.gov/nchs/nhanes/tutorials/default.aspx), using R software (http://www.R-project.org, The R Foundation R.3.4.3). And MedCalc version 13.0 for Windows (MedCalc Software, Mariakerke, Belgium) was used for significance tests in AUC comparison.

Normally distributed data were expressed as mean ± standard deviation (SD), while abnormally distributed data were expressed as the median of the interquartile range (IQR) (25%, 75%). Characteristics were analyzed between the MAFLD group and the non-MAFLD group using Student's *t*-test, chi-square test or Mann-Whitney U test, as well as between the NAFLD group and the non-NAFLD group. Three logistic models were employed to estimate the odds ratio (OR) with a 95% confidence interval (CI) for MAFLD/NAFLD using nine indirect indexes (BMI, WC, VAI, LAP, WHtR, TyG, TyG-BMI, TyG-WC and TyG-WHtR) and MAFLD/NAFLD as continuous variables (per inter-quartile range (IQR) increment). Model 1 contained only independent variables. Model 2 was adjusted for gender, age, ethnicity, FIPR and education level. Model 3 was further adjusted for hypertension, high cholesterol and diabetes history. Results were presented with odds ratios (ORs) and confidence intervals (95% CIs). The receiver operating characteristic (ROC) curve and the area under curve (AUC) were used to assess the predictive performance of the nine indexes for MAFLD/NAFLD. DeLong et al's non-parametric method was used to compare the AUC between TyG-WC and other indexes. The best cutoff values of the nine indexes for predicting MAFLD/NAFLD were determined based on the maximum value of the sum of sensitivity and specificity. Statistical significance was set to $P<0.05$.

3 Results

3.1 Characteristics of the participants

Of all 5,569 individuals aged ≥20 years in cycle 2017-2018 of NHANES, we excluded those missing important data (Figure 1). At last, we included 809 participants with complete ultrasound and required data for the evaluation of MAFLD/NAFLD.

The demographic and clinical characteristics of participants, grouped as non-MAFLD, MAFLD, non-NAFLD and NAFLD were shown in Table 1. Among all the 809 participants, there were 478 participants with MAFLD and 499 participants with NAFLD, respectively. The proportion of male and female was 50.43% and 49.57%, respectively. The mean age was 46.0 (33.0, 60.0) years. Participants with and without MAFLD/NAFLD had statistically different baseline characteristics, except for FIPR and education level. However, there was no statistical difference in gender between the two groups with and without MAFLD. Participants with MAFLD/NAFLD were more likely to be older and have hypertension/high cholesterol/diabetes. More importantly, participants with MAFLD/NAFLD had higher BMI/WC/VAI/LAP/WHtR/TyG/TyG-BMI/TyG-WC/TyG-WHtR levels.

3.2 Associations between nine indirect indexes and NAFLD/MAFLD

Table 2 showed the multi-variate adjusted ORs and 95% CIs of MAFLD/NAFLD risks in relation to the quartile increment of nine indexes levels. After adjusting for gender, age, ethnicity, FIPR and education level, all those nine indexes were positive correlated with the risks of MAFLD/NAFLD. For MAFLD, TyG-WC presented the highest OR (OR = 28.435, 95% CI = 12.121 to 66.705), followed by TyG-WHtR (OR = 26.863, 95% CI = 12.417 to 58.115), TyG-BMI (OR = 17.196, 95% CI = 7.193 to 41.110), LAP (OR = 16.609, 95% CI = 7.927 to 34.797), WC (OR = 15.449, 95% CI = 7.440 to 32.077), WHtR (OR = 15.005, 95% CI = 8.052 to 27.964), BMI (OR = 10.986, 95% CI = 5.317 to 22.698), TyG (OR = 5.901, 95% CI = 3.825 to 9.102), and VAI (OR = 4.651, 95% CI = 2.966 to 7.295). Similar results were found after adjusting for all the covariates.

For NAFLD, TyG-WC presented the highest OR (OR = 12.742, 95% CI = 6.576 to 24.689), followed by TyG-WHtR (OR = 12.202, 95% CI = 6.830 to 21.798), LAP (OR = 9.731, 95% CI = 5.318 to 17.807), TyG-BMI (OR = 8.278, 95% CI = 4.199 to 16.321), WC (OR = 8.204, 95% CI = 4.491 to 14.985), WHtR (OR = 7.939, 95% CI = 4.797 to 13.140), BMI (OR = 6.047, 95% CI = 3.315 to11.032), TyG (OR = 4.896, 95% CI = 3.164 to 7.577), VAI (OR = 3.706, 95% CI = 2.309 to 5.948).

3.3 Nine indirect indexes for predicting MAFLD/NAFLD

Table 3 and Figure 2 showed the AUC values (95% CI) of the 9 indexes for screening American adults with MAFLD/NAFLD. For MAFLD, TyG-WC presented the highest AUC for male (0.900, 95% CI: 0.867-0.927) and overall (0.869, 95% CI: 0.843-0.891). The optimum cutoff value of TyG-WC was 789.868 (specificity 92.43%, sensitivity 72.61%) for male. However, TyG-WHtR presented the highest AUC for female (0.845, 95% CI: 0.806-0.879), with an optimum cutoff value of 4.821 (specificity: 86.78%, sensitivity: 69.54%). Table 3 also showed negative predictive value (NPV) and positive predictive value (PPV) of the nine indexes.

Similar results were found for NAFLD. TyG-WHtR presented best predictive performance for female, while TyG-WC presented best predictive performance for male (Table 3).

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TABLE 1 Basic characteristics of participants by MAFLD and NAFLD in NHANES 2017-2018.

Variables	Total (*n*=809)		*p*	Total (*n*=809)		*p*
	non-MAFLD (*n* = 331)	MAFLD (*n* = 478)		non-NAFLD (*n* = 310)	NAFLD (*n* = 499)	
Age	40.00 (28.50, 57.00)	50.00 (37.00, 61.00)	< 0.001	41.00 (29.25, 57.00)	49.00 (36.00, 61.00)	< 0.001
Gender, *n* (%)			0.177			0.032
Female	174 (52.57)	227 (47.49)		169 (54.52)	232 (46.49)	
Male	157 (47.43)	251 (52.51)		141 (45.48)	267 (53.51)	
Ethnicity, *n* (%)			< 0.001			< 0.001
Mexican American	33 (9.97)	77 (16.11)		30 (9.68)	80 (16.03)	
Non-Hispanic Black	105 (31.72)	93 (19.46)		101 (32.58)	97 (19.44)	
Non-Hispanic White	90 (27.19)	164 (34.31)		81 (26.13)	173 (34.67)	
Other	103 (31.12)	144 (30.13)		98 (31.61)	149 (29.86)	
FIPR	2.16 (1.20, 4.27)	2.13 (1.22, 4.13)	0.884	2.20 (1.18, 4.25)	2.11 (1.22, 4.155)	0.880
Education, *n* (%)			0.733			0.654
College or above	195 (58.91)	275 (57.65)		186 (60.00)	284 (57.03)	
High school or equivalent	78 (23.57)	108 (22.64)		70 (22.58)	116 (23.29)	
Less than high school	58 (17.52)	94 (19.71)		54 (17.42)	98 (19.68)	
BMI (kg/m2)	24.00 (21.50, 26.90)	30.80 (27.53, 35.10)	< 0.001	24.15 (21.60, 27.28)	30.30 (27.00, 34.95)	< 0.001
WC	85.50 (78.45, 94.10)	104.40 (95.60, 115.45)	< 0.001	85.70 (78.53, 94.60)	103.70 (94.10, 115.00)	< 0.001
VAI	1.00 (0.67, 1.52)	1.83 (1.27, 2.90)	< 0.001	1.01 (0.67, 1.56)	1.77 (1.23, 2.79)	< 0.001
LAP	21.23 (13.19, 37.72)	58.84 (40.33, 86.40)	< 0.001	21.92 (13.58, 38.86)	57.08 (37.85, 83.82)	< 0.001
WHtR	0.51 (0.47, 0.57)	0.63 (0.57, 0.69)	< 0.001	0.52 (0.47, 0.57)	0.62 (0.57, 0.69)	< 0.001
TyG	8.27 (8.00, 8.62)	8.78 (8.47, 9.18)	< 0.001	8.29 (7.99, 8.64)	8.76 (8.43, 9.16)	< 0.001
TyG-BMI	200.81 (174.19, 227.81)	270.06 (240.02, 314.40)	< 0.001	202.30 (174.18, 231.15)	266.78 (236.94, 312.21)	< 0.001
TyG-WC	707.22 (637.65, 806.62)	927.21 (836.70, 1026.31)	< 0.001	708.82 (639.24, 808.82)	916.18 (824.10, 1019.42)	< 0.001
TyG-WHtR	4.23 (3.83, 4.83)	5.51 (5.00, 6.18)	< 0.001	4.29 (3.84, 4.89)	5.46 (4.94, 6.16)	< 0.001
Hypertension, *n* (%)			< 0.001			< 0.001
no	278 (84.50)	301 (62.97)		258 (83.77)	321 (64.33)	
yes	51 (15.50)	177 (37.03)		50 (16.23)	178 (35.67)	
High cholesterol, *n* (%)			0.002			0.011
no	274 (83.03)	348 (73.42)		255 (82.26)	367 (74.29)	
yes	56 (16.97)	126 (26.58)		55 (17.74)	127 (25.71)	
Diabetes, *n* (%)			< 0.001			< 0.001
no	311 (93.96)	384 (80.34)		290 (93.55)	405 (81.16)	
yes	20 (6.04)	94 (19.66)		20 (6.45)	94 (18.84)	

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; FIPR, family income-poverty ratio; BMI, body mass index; WC, waist circumference; VAI, visceral adiposity index; LAP, lipid accumulation product; WHtR, waist-to-height ratio; TyG, triglyceride and glucose index.

3.4 Gender difference in AUC values between TyG-WC and other indexes

TyG-WC presented the largest AUC in overall population both with NAFLD and MAFLD. We compared the AUC values between TyG-WC and other eight indexes to explore possible gender differences. Table 4 showed the gender differences in AUC values between TyG-WC and other indexes for MAFLD/NAFLD. Similar results were found for both MAFLD and NAFLD. For female, the AUC value of TyG-WC was statistically different from that of WC (P<0.05), but not statistically different from that of BMI, LAP, TyG-BMI and TyG-WHtR. For male, the AUC value of TyG-WC was statistically different from that of BMI and WC (P<0.05), but not statistically different from that of LAP, TyG-BMI and TyG-WHtR.

TABLE 2 Multi-variate adjusted ORs (95% CIs) of NAFLD and MAFLD in relation to quartile increment of nine predictive indexes among participants in NHANES 2017-2018.

Variables	Model 1	*p-Value*	Model 2	*p-Value*	Model 3	*p-Value*
MAFLD						
BMI	9.925[4.677,21.063]	<0.001	10.986[5.317,22.698]	0.001	10.847[5.195,22.650]	0.008
WC	16.011[7.257,35.321]	<0.001	15.449[7.440,32.077]	<0.001	15.638[7.426,32.935]	0.005
VAI	4.000[2.699,5.929]	<0.001	4.651[2.966,7.295]	0.001	4.399[2.698,7.172]	0.010
LAP	15.372[7.152,33.039]	<0.001	16.609[7.927,34.797]	<0.001	15.931[7.720,32.876]	0.005
WHtR	11.323[6.324,20.277]	<0.001	15.005[8.052,27.964]	<0.001	15.399[8.214,28.870]	0.003
TyG	6.178[3.958,9.644]	<0.001	5.901[3.825,9.102]	<0.001	5.768[3.608,9.223]	0.005
TyG-BMI	16.132[6.316,41.202]	<0.001	17.196[7.193,41.110]	0.001	17.118[7.165,40.895]	0.008
TyG-WC	29.436[11.649,74.379]	<0.001	28.435[12.121,66.705]	<0.001	28.877[12.298,67.805]	0.005
TyG-WHtR	19.412[9.256,40.710]	<0.001	26.863[12.417,58.115]	<0.001	27.798[12.960,59.623]	0.003
NAFLD						
BMI	5.775[3.145,10.603]	<0.001	6.047[3.315,11.032]	0.001	5.806[3.106,10.851]	0.012
WC	8.714[4.531,16.759]	<0.001	8.204[4.491,14.985]	<0.001	7.921[4.232,14.826]	0.007
VAI	3.173[2.077,4.846]	<0.001	3.706[2.309,5.948]	0.002	3.557[2.129,5.942]	0.017
LAP	8.779[4.768,16.166]	<0.001	9.731[5.318,17.807]	<0.001	9.350[5.087,17.188]	0.006
WHtR	6.234[3.883,10.008]	<0.001	7.939[4.797,13.140]	<0.001	7.767[4.584,13.160]	0.005
TyG	4.997[3.262,7.656]	<0.001	4.896[3.164,7.577]	<0.001	4.826[2.962,7.861]	0.008
TyG-BMI	8.178[4.005,16.699]	<0.001	8.278[4.199,16.321]	0.001	8.113[4.084,16.116]	0.009
TyG-WC	13.343[6.579,27.061]	<0.001	12.742[6.576,24.689]	<0.001	12.642[6.453,24.766]	0.005
TyG-WHtR	9.329[5.363,16.227]	<0.001	12.202[6.830,21.798]	<0.001	12.283[6.782,22.245]	0.004

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; VAI, visceral adiposity index; LAP, lipid accumulation product; WHtR, waist-to-height ratio; TyG, triglyceride and glucose index. Model 1 included only independent variables; model 2 was additionally adjusted for gender, age, ethnicity, FIPR and education level; and model 3 was further adjusted for the disease history (hypertension, high cholesterol and diabetes).

Therefore, TyG-WC, TyG-BMI and TyG-WHtR might have better predictive performance in identifying MAFLD and NAFLD compared to BMI, WC and LAP.

4 Discussion

As the most common chronic liver disease, MAFLD/NAFLD has affected the life of about 1/4 adults worldwide. Increasing studies are exploring easy-to-use, practical, and reliable predictors of MAFLD/NAFLD, which is also one of the urgent needs in clinical practice. Obesity is an independent risk factor of NAFLD. A Meta-analysis showed that the risk of NAFLD in obese individuals was 3.5 times higher than those with normal BMI, and the severity of NAFLD tended to increase in individuals with higher BMI (33). Previous studies suggested that inflammatory mediators such as lipocalin, leptin, and tumor necrosis factor-α secreted by adipocytes (34), especially lipocalin and leptin, could influence the development of NAFLD by regulating hepatic fat accumulation, IR, and fibrosis (35). Obesity-associated IR is considered to be one of the important pathogenic mechanisms of NAFLD (36).BMI, WC, VAI, LAP and WHtR are obesity-associated indexes, TyG is a reliable IR index, and TyG-BMI, TyG-WC and TyG-WHtR are composite indicators combining TyG and anthropometric parameters. Which of the nine indexes will be most closely linked to NAFLD/MAFLD remains to be determined.

We screened nine associated indexes and compared their performance in predicting MAFLD/NAFLD in adults based on previous studies. To the best of our knowledge, this was the first study to investigate the association between indirect indexes (BMI/WC/VAI/LAP/WHtR/TyG/TyG-WC/TyG-BMI/TyG-WHtR) and MAFLD/NAFLD in the U.S. population by different gender groups. Further assessment has been completed to assess the diagnostic utility of nine indexes for MAFLD/NAFLD. We found that all nine indexes were significantly associated with risks of MAFLD/NAFLD. ROC analysis showed that TyG-WC was the best predictor, followed by TyG-WHtR and TyG-BMI, for MAFLD/NAFLD in male participants. TyG-WHtR was the best predictor for MAFLD/NAFLD in female participants. It is notable that these findings highlight the potential impact of gender on the reliability of assessed indexes.

4.1 Relationship between BMI/WC/WHtR and MAFLD/NAFLD

BMI is the most commonly used clinical indicator of whole-body adiposity, while WC is more suitable for assessing central obesity. Our

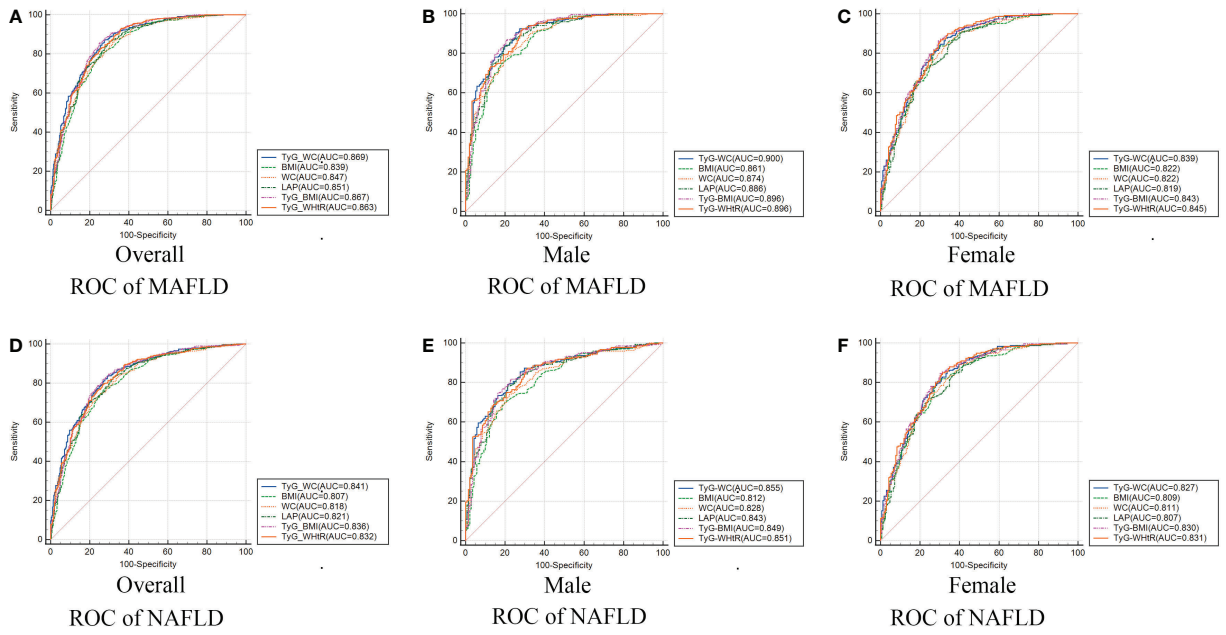

FIGURE 2
Receiver operating characteristic curves of TyG-WC and other indexes in overall (A/D), male (B/E), female (C/F) for identifying MAFLD/NAFLD. MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; TyG, triglyceride and glucose index; WC, waist circumference; BMI, body mass index; LAP, lipid accumulation product; WHtR, waist-to-height ratio.

TABLE 3 Selected parameters for predicting MAFLD/NAFLD and the corresponding AUC, optimal cut-off values, their sensitivity and specificity, PPV and NPV.

	Gender	Variable	AUC (95%CI)	Cut-off Values	Specificity (%)	Sensitivity (%)	PPV	NPV	*p*-Value
MAFLD	Female	BMI	0.822[0.781,0.859]	26.500	81.94	72.41	0.795	0.754	<0.0001
		WC	0.822[0.781,0.859]	88.600	85.46	66.09	0.767	0.777	<0.0001
		VAI	0.733[0.687,0.776]	1.225	81.5	56.32	0.709	0.700	<0.0001
		LAP	0.819[0.778,0.856]	33.125	86.34	63.79	0.757	0.782	<0.0001
		WHtR	0.833[0.793,0.868]	0.574	84.58	70.69	0.790	0.778	<0.0001
		TyG	0.725[0.679,0.769]	8.535	67.4	70.69	0.750	0.624	<0.0001
		TyG-BMI	0.843[0.804,0.878]	225.138	86.34	70.69	0.794	0.799	<0.0001
		TyG-WC	0.839[0.800,0.874]	755.391	87.67	67.24	0.777	0.807	<0.0001
		TyG-WHtR	0.845[0.806,0.879]	4.821	86.78	69.54	0.788	0.801	<0.0001
	Male	BMI	0.861[0.824,0.893]	27.400	74.5	82.17	0.870	0.668	<0.0001
		WC	0.874[0.838,0.904]	96.300	78.09	79.62	0.860	0.694	<0.0001
		VAI	0.800[0.758,0.838]	1.269	71.71	77.71	0.837	0.632	<0.0001
		LAP	0.886[0.852,0.916]	36.271	83.27	80.89	0.874	0.752	<0.0001
		WHtR	0.870[0.833,0.901]	0.545	82.07	76.43	0.848	0.727	<0.0001
		TyG	0.790[0.748,0.829]	8.527	74.9	72.61	0.814	0.644	<0.0001
		TyG-BMI	0.896[0.863,0.924]	228.023	86.45	79.62	0.871	0.786	<0.0001
		TyG-WC	0.900[0.867,0.927]	789.868	92.43	72.61	0.844	0.857	<0.0001
		TyG-WHtR	0.896[0.862,0.923]	4.476	92.43	71.34	0.838	0.855	<0.0001
	Overall	BMI	0.839[0.811,0.863]	26.700	80.33	74.32	0.819	0.723	<0.0001
		WC	0.847[0.820,0.871]	90.300	87.24	67.07	0.793	0.784	<0.0001

(Continued)

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TABLE 3 Continued

	Gender	Variable	AUC (95%CI)	Cut-off Values	Specificity (%)	Sensitivity (%)	PPV	NPV	*p*-Value
		VAI	0.759[0.728,0.789]	1.186	79.71	62.84	0.756	0.682	<0.0001
		LAP	0.851[0.825,0.875]	33.286	86.61	70.09	0.807	0.784	<0.0001
		WHtR	0.842[0.815,0.866]	0.559	81.38	73.72	0.817	0.733	<0.0001
		TyG	0.758[0.727,0.788]	8.535	71.34	71.6	0.784	0.634	<0.0001
		TyG-BMI	0.867[0.841,0.889]	223.282	88.28	72.21	0.821	0.810	<0.0001
		TyG-WC	0.869[0.843,0.891]	790.927	87.03	72.51	0.821	0.795	<0.0001
		TyG-WHtR	0.863[0.838,0.886]	4.811	82.64	74.62	0.825	0.749	<0.0001
NAFLD	Female	BMI	0.809[0.767,0.846]	26.500	80.17	71.6	0.795	0.725	<0.0001
		WC	0.811[0.769,0.848]	86.400	89.22	59.76	0.753	0.802	<0.0001
		VAI	0.722[0.676,0.766]	1.225	80.6	56.21	0.716	0.679	<0.0001
		LAP	0.807[0.765,0.845]	40.826	71.98	75.74	0.803	0.663	<0.0001
		WHtR	0.820[0.779,0.856]	0.574	82.76	69.82	0.790	0.747	<0.0001
		TyG	0.715[0.668,0.759]	8.535	65.95	69.82	0.750	0.599	<0.0001
		TyG-BMI	0.830[0.789,0.865]	225.138	84.48	69.82	0.794	0.766	<0.0001
		TyG-WC	0.827[0.786,0.863]	755.391	85.78	66.27	0.777	0.772	<0.0001
		TyG-WHtR	0.831[0.791,0.867]	4.821	84.91	68.64	0.788	0.768	<0.0001
	Male	BMI	0.812[0.771,0.849]	27.400	70.04	80.14	0.870	0.586	<0.0001
		WC	0.828[0.787,0.863]	96.300	73.78	78.01	0.864	0.611	<0.0001
		VAI	0.769[0.725,0.809]	1.269	67.79	75.89	0.842	0.554	<0.0001
		LAP	0.843[0.804,0.877]	36.271	78.28	78.72	0.874	0.657	<0.0001
		WHtR	0.823[0.783,0.859]	0.545	77.53	74.47	0.852	0.636	<0.0001
		TyG	0.767[0.722,0.807]	8.512	73.03	70.21	0.823	0.579	<0.0001
		TyG-BMI	0.849[0.811,0.883]	228.023	81.27	77.3	0.871	0.685	<0.0001
		TyG-WC	0.855[0.817,0.888]	831.409	79.03	78.72	0.876	0.665	<0.0001
		TyG-WHtR	0.851[0.813,0.884]	4.476	87.27	68.79	0.841	0.741	<0.0001
	Overall	BMI	0.807[0.779,0.834]	26.700	76.95	72.58	0.819	0.662	<0.0001
		WC	0.818[0.790,0.844]	94.400	74.75	74.52	0.825	0.647	<0.0001
		VAI	0.735[0.703,0.765]	1.186	76.95	61.29	0.762	0.623	<0.0001
		LAP	0.821[0.793,0.847]	33.286	82.97	68.06	0.807	0.713	<0.0001
		WHtR	0.810[0.781,0.836]	0.559	78.16	72.26	0.819	0.673	<0.0001
		TyG	0.741[0.710,0.771]	8.535	68.94	70.65	0.791	0.586	<0.0001
		TyG-BMI	0.836[0.808,0.861]	227.600	81.96	73.23	0.831	0.716	<0.0001
		TyG-WC	0.841[0.814,0.865]	790.927	83.57	70.97	0.823	0.729	<0.0001
		TyG-WHtR	0.832[0.804,0.857]	4.811	79.36	73.23	0.827	0.688	<0.0001

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; VAI, visceral adiposity index; LAP, lipid accumulation product; WHtR, waist-to-height ratio; TyG, triglyceride and glucose index;PPV, positive predictive value; NPV, Negative predictive value.

findings showed that, in both male and female participants, WC presented significantly better predictive performance for MAFLD/NAFLD than BMI, which suggested that abdominal obesity might be a more accurate and important index of steatosis than overweight measured by BMI. Our findings also showed that it was clinically valuable to include people with normal BMI but abnormal metabolism in the diagnosis of MAFLD.

Previous studies have found that WC may better reflect the risk of obesity-associated diseases compared to BMI (37). Li et al. found that WC was more effective than BMI in predicting metabolic syndrome

TABLE 4 Gender differences in AUC values between TyG-WC and other indexes.

MAFLD	Difference between Area (95%CI)	*p*-Value	NAFLD	Difference between Area (95%CI)	*p*-Value
Female			Female		
TyG-WC VS BMI	0.017[-0.011,0.045]	0.236	TyG-WC VS BMI	0.018[-0.010,0.046]	0.210
TyG-WC VS WC	0.017[0.002,0.032]	0.028	TyG-WC VS WC	0.016[0.001,0.031]	0.037
TyG-WC VS LAP	0.020[0.000,0.040]	0.051	TyG-WC VS LAP	0.020[0.000,0.040]	0.054
TyG-WC VS TyG-BMI	0.004[-0.016,0.024]	0.686	TyG-WC VS TyG-BMI	0.003[-0.018,0.023]	0.807
TyG-WC VS TyG-WHtR	0.006[-0.004,0.016]	0.258	TyG-WC VS TyG-WHtR	0.004[-0.006,0.015]	0.423
Male			Male		
TyG-WC VS BMI	0.039[0.013,0.064]	0.003	TyG-WC VS BMI	0.043[0.016,0.070]	0.002
TyG-WC VS WC	0.026[0.010,0.042]	0.002	TyG-WC VS WC	0.028[0.011,0.045]	0.001
TyG-WC VS LAP	0.014[-0.004,0.031]	0.126	TyG-WC VS LAP	0.012[-0.006,0.030]	0.193
TyG-WC VS TyG-BMI	0.004[-0.012,0.020]	0.663	TyG-WC VS TyG-BMI	0.006[-0.011,0.023]	0.493
TyG-WC VS TyG-WHtR	0.004[-0.006,0.015]	0.406	TyG-WC VS TyG-WHtR	0.004[-0.007,0.016]	0.429
Overall			Overall		
TyG-WC VS BMI	0.030[0.011,0.049]	0.002	TyG-WC VS BMI	0.033[0.013,0.053]	0.001
TyG-WC VS WC	0.022[0.011,0.033]	0.000	TyG-WC VS WC	0.022[0.011,0.033]	0.000
TyG-WC VS LAP	0.017[0.003,0.032]	0.015	TyG-WC VS LAP	0.019[0.005,0.034]	0.009
TyG-WC VS TyG-BMI	0.002[-0.012,0.016]	0.777	TyG-WC VS TyG-BMI	0.005[-0.009,0.019]	0.502
TyG-WC VS TyG-WHtR	0.005[-0.005,0.016]	0.328	TyG-WC VS TyG-WHtR	0.009[-0.002,0.020]	0.119

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; VAI, visceral adiposity index; LAP, lipid accumulation product; WHtR, waist-to-height ratio; TyG, triglyceride and glucose index.

in patients with T2DM (38). A prospective cohort study of 11,714 participants suggested that increased WC might result in blood pressure elevation even without increase in BMI (39). In addition, Hou et al. found a stronger correlation between WC and diabetes compared to BMI (40). For a specific BMI, a large WC meant two-to three-fold of the risk of developing diabetes and cardiovascular disease (CVD) in the future (41).

Some studies have explored the relationship between WC and MAFLD/NAFLD. Similar to our findings, Motamed et al. (42) found that WC presented excellent performance in the diagnosis of NAFLD (AUC: 0.8533, 95%CI: 0.8419-0.8646)and almost the same predictive power as fatty liver index (FLI), a widely used index for the diagnosis and evaluation of fatty liver development in a number of studies (43, 44).

We found that WC had higher predictive power than BMI, which may be related to the following factors. First, not all patients with NAFLD have an excessive BMI. In all, about 40% people NAFLD worldwide are classified as non-obese and nearly a fifth are lean (45). Second, the distribution of abdominal fat can be used as a marker of ectopic fat in various sites. According to previous study, those with a predominance of abdominal fat and a large WC have more visceral/intra-abdominal fat, expanded (hypertrophic) subcutaneous adipose cells, as well as dysfunctional and inflammatory adipose tissue (45), and thus are more likely to develop metabolic disorders. Third, it is known that unhealthy dietary patterns have a significant role in the development of MAFLD/NAFLD. Interestingly, Ghaemi et al. found that the indirect effect of diet through abdominal circumference was 28 times more than the direct effect on NAFLD and that WC is a powerful mediator in the association between dietary patterns and NAFLD (46), indicating that WC was of great importance in the development of NAFLD.

Notably, WHtR presented the best predictive performance for MAFLD/NAFLD in female participants. A possible explanation is that WHtR is an adjusted indicator with WC and height, so it can better indicate abdominal obesity than WC.

As easy-to-use and cheap indexes, WC and WHtR are important indicators for assessing central obesity and reliable indexes for efficient screening of individuals at high risk of MAFLD/NAFLD.

4.2 The relationship between VAI/LAP and MAFLD/NAFLD

As important indexes of visceral adiposity, VAI and LAP had good performance in diagnosing MAFLD/MAFLD in this study, with AUCs of about 0.7 and 0.8, respectively. And elevated VAI and LAP levels were associated with higher risks of MAFLD and NAFLD after

adjusting for all covariates, which was consistent with previous findings by Vural and Zhang et al. (47, 48).

Numerous studies have shown the close link between VAI/LAP and metabolic disorders. According to Dong et al. (49), VAI performed better than traditional adiposity index in predicting an unhealthy metabolic phenotype in Chinese children and adolescents (BMI, WC, and WHtR). However, there is a strong correlation between VAI and abnormalities in lipid and glucose levels in obese individuals (50). A 10-year prospective cohort study has shown a link between LAP and incident cardiovascular disease.

Unexpectedly, we found that MAFLD was also strongly correlated with VAI and LAP. Possible explanations could be: First, people with more visceral adipose tissue (VAT) had higher levels of inflammatory cytokines, including C-reactive protein, tumor necrosis factor-α, and interleukin-6, which may cause IR and metabolic problems (51). Second, the enhanced lipolysis in VAT causes an excess of free fatty acids (FFAs) to be released into the portal vein. FFAs with high concentrations can cause IR and intracellular inflammation (52). Third, the elevated FFAs load in NAFLD may impede a β-oxidation, which takes place in the liver mitochondria, leading to the production of reactive oxygen species (53). Oxidative stress as a result causes the initiation and development of fibrosis, inflammation, and liver damage.

It is notable that LAP seems to have a better predictive performance than VAI according to our findings. LAP can be more easily calculated with WC and TG, so it can be widely used in clinical practice.

4.3 Relationship between TyG/TyG-BMI/TyG-WC/TyG-WHtR and MAFLD/NAFLD

TyG index has been widely explored in cardiovascular diseases recently. It is considered as a reliable index to predict adverse cardiovascular events and progression of coronary artery calcification in patients with acute coronary syndrome and diabetes (19, 54). Our findings suggested that TyG was strongly linked to NAFLD, with a 4~6-fold increase in MAFLD/NAFLD risk as each quartile increment in TyG. According to ROC analysis, the optimal cut-off point of TyG for MAFLD was 8.535 and the AUC was 0.758 (95% CI 0.727-0.788), which were generally consistent with the previous findings by Zhang et al. (21). Moreover, we further explored the relationship between TyG and MAFLD/NAFLD in different gender subgroups.

We also found that TyG presented lower performance in predicting MAFLD/NAFLD compared to the other eight indexes. TyG is considered as a novel indicator of IR, but previous study found that a significant number patients with fatty liver remained insulin sensitive and 37% of these patients presented no metabolic syndrome, prediabetes or diabetes (55). We therefore speculate that this may be a reason why the TyG has lower predictive performance than other indexes.

Interestingly, we found that TyG-BMI, TyG-WC and TyG-WHtR had higher predictive performance for MAFLD/NAFLD in overall population, which was similar to the findings by Sheng et al. (56). A possible explanation could be that TyG-BMI, TyG-WC and TyG-BMI were adjusted indexes with indicators of insulin sensitivity and obesity including glucose, insulin level, BMI, WC and WHtR, which were also suggested with good predictive performance for T2DM in previous study (57). Our findings further confirmed the significant contribution of obesity and IR in the development of MAFLD/NAFLD.

4.4 Strengths and limitations

Strengths: For the first time, we explored the differences in the performance of nine indexes in predicting both MAFLD and NAFLD, providing a reliable reference for efficient and accurate screening in clinical settings. Furthermore, there are few related clinical studies on MAFLD, so our findings contribute some evidence to the scant research. Last, the study findings were based on the high-quality anthropometric and laboratory data from the NHANES database, which was comprehensive and representative of the population on a national level.

Limitations: First, rather than using the gold standard in histology, the diagnosis of hepatic steatosis was based on imaging (FibroScan). Second, despite the fact that we adjusted multiple covariates in the study, there may still be other potential confounders, such as physical activity and food intakes. Third, in this cross-sectional study, we were unable to confirm a cause-effect relationship between the risk of MAFLD/NAFLD and the 9 indexes (BMI, WC, VAI, LAP, WHtR, TyG, TyG-WC, TyG-BMI and TyG-WHtR). More large-scale and prospective cohort studies should be encouraged in the future.

5 Conclusion

Our study suggests that BMI/WC/VAI/LAP/WHtR/TyG/TyG-WC/TyG-BMI/TyG-WHtR are important reference indexes for identifying the risks of MAFLD/NAFLD. TyG-WC presents the best predictive performance in male, while TyG-WHtR presents the best predictive performance in female. Further prospective studies are needed before definite conclusions about the best predictor of MAFLD/NAFLD can be made.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: Centers for disease control and prevention. 2017: National Health and Nutrition Examination Survey (NHANES). U.S. Department of health and human services. Available from https://wwwn.cdc.gov/nchs/data/nhanes/2017-2018/manuals/2017_MEC_Laboratory_Procedures_Manual.pdf accessed 31 March 2020.

Ethics statement

The studies involving human participants were reviewed and approved by The Research Ethics Review Board of the National

Center for Health Statistics examined and approved the NHANES protocol. Before taking part, each participant completed a written statement of informed consent. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, HP and WL. Methodology, HP, LP and SR. Formal analysis, MW, SH and MZ. Data curation, ZC, ZY, LX and QY. Writing—original draft preparation, HP, LP and SR. Writing—review and editing, WL. Visualization, MW, SH and MZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Significant fibrosis assessed by liver biopsy among Chinese bariatric surgery patients: A prospective cross-sectional study

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Background: Fibrosis stages affect clinical prognoses related to nonalcoholic fatty liver disease (NAFLD). However, data on the prevalence and clinical features of significant fibrosis are scarce in Chinese bariatric surgery patients. We aimed to investigate the prevalence of significant fibrosis in bariatric surgery patients and to identify its predictors.

Methods: We prospectively enrolled the patients performing intra-operative liver biopsies during bariatric surgery from a bariatric surgery center in a university hospital between May 2020 and January 2022. Anthropometric characteristics, co-morbidities, laboratory data and pathology reports were collected and analyzed. The performance of non-invasive models was evaluated.

Results: Of 373 patients, 68.9%% had non-alcoholic steatohepatitis (NASH) and 60.9% exhibited fibrosis. Significant fibrosis was present in 9.1% of patients, advanced fibrosis in 4.0%, and cirrhosis in 1.6%. Multivariate logistic regression showed that increasing age (odds ratio [OR], 1.06; p=0.003), presence of diabetes (OR, 2.62; p=0.019), elevated c- peptide (OR, 1.26; p=0.025) and elevated aspartate aminotransferase (AST) (OR, 1.02; p=0.004) were independent predictors of significant fibrosis. The non-invasive models, AST to Platelet ratio (APRI), Fibrosis-4 (FIB-4), and Hepamet fibrosis scores (HFS) provided greater accuracy for predicting significant fibrosis, compared to the NAFLD Fibrosis Score (NFS) and BARD score.

Conclusion: More than two-thirds of bariatric surgery patients had NASH and the prevalence of significant fibrosis was high. Elevated levels of AST and c- peptide, advanced age and diabetes indicated a higher risk of significant fibrosis. Non-invasive models, APRI, FIB-4 and HFS can be used to identify significant liver fibrosis in bariatric surgery patients.

KEYWORDS

non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, fibrosis, bariatric surgery, obesity

Introduction

Nonalcoholic fatty liver disease (NAFLD), now known as metabolic-associated fatty liver disease (MAFLD), has become the most common cause of chronic liver disease worldwide (1, 2). Epidemiological research estimates a 25% prevalence in the general population, rising to 90% in patients undergoing bariatric surgery (3, 4). Some risk factors, including obesity, diabetes, hypertension, hyperlipidemia and metabolic syndrome are established indicators of NAFLD development (5). Thus, it is anticipated that as the prevalence of obesity and diabetes increases, so will that of NAFLD. Non-alcoholic steatohepatitis (NASH) is the active form of NAFLD. It is characterized by hepatocyte ballooning and lobular necroinflammation (which can occur with or without fibrosis) and may silently progress towards cirrhosis, end-stage liver disease, and even hepatocellular carcinoma (6, 7).

A strong correlation has been demonstrated between the degree of fibrosis and liver-specific morbidity and overall mortality in NAFLD patients (8). Furthermore, patients with significant fibrosis are most likely to experience complications and further progression of the hepatic disease (9). Unfortunately, most patients with fibrosis are asymptomatic and have normal transaminases. Thus, we need to detect risk factors for liver fibrosis, especially significant fibrosis, because distinguishing between NAFLD with or without significant fibrosis has important clinical significance for determining the prognosis (10, 11). Abdominal ultrasound is effective in detecting fatty liver but not liver fibrosis. To date, histologic evaluation of the liver biopsy remains the gold standard for diagnosing NASH and assessing the stage of fibrosis (6, 12). Nevertheless, liver biopsy is not a routine procedure due to its invasiveness, high costs, sampling variability and various potential complications. There are several non-invasive scoring systems specifically designed to identify the presence of advanced fibrosis which include: the aspartate aminotransferase (AST)-to-platelet ratio index (APRI) (13), BARD scoring system (14), NAFLD Fibrosis Score (NFS) (15) and Fibrosis-4 (FIB-4) score (16). Some studies have shown that these non-invasive scoring systems were assessed to detect advanced fibrosis in morbid obesity or diabetes, but the application of these scores was from white and non-Asian populations (17, 18). Little is known about the reliability of non-invasive scoring systems to detect significant liver fibrosis in Chinese bariatric patients. (Reviewer #1). In addition, these scoring systems were developed using data from viral hepatitis patients and have yet to be validated for Chinese bariatric surgery patients. Thus, we determined to test the hypothesis that these algorithms were able to identify significant liver fibrosis among bariatric surgery patients.

Currently, research data reporting on the prevalence and clinical characteristics of fibrosis mainly originate from Western countries (3, 19, 20). However, there has yet to be a study that specifically evaluates the prevalence of significant fibrosis (and its associated predictors) in the Chinese population. In fact, China is one of the countries with the largest population of obesity, and the obesity phenotype is mainly moderate obesity (21). In addition, given that Chinese eating habits and lifestyles are particularly distinctive compared with those of other nationalities, the prevalence of significant fibrosis may vary considerably compared with data published to date. Determining potential risk factors for significant fibrosis may help clinicians perform risk stratification of bariatric surgery patients with NAFLD, facilitating early identification of high-risk populations. Thus, this study aimed to evaluate prevalence and clinical predictors of hepatic fibrosis (confirmed by biopsy) experienced by Chinese bariatric surgery patients. In addition, we look to validate the reliability of the aforementioned, non-invasive fibrosis scoring algorithms.

Materials and methods

Study population

This is a prospective, observational study of a cohort of Chinese bariatric surgery patients. In this study, patients were recruited from a bariatric surgery center in a tertiary university hospital during the period May 2020-January 2022. Then inclusion criteria for this study were as follows: (1) patients who met metabolic surgery standard: body mass index (BMI) ≥ 32.5 kg/m^2 or BMI ≥ 27.5 kg/m^2 with poor weight loss by medications or lifestyle modification and with at least two components of metabolic syndrome or with comorbidities (22); (2) patients who had consented to a trans-operative liver biopsy. The exclusion criteria were:(1) patient had any history of alcoholism (average daily consumption of alcohol of 30 g/day for men and 20 g/day for women); (2) patients tested positive for viral hepatitis (B or C); (3) patients had incomplete pathology reports. (4) patients with diabetes take insulin treatment; (5) patients underwent preoperative weight loss or very low-calorie diets. (Reviewer #3) The study was approved by our hospital ethics committee (2019-024). Written informed consent was obtained from each participant or legal representatives before bariatric surgery.

Clinical and laboratory data

Clinical and laboratory data was sourced from a prospectively collected database (KY-2020-021). Demographic data (gender, age), anthropometric data (weight, BMI, waist circumference, hip circumference, waist to hip ratio) and the presence of co-morbidity (Metabolic syndrome, hypertension, type-2 diabetes mellitus (T2DM)) were analyzed. BMI was calculated by dividing body weight by the square of body height. Metabolic syndrome was defined as the presence of at least 3 of the 5 following criteria (23): (1) abdominal obesity (waist circumference ≥ 90 cm in man and ≥ 80 cm in women); (2) blood pressure ≥130/85 mmHg or taking antihypertensive drug; (2) serum triglycerides ≥1.7 mmol/L, or taking lipid-lowering drugs; (4) serum high-density lipoprotein cholesterol (HDL-c) <1.0 mmol/L for man and <1.3 mmol/L for women, or drug treatment for reduced HDL-c; (5) fasting plasma glucose (FPG) ≥5.6 mmol/L), or drug treatment for elevated glucose. Hypertension was diagnosed as patients with systolic/diastolic pressures ≥ 140/90 mmHg, or taking antihypertensive drugs. T2DM was defined in accordance with the clinical classification and diagnosis of diabetes (24).

We also collected the following biochemical parameters: FPG; fasting plasma C-peptide; fasting plasma insulin; glycated hemoglobin (HbA1c); serum uric acid (SUA); creatinine; blood urea nitrogen

(BUN); aminotransferase (ALT); aspartate aminotransferase (AST); γ-glutamyl transpeptidase (GGT); alkaline phosphatase (ALP); total bilirubin; direct bilirubin; indirect bilirubin; albumin; total cholesterol; triglycerides; HDL-C; low-density lipoprotein cholesterol (LDL-C)], and routine blood data pertaining to red blood cell (RBC), white blood cells (WBC) and platelet. Standard laboratory methods were used to carry out each of these biochemical tests. In addition, we also calculated homeostatic model assessment of insulin resistance (HOMA-IR) (insulin (mU/L) x FPG (mmol/L)/ 22.5) to indirectly assessed insulin resistance.

In addition, certain non-invasive fibrosis scores were computed using the relevant published formulas: APRI (AST to platelet ratio index) (13); FIB-4 (age, ALT, AST, platelet) (16); NFS (age, BMI, diabetes status, platelet, albumin) (15); BARD (BMI, AST/ALT ratio, T2DM) (14); Hepamet Fibrosis Score (HFS) was computed using a free web page: https://www.hepamet-fibrosis-score.eu/ (25)..

Histopathological evaluation

Liver specimens were obtained, in the form of a wedge biopsy from the left lobe of the liver, by the surgeon performing the bariatric surgery. Liver tissue specimens were routinely formalin-fixed, paraffin-embedded and then stained with hematoxylin-eosin. The biopsy specimen was at least 10 mm long or not less than 10 portal tracts. All histological examinations were performed by the same experienced pathologist, blinded for clinical and laboratory data. Histopathological analysis was performed according to the steatosis, activity, and fibrosis (SAF) score (26). Fibrosis was graded as 0–4 stages (27): F0 = no fibrosis, F1 = perisinusoidal or periportal fibrosis, F2 = perisinusoidal and portal/periportal fibrosis, F3 = bridged fibrosis, and F4 = cirrhosis. NASH was defined as steatosis (5% of hepatocytes), hepatocellular ballooning and lobular inflammation. Significant liver fibrosis was defined as stage 2 fibrosis or above.

Statistical analysis

Statistical analysis was performed using SPSS version 26.0 (SPSS Inc. Chicago, IL, USA), and MedCalc version 19.4.0 (Ostend, Belgium). Continuous data were presented as mean ± standard deviation (SD), whilst categorical data was given as a number (frequency or percentage). Pairwise comparisons of continuous data were performed using the t-test or Mann–Whitney test, whereas categorical data were compared using a chi-square test or Fisher's exact test. Normality was assessed using the Kolmogorov-Smirnov test. To identify the predictive factors related to significant fibrosis, univariate logistic regression models were performed to identify each possible predictor. Then, multicollinearity was assessed using the variance inflation factor (VIF) method, with a VIF≥5 indicating the presence of multicollinearity, and no significant collinear variables were found. Finally, independent variables with statistically significant (P <0.05) were introduced into a multivariable logistic regression (backward selection method). An odds ratio (OR) with a 95% confidence interval (CI) was calculated.

In order to evaluate the performance of non-invasive scoring systems for detecting significant fibrosis, we calculated the area under the curve (AUC) of receiver operating characteristic curves (ROC) (AUROC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) along with their 95% CI. ROC curves were compared using the methods of Hanley & McNeil (28). Statistical significance was defined as a $p<0.05$.

Results

Clinical baseline characteristics

Of the 417 consecutive patients who underwent bariatric surgery between May 2020 and January 2022, 44 patients exhibited criteria (detailed in methods) that meant they were excluded from our study. In total, 373 patients were recruited into this study, including 126 (33.7%) male patients and 247 (66.3%) female patients. Flow diagram of the study is shown in Figure 1.

The mean age and BMI of the study population were 30.9 ± 9.0 years and 39.4 ± 7.6 kg/m^2, respectively. Patients with significant fibrosis tended to be older. They also exhibited: a higher prevalence of T2DM; higher levels of fasting plasma glucose, c-peptide, HbA1c, ALT, AST, GGT, and lower platelet counts compared to patients without significant fibrosis ($p<0.05$). When the non-invasive scoring systems were applied to our data, the results revealed that the significant fibrosis group had significantly higher scores than the patients assigned to the non-significant fibrosis group. A more detailed description of the study population is displayed in Table 1.

Prevalence of steatosis and significant fibrosis

Of those 373 patients, 89.0% (332/373) of patients fulfilled the NAFLD criteria and 68.9% met the NASH criteria. The overall prevalence of significant fibrosis (F≥2) was 9.1%. Our analysis showed that patients with T2DM have a significantly higher prevalence of significant fibrosis than those without T2DM ($\chi 2$ = 13.407, p=0.003). The prevalence of significant fibrosis increased significantly as age increased. We determined the frequency of fibrosis as 7.0% in individuals with age < 30 years rising to 25% in patients with an age ≥50 years ($\chi 2$ = 10.315, p=0.016). However, when patients were stratified according to gender, MS or BMI, there was no

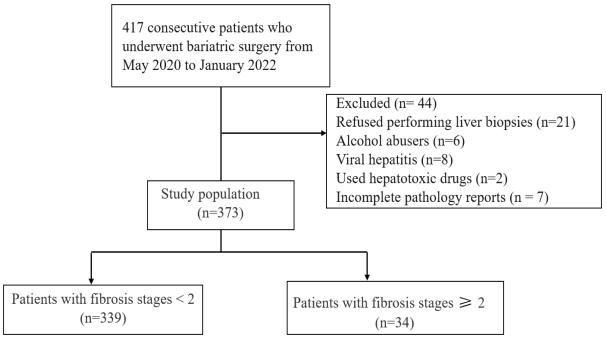

FIGURE 1
A flowchart illustrating the study approach.

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TABLE 1 Baseline characteristics of all patients and those with significant fibrosis and without significant fibrosis.

Variables	Total cohort (n=373)	Fibrosis stages F < 2 (n=339)	Fibrosis stages F ≥2 (n=34)	P value
Demographic characteristics				
Male, n (%)	126 (33.7%)	112 (33.0%)	14 (41.2%)	0.339
Age (years)	30.9 ± 9.0	30.4 ± 8.5	35.8 ± 12.0	0.018
Weight (kg)	109.5 ± 26.6	109.3 ± 26.7	111.3 ± 25.8	0.679
BMI (kg/m2)	39.4 ± 7.6	39.3 ± 7.6	40.3 ± 6.7	0.494
Waist circumference (cm)	120.1 ± 17.5	119.5 ± 17.6	125.4 ± 14.9	0.101
Hip circumference (cm)	123.2 ± 13.8	123.2 ± 13.9	123.3 ± 12.9	0.970
WHR	0.97 ± 0.09	0.97 ± 0.09	1.01 ± 0.10	0.006
Comorbidities				
Metabolic syndrome, n (%)	280 (75.1%)	252 (74.3%)	28 (82.4%)	0.303
Hypertension, (%)	106 (28.4%)	96 (28.3%)	10 (29.4%)	0.892
T2D, n (%)	124(33.2%)	102(30.1%)	22 (64.7%)	0.000
Laboratory data				
FPG (mmol/l)	6.6 ± 3.1	6.5 ± 2.8	8.3 ± 5.1	0.012
Insulin	23.6 ± 18.0	23.5 ± 18.3	24.7 ± 14.4	0.701
C-peptide	3.7 ± 1.6	3.6 ± 1.5	4.6 ± 2.1	0.010
HbA1c (%)	6.4 ± 1.6	6.3 ± 1.5	7.3 ± 1.9	0.000
HOMA-IR	7.1 ± 6.7	6.9 ± 6.6	8.7 ± 7.2	0.134
BUN (mmol/L)	4.5 ± 1.2	4.5 ± 1.7	4.8 ± 1.6	0.196
Creatinine (μmol/L)	62.5 ± 16.7	62.3 ± 16.0	66.6 ± 22.5	0.625
SUA(μmol/L)	450.7 ± 123.7	447.8 ± 122.9	479.1 ± 130.1	0.162
ALT (U/L)	59.1 ± 50.1	57.3 ± 50.5	76.8 ± 43.6	0.030
AST (U/L)	35.4 ± 27.0	33.3 ± 25.3	55.9 ± 34.2	0.000
AST/ALT	0.70 ± 0.27	0.69 ± 0.27	0.76 ± 0.25	0.152
GGT (U/L)	48.3 ± 38.8	45.8 ± 36.3	72.1 ± 53.7	0.001
ALP	83.2 ± 24.7	82.5 ± 23.9	91.1 ± 30.3	0.148
Total bilirubin (mmol/l)	11.7 ± 5.2	11.7 ± 5.0	12.4 ± 6.6	0.457
Direct bilirubin	3.3 ± 1.7	3.3 ± 1.7	3.5 ± 2.3	0.451
Indirect bilirubin	8.4 ± 3.8	8.4 ± 3.8	8.9 ± 4.6	0.513
Albumin, g/dL	42.4 ± 3.9	42.3 ± 3.2	43.2 ± 7.7	0.448
Total cholesterol (mg/dL)	5.2 ± 1.0	5.2 ± 1.0	5.0 ± 1.1	0.242
Triglycerides (mg/dL)	2.0 ± 1.9	2.0 ± 2.0	2.1 ± 0.9	0.853
HDL-c (mg/dL)	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	0.211
LDL-c (mg/dL)	3.1 ± 0.8	3.1 ± 0.7	3.1 ± 0.9	0.954
WBC (10^{12}/L)	8.4 ± 3.7	8.4 ± 3.7	8.4 ± 2.6	0.993
Platelets (109/L)	284.1 ± 67.0	286.6 ± 65.3	258.9 ± 78.9	0.042
Hepatic fibrosis index				
APRI	0.13 ± 0.11	0.12 ± 0.10	0.24 ± 0.18	0.000
FIB-4	0.56 ± 0.65	0.50 ± 0.25	1.35 ± 1.87	0.000
NFS	-2.2 ± 1.57	-2.33 ± 1.43	-0.96 ± 2.23	0.001

(Continued)

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TABLE 1 Continued

Variables	Total cohort (n=373)	Fibrosis stages F < 2 (n=339)	Fibrosis stages F ≥2 (n=34)	P value
BARD	1.89 ± 0.98	1.82 ± 0.94	2.50 ± 1.21	0.001
HFS	0.05 ± 0.08	0.04 ± 0.05	0.18 ± 0.18	0.000

BMI, body mass index; WHR, waist to hip ratio; T2D, type 2 diabetes; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HOMA-IR, Homeostatic model assessment of insulin resistance; BUN, blood urea nitrogen; SUA, serum uric acid; ALT, aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; WBC, white blood cells; APRI, AST to Platelet Ratio Index; FIB-4, Fibrosis-4 score; NFS, NAFLD fibrosis score; HFS, Hepamet fibrosis score.

statistically significant correlation between the occurrence of significant fibrosis and any of these factors. In addition, we observed a 4.0% prevalence of advanced fibrosis (F≥3) and a 1.6% prevalence of cirrhosis in bariatric surgery patients. (Table 2, Figure 2).

TABLE 2 Liver biopsy characteristics of patients.

Liver histology	N (%)
Steatosis grade	
0	41(11.0%)
1	121(32.4%)
2	114 (30.6%)
3	97 (26.0%)
Lobular inflammation grade	
0	25 (6.7%)
1	165 (44.2%)
2	183 (49.1%)
3	0 (0)
Ballooning grade	
0	81(21.7%)
1	258 (69.2%)
2	34 (9.1%)
Fibrosis stage	
0	146 (39.1%)
1	193 (51.7%)
2	19 (5.1%)
3	9 (2.4%)
4	6 (1.6%)
NAFLD	332 (89.0%)
NASH	257 (68.9%)
Fibrosis (F≥1)	227 (60.9%)
Significant fibrosis (F≥2)	34 (9.1%)
Advanced fibrosis (F≥3)	16 (4.0%)

NAFLD, Non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Clinical predictors for significant fibrosis

To explore the predictive factors of significant fibrosis, clinical variables associated with significant fibrosis were evaluated using univariate analysis. Further analysis using a multivariable logistic regression model was performed based on variables with $P < 0.05$ in the univariate analysis (age, WHR, T2DM, FPG, c-peptide, HbA1c, AST, GGT, platelets). The results revealed that: age (OR], 1.06; 95% CI, 1.02-1.11, p=0.003); T2DM (OR, 2.62; 95% CI, 1.17-5.88, p=0.019); c- peptide (OR, 1.26; 95% CI, 1.03-1.55, p=0.025) and AST (OR, 1.02; 95% CI, 1.01-1.03, p=0.004) were detected as independent predictors of significant fibrosis. (Table 3)

Comparison of non-invasive scoring systems

To validate the reliability of non-invasive scoring algorithms for the diagnosis of significant fibrosis, we calculated the AUROC for the results of the five non-invasive scoring systems that were applied to our data. This yielded AUROC ranging from 0.652 to 0.781. The HFS had the best predictive performance, with an AUROC of 0.781, followed by the FIB-4 (0.745), APRI (0.759), NFS (0.657) and BARD (0.652) (Figure 3, Table 4). Pairwise comparison of the AUROC of different scoring systems demonstrated that there were significant differences between these non-invasive scoring systems, including APRI vs NFS, BRAD vs FIB-4, BRAD vs HFS, FIB-4 vs NFS and HFS vs NFS (all $P < 0.05$); while no significant differences between other non-invasive scoring systems were detected (all $P > 0.05$).

Discussion

The presence of fibrosis in NAFLD patients affects clinical prognoses. NAFLD has got widespread attention in bariatric surgery patients, but there are still scant studies into the prevalence of significant fibrosis. For this reason, we first examined the prevalence and potential risk factors of significant fibrosis among Chinese bariatric surgery patients. Our results indicated an overall prevalence of significant fibrosis, advanced fibrosis and cirrhosis of 9.1%, 4.0% and 1.6%, respectively. Specifically, the odds of having significant fibrosis were independently associated with the presence of T2DM, increasing age, and elevated AST, c-peptide levels. Furthermore, we also validated the reliability of non-invasive scoring systems and found that APRI, FIB-4 and HFS showed appropriate AUROC (>0.70) for predicting significant fibrosis, but

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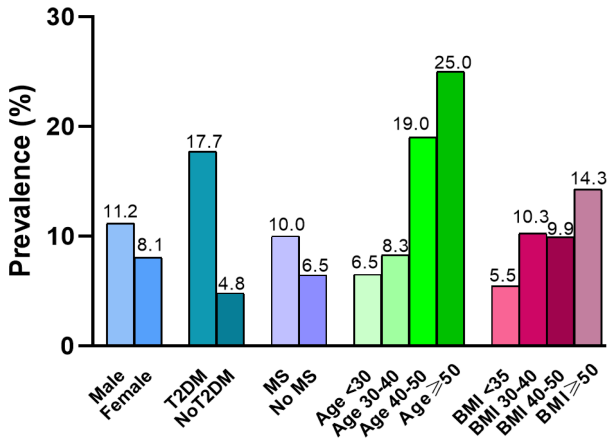

FIGURE 2
Prevalence of significant fibrosis stratified by gender, age, T2DM, MS and BMI.

TABLE 3 Univariate and multivariate logistic regression analyses were used to identify independent factors associated with significant fibrosis.

	Univariate analysis		Multivariate analysis	
	Crude OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
Male, n (%)	1.44 (0.70-2.95)	0.323	–	–
Age (years)	1.06 (1.02-1.10)	0.001	1.06 (1.02-1.11)	0.003
Weight (kg)	1.00 (0.99-1.02)	0.678	–	–
BMI (kg/m2)	1.02 (0.97-1.06)	0.494	–	–
Waist circumference (cm)	1.02 (0.99-1.04)	0.101	–	–
Hip circumference (cm)	1.00 (0.98-1.03)	0.970	–	–
WHR#	1.83 (1.20-2.80)	0.005	–	–
Comorbidities			–	–
Metabolic syndrome, n (%)	1.61 (0.65-4.02)	0.307	–	–
Hypertension, (%)	1.05 (0.48-2.28)	0.901	–	–
T2DM, n (%)	4.26 (2.03-8.93)	0.000	2.62 (1.17-5.88)	0.019
Laboratory data			–	–
FPG (mmol/l)	1.14 (1.05-1.24)	0.003	–	–
Insulin	1.00 (0.99-1.02)	0.701	–	–
C- peptide	1.33 (1.11-1.60)	0.002	1.26 (1.03-1.55)	0.025
HbA1c (%)	1.31 (1.11-1.56)	0.002		
HOMA-IR	1.03 (0.99-1.07)	0.144	–	–
BUN (mmol/L)	1.18 (0.92-1.53)	0.198	–	–
Creatinine (μmol/L)	1.01 (0.99-1.03)	0.449	–	–
SUA(μmol/L)	1.00 (1.00-1.01)	0.162	–	–
ALT (U/L)	1.01 (1.00-1.01)	0.038	–	–
AST (U/L)	1.02 (1.01-1.03)	0.000	1.02 (1.01-1.03)	0.004
AST/ALT	2.35 (0.73-7.60)	0.154		
GGT (U/L)	1.01 (1.01-1.02)	0.001	–	–
ALP	1.00 (1.00-1.03)	0.055	–	–
Total bilirubin (mmol/l)	1.03 (0.96-1.09)	0.456	–	–
Direct bilirubin	1.08 (0.89-1.30)	0.451	–	–
Indirect bilirubin	1.04 (0.96-1.13)	0.362	–	–
Albumin, g/dL	1.05 (0.97-1.13)	0.222	–	–
Total cholesterol (mg/dL)	0.80 (0.56-1.16)	0.242	–	–
Triglycerides (mg/dL)	1.02 (0.86-1.20)	0.853	–	–
HDL-c (mg/dL)	0.33 (0.06-1.86)	0.210	–	–
LDL-c (mg/dL)	0.99 (0.62-1.57)	0.954	–	–
WBC (10^{12}/L)	0.99 (0.91-1.10)	0.993	–	–
Platelets (10^{9}/L)	0.99 (0.98-1.00)	0.020	–	–

(Continued)

TABLE 3 Continued

OR, odds ratio; CI, confidence interval. BMI, body mass index; WHR, waist to hip ratio; T2D, type 2 diabetes; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HOMA-IR, Homeostatic model assessment of insulin resistance; BUN, blood urea nitrogen; SUA, serum uric acid; ALT, aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; WBC, white blood cells.
#Per 0.1 increase 10.

BRAD and NFS score revealed poorly predictive performance compared to the other scores.

Previous studies reported the prevalence of biopsy-proven NASH during bariatric surgery, ranging from 2.6% to 98% (4). Some potential explanations for the discrepancy in prevalence are different histological scoring systems, selection bias, race-based differences and variability of observations among pathologists. In this study, we observed 68.9% population had NASH and 60.9% had fibrosis, which was similar to those from Japan (77.5%) and Taiwan (71.3%) (4). In contrast, a study with 1000 patients who underwent routine liver biopsies during bariatric surgery showed the rate of NASH/fibrosis was only 14.3% (29). Another large-scale study including 2557 bariatric surgery patients also discovered that only 30.9% and 29.3% of individuals had NASH and fibrosis respectively (30). Obviously, our results were significantly higher than those from two studies (29, 30), as well as those from the USA (24.1-58.6%) and Australia (18.4-24.8%) (4). This discrepancy may be due to racial differences, as Asian populations (even individuals with relatively low BMI) have an elevated risk of metabolic disease due to differing body fat percentages and body composition (31). In addition, 9.1% of patients were found to have significant fibrosis, 4.0% had advanced fibrosis and 1.6% had cirrhosis. Our findings are in agreement with the study by Udelsman BV, which found that in a cohort of bariatric

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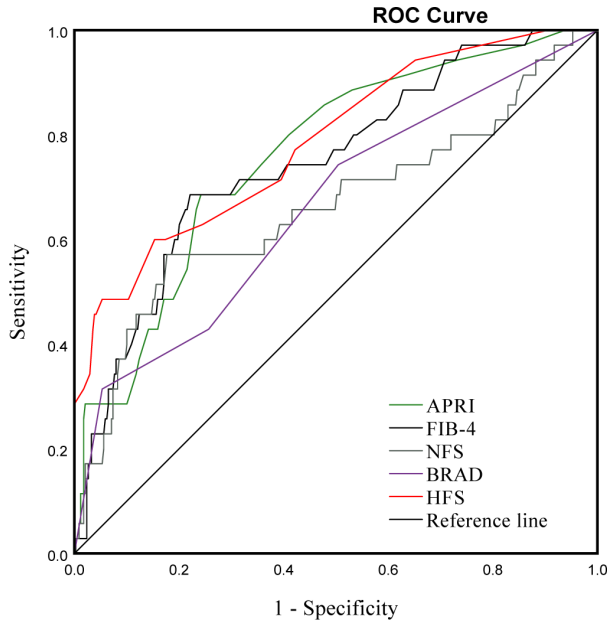

FIGURE 3
ROC curve for APRI, FIB-4, NFS, BARD and HFS in bariatric surgery patients with and without significant fibrosis.

surgery patients, 7.8% had significant fibrosis and 3.6% had advanced fibrosis (30). However, another retrospective study of 330 patients undergoing routine liver biopsy during bariatric surgery showed an increased prevalence of significant fibrosis, although results for advanced fibrosis, and cirrhosis were more similar to our findings (20.9%, 4.2% and 1.5%, respectively) (32).

Significant fibrosis is an established risk factor for cirrhosis and overall mortality (33). Research has shown that advanced fibrosis can persist for many years despite substantial weight loss following bariatric surgery (34). Accordingly, the early identification of clinically significant fibrosis could potentially improve patient outcomes. Several independent predictors of advanced fibrosis have been reported in prior studies (9, 19, 25, 35), including increasing age, T2DM, HOMA-IR, hypertension, elevated AST, and decreased platelets. Of those predictors, T2DM is one of the most useful predictors of liver fibrosis. In this study, patients with T2DM have a higher prevalence of significant fibrosis than patients without T2DM. Glucose metabolism-related indicators, such as T2DM and c-peptide, were found to be strongly associated using multivariate logistic regression models. However, hypertension and MS were not accepted as predictors of significant fibrosis, in line with previous study (4, 36). In addition, our study found that increasing age and elevated AST were independently associated with significant fibrosis, as has been mentioned above predictors.

Current guidelines recommend utilizing non-invasive scoring systems to identify at-risk NASH or fibrosis (37). Among such non-invasive scoring systems, the APRI, FIB-4, BRAD and NFS are widely used to detect liver fibrosis (38). HFS was recently developed based on an international multicenter study with 2452 participants and provided superior performance to detect patients with advanced fibrosis with an AUROC of 0.85, the sensitivity of 74%, and specificity of 97.2%, when compared with the FIB-4 and NFS systems (25). Another international multicenter retrospective study of 379 biopsy-proven NAFLD patients showed HFS and FIB-4 had higher AUROC for identifying significant fibrosis (0.744 and 0.725, respectively) than that of the no NFS, but no statistical differences were found between HFS and FIB-4 AUROC (39). Similarly, a retrospective study including 222 patients with biopsy-proven NAFLD demonstrated that the HFS(AUROC,0.758) was marginally less superior than FIB-4(AUROC,0.796) in detecting advanced fibrosis (40). In this study, APRI, FIB-4 and HFS all showed sufficient prediction accuracy (all AUROC ≥0.70), but there were no significant differences between APRI, FIB-4 and HFS AUROC. Compared to other scoring systems, BRAD and NFS scores did not exhibit satisfactory diagnostic performance in detecting significant fibrosis. In this prospective derivation and global validation study, the accuracies of BRAD and NFS for predicting significant fibrosis were 0.58 (0.54–0.62) and 0.66 (0.62–0.70), respectively (7). In the study by Zambrano-Huailla R et al, NFS was unable to effectively detect significant fibrosis in patients with NAFLD, with an AUROC of 0.581 (39). Thus, the role of the BRAD and NFS in predicting significant fibrosis in bariatric surgery patients should be further explored. Based on the current results, we can use non-invasive scores (APRI, FIB-4 and HFS) to monitor these patients with fibrosis closely. (Reviewer #2)

TABLE 4 Performance of the APRI, FIB-4, NFS, BARD and HFS for the detection of significant fibrosis.

	APRI	FIB-4	NFS	BARD	HFS
Cutoff value	0.43	0.46	0.38	0.24	0.43
AUC (95% CI)	0.759 (0.712-0.801)	0.745 (0.697-0.788)	0.657 (0.607-0.705)	0.652 (0.601-0700)	0.781 (0.735-0.822)
Sensitivity (95% CI)	67.6 (49.5-82.6)	67.7 (49.5-82.6)	55.9 (37.9-72.8)	29.4 (15.1-47.5)	58.8 (40.7-75.4)
Specificity (95% CI)	75.8 (70.9-80.3)	77.9 (73.1-82.2)	82.3 (77.8-86.2)	94.7 (91.7-96.8)	84.7 (80.4-88.3)
LR (+) (95% CI)	2.8 (2.1-3.8)	3.1 (2.3-4.3)	3.2 (2.2-4.6)	5.5 (2.8-11.0)	3.8 (2.6-5.6)
LR (-) (95% CI)	0.4 (0.3-0.7)	0.4 (0.3-0.7)	0.5 (0.4-0.8)	0.8 (0.6-0.9)	0.5 (0.3-0.7)
PPV (95% CI)	21.9 (17.2-27.4)	23.5 (18.4-29.4)	24.1 (17.8-31.6)	35.7 (21.8-52.5)	27.8 (20.9-35.9)
NPV (95% CI)	95.9 (93.5-97.4)	96.0 (93.6-97.5)	94.9 (92.7-96.5)	93.0 (91.5-94.3)	95.3 (93.2-96.8)

AUC, area under the curve; CI, confidence interval; APRI, AST to Platelet Ratio Index; FIB-4, Fibrosis-4 score; NFS, NAFLD fibrosis score; HFS, Hepamet fibrosis score; LR likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

The strength of our study is that it was the first to prospectively evaluate the prevalence and clinical predictors of biopsy-confirmed significant fibrosis among Chinese bariatric surgery patients. However, we acknowledged there were several limitations in the current study. Firstly, this was a single-center cross-section study, limiting our study's generalizability. Secondly, the biopsy samples were only from the left lobe of the liver, which may lead to misclassification of liver fibrosis severity as, in terms of histology, severity varies depending on the specific area of the liver being biopsied (41). Thirdly, some drugs, such as lipid-lowering drugs, antihypertensive drugs and antidiabetic drugs, may influence the results. Finally, we could not evaluate the application of this test in bariatric patients, because our hospital lacked "FibroScan". (**Reviewer #1)** Therefore, multicenter studies with larger sample sizes should be undertaken to better evaluate the prevalence of fibrosis and its predictive factors in Chinese bariatric surgery patients.

Conclusions

Our study showed more than two-thirds of bariatric surgery patients had NASH, and the prevalence of significant fibrosis was high. Risk factors for significant fibrosis include increasing age, presence of T2DM, elevated AST and c-peptide levels. Non-invasive models (including APRI, FIB-4 and HFS) can help clinicians to identify significant liver fibrosis in bariatric surgery patients. Further multicenter studies with larger sample sizes on liver fibrosis are warranted in bariatric surgery 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 Ethics Research Committee of the First Affiliated Hospital of Jinan University (2019-024). The patients/participants provided their written informed consent to participate in this study.

Author contributions

YS and WC: conceptualization and writing-original draft preparation. YS, SD, and WC: methodology, data curation, and formal analysis. CW and ZD: resources, supervision, and project administration. CW, ZD, and WC: writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Association between weight-adjusted-waist index with hepatic steatosis and liver fibrosis: a nationally representative cross-sectional study from NHANES 2017 to 2020

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Background: The negative effects of obesity on hepatic steatosis and fibrosis have received considerable attention in recent years. The weight-adjusted-waist index (WWI) reflects weight-independent centripetal obesity. Herein, we provide the first investigation of a link between WWI, hepatic steatosis, and liver fibrosis.

Methods: We used data from the National Health and Nutrition Examination Survey 2017-2020 to conduct a cross-sectional study. The linear relationship between WWI, controlled attenuation parameters, and liver stiffness measurements (LSM) was investigated using multivariate linear regression models. The nonlinear relationship was described using fitted smoothed curves and threshold effect analyses. Subgroup analyses were performed based on gender, age, body mass index, diabetes, hypertension, drinking, and smoking.

Results: This population-based study included 7,594 people, 50.74% of whom were men and 49.26% of whom were women. Multivariate linear regression analysis revealed a significant positive relationship between WWI and hepatic steatosis [CAP, β=7.60, 95% confidence interval (CI) (4.42, 10.78), P<0.0001]. This positive association was stronger when excessive alcohol intake was present compared to when it was absent (P for interaction = 0.031), and when hypertension was present compared to when it was not (P for interaction = 0.014). The linear relationship between WWI and liver fibrosis was not statistically significant on multiple regression analysis [LSM, β=0.03, 95% CI (-0.26, 0.32), P=0.84]. However, a U-shaped association was seen between WWI

and LSM, with a negative correlation when WWI< 10.92 and a positive correlation when WWI > 10.92.

Conclusion: We report a strong association between WWI and hepatic steatosis, and suggest that it may potentially be used as a simple anthropometric index to predict hepatic steatosis.

KEYWORDS

WWI, steatosis, and fibrosis weight-adjusted-waist index, hepatic steatosis, liver fibrosis, NAFLD, NHANES, VCTE

1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, which affects up to 40% of adults and children (1, 2). NAFLD can progress from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH) and then to liver fibrosis, cirrhosis, and hepatocellular carcinoma (3, 4). NAFLD has been linked to obesity and obesity-related metabolic disorders such as glucose intolerance, type 2 diabetes (T2D), and dyslipidemia (5).

Excessive hepatic fat accumulation not only leads to local changes, such as hepatocyte dysfunction, proinflammatory immune response activation, and fibrogenesis, but also triggers a series of extrahepatic metabolic disorders, including cardiovascular events and T2D. Furthermore, observational studies have highlighted that both hepatic steatosis and fibrosis are associated with a higher risk of all-cause mortality. Therefore, determining the level of liver steatosis is critical in the evaluation and clinical prognosis of patients with NAFLD (6). Although pathological biopsy remains the gold standard for evaluating the severity of hepatic steatosis and liver fibrosis, vibration controlled transient elastography (VCTE) is a non-invasive alternative that is increasingly being used. Recent observational studies have suggested that VCTE has robust accuracy in estimating the grade of hepatic steatosis and the stage of liver fibrosis (7, 8). However, the use of VCTE is limited by its popularity and high learning curve (9).

Weight-adjusted-waist index (WWI) was first postulated in 2018 as an anthropometric measure of central obesity that reflects both fat and muscle mass components, regardless of the body mass index (BMI) (10, 11). The links between WWI and various cardiovascular events have since been well established (11–14). However, the relationship between WWI and these hepatic indicators has not been defined.

In the present study, we used the National Health and Nutrition Examination Survey (NHANES) to investigate the relationship between WWI and hepatic steatosis and liver fibrosis in the US population.

2 Materials and methods

2.1 Data and sample sources

The NHANES is a cross-sectional survey conducted every two years to assess the nutritional and physical health of the general public in the United States (15, 16). Through interviews and related tests, demographics, dietary, and health-related information are collected (12, 13). The survey is approved by the Center for Disease Control and Prevention Research Ethics Review Board, and all survey participants provide written informed consent to participate (17).

In the present study, we used the 2017–2020 pre-coronavirus-19 pandemic data from the NHANES database. Out of the 15,560 individuals who participated, we excluded those with hepatitis B or C infection (n=215), missing VCTE data (n=434), unreliable VCTE estimation (liver stiffness interquartile range/median≥30%, n = 201), those younger than 18 years old (n=4,123), and incomplete data on weight and waist circumference (WC) (n=2993). The final analyses included 7,594 participants (Figure 1).

2.2 Definition of weight-adjusted-waist index

WWI is a novel index that estimates central obesity based on WC and weight. WC and weight were measured in a mobile examination center (MEC), where laboratory tests were carried out under controlled conditions (14). WWI was included as an exposure variable in our study and calculated as follows:

$$WWI(cm/kg^2) = WC/Weight^2$$

2.3 Measurement of hepatic steatosis and liver fibrosis

Hepatic steatosis and liver fibrosis was detected with VCTE. Specifically, controlled attenuation parameter (CAP) and liver stiffness measurement (LSM) represented the levels of steatosis and fibrosis, respectively. We only included participants with a reliable VCTE estimation (interquartile range/median of LSM ≤ 30%).

2.4 Covariates

Based on a review of the literature, we summarized potential confounding covariates between WWI and hepatic steatosis and liver

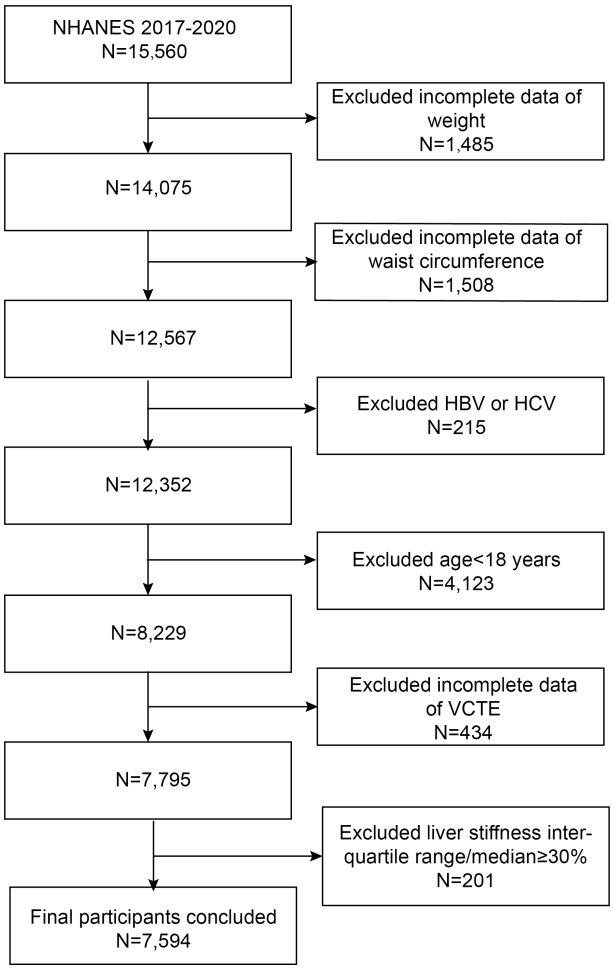

FIGURE 1
Flowchart of the sample selection from NHANES 2017–2020. NHANES, National Health and Nutrition Examination Survey; HBV/HCV, participants with infection of hepatitis B virus and hepatitis C virus; VCTE, vibration controlled transient elastography.

fibrosis in our multivariable-adjusted model (10, 11, 18, 19). Gender, age, race, family income-to-poverty ratio, and education level were all demographic covariates in our study. Anthropometric and laboratory covariates included BMI, direct high-density lipoprotein cholesterol (HDL, mmol/L), low-density lipoprotein cholesterol (LDL, mmol/L), triglycerides (TG, mmol/L), total cholesterol (TC, mmol/L), serum uric acid (μmol/L), alanine aminotransferase (ALT, IU/L), alkaline phosphatase (ALP, IU/L), and aspartate aminotransferase (AST, IU/L). Medical history covariates included the presence or absence of T2D, excess alcohol consumption (≥ 4 drinks per day), smoking, or cardiovascular disease (CVD, defined as history of coronary artery disease, congestive heart failure, heart attack, stroke, or angina pectoris).

2.5 Statistical analysis

Weighted Student's t-tests for continuous variables and weighted chi-squared tests for categorical variables were used to assess differences between WWI quartiles. The NHANES used an inferential statistics method to represent a large, nationally representative sample due to its complex multistage probability sampling design. Thus, using linear regression analyses, we summarized continuous variables as means with standard errors (SE) and categorical parameters as proportions using logistic regression analyses. Weighted multivariable regression models were used in three different models to investigate the relationship between WWI and hepatic steatosis and liver fibrosis. No covariates were adjusted in Model 1. Model 2 was adjusted for gender, age, and race. Model 3 was adjusted for gender, age, race, education level, BMI, excess alcohol consumption, HDL, LDL, TG, total cholesterol, serum uric acid, ALT, ALP, AST, CVD, and T2D status.

We performed a sensitivity analysis after categorizing the WWI into quartiles to assess robustness. A generalized additive model (GAM) and smooth curve fitting were used to address non-linearity. When a non-linear correlation was observed, a two-piecewise linear regression model (segmented regression model) was used to fit each interval and calculate the threshold effect. Subgroup analyses were then performed based on gender, age, BMI, T2D, hypertension, excess alcohol consumption, smoking, and CVD. A log-likelihood ratio test was used to determine whether a threshold existed by comparing a one-line model (non-segmented) to a two-piecewise linear regression model. The inflection point (K) was determined using a two-step recursive method (14). Furthermore, a subgroup analysis of the correlations between WWI and hepatic steatosis and liver fibrosis was carried out using stratified multivariable logistic regression models with stratified covariates such as gender, age, BMI, and T2D. A two-sided P value ≤ 0.05 was considered statistically significant. R (version 4.1.3) and EmpowerStats, two statistical computing and graphical programs, were used to conduct the statistical studies (version 2.0).

3 Results

3.1 Baseline characteristics

Table 1 summarizes the demographic profiles of the 7,594 participants. These participants had a mean ± SD age of 42.59 ± 20.99 years; 50.74% were men, and 49.26% were women. The WWI ranges for the first, second, third, and fourth quartiles were 8.04-10.45, 10.45-11.05, 11.05-11.64, and 11.64-14.14, respectively. Compared with participants in the lowest WWI quartile, those in the highest quartile were more likely to be male, older, excess alcohol consumers, or have CVD, lower education level, lower socioeconomic status, higher TG levels, higher BMI, higher total cholesterol, higher LDL, higher ALT, higher ALP, and higher serum uric acid.

3.2 Association between the weight-adjusted-waist index and hepatic steatosis (CAP)

We first estimated the association between WWI and the severity of liver fibrosis without adjusting for any covariates. Higher WWI was associated with a higher grade of hepatic steatosis. After full adjustment

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TABLE 1 Weighted characteristics of the study population based on controlled attenuated parameter (CAP) and median liver stiffness measurement (LSM).

Weight-adjusted-waist index (WWI)	Q1 N=1,899 (8.04-10.44)	Q2 N=1,898 (10.44-11.05)	Q3 N=1,898 (11.05-11.64)	Q4 N=1,899 (11.64-14.14)	*P*-value
Age (years), (%)					<0.001
18-39	1,263 (67%)	693 (37%)	433 (23%)	266 (14%)	
40-59	184 (9.7%)	444 (23%)	775 (41%)	1,098 (58%)	
≥60	452 (24%)	761 (40%)	690 (36%)	535 (28%)	
Sex, (%)					<0.001
Male	754 (40%)	857 (45%)	963 (51%)	1,279 (67%)	
Female	1,145 (60%)	1,041 (55%)	935 (49%)	620 (33%)	
Race, (%)					<0.001
Mexican American	155 (8.2%)	227 (12%)	293 (15%)	256 (13%)	
Other Hispanic	162 (8.5%)	204 (11%)	202 (11%)	226 (12%)	
Non-Hispanic White	576 (30%)	603 (32%)	642 (34%)	785 (41%)	
Non-Hispanic Black	632 (33%)	487 (26%)	450 (24%)	391 (21%)	
Other Races	374 (20%)	377 (20%)	311 (16%)	241 (13%)	
Education level, (%)					<0.001
Less than high school	182 (9.6%)	284 (15%)	392 (21%)	435 (23%)	
High school or above high school	1,460 (77%)	1,541 (81%)	1,471 (78%)	1,439 (76%)	
Others	257 (14%)	73 (3.8%)	35 (1.8%)	25 (1.3%)	
BMI (kg/m^2), (%)					<0.001
Normal weight	1,061 (56%)	522 (28%)	321 (17%)	169 (8.9%)	
Overweight	243 (13%)	674 (36%)	930 (49%)	1,246 (66%)	
Obese	594 (31%)	698 (37%)	644 (34%)	482 (25%)	
Diabetes, (%)					<0.001
Yes	61 (0.8%)	239 (3.1%)	436 (5.7%)	681 (8.9%)	
No	1,838 (24.2%)	1,659 (21.8%)	1,462 (19.3%)	1,218 (16.3%)	
Hypertension, (%)					<0.001
Yes	267 (14%)	581 (31%)	806 (42%)	1,038 (55%)	
No	1,632 (86%)	1,317 (69.02%)	1,091 (57.01%)	861 (45.02%)	

(Continued)

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TABLE 1 Continued

Weight-adjusted-waist index (WWI)	Q1 N=1,899 (8.04-10.44)	Q2 N=1,898 (10.44-11.05)	Q3 N=1,898 (11.05-11.64)	Q4 N=1,899 (11.64-14.14)	*P*-value
Smoking, (%)					<0.001
Yes	1,307 (69%)	1,160 (61%)	1,067 (56%)	1,089 (57%)	
No	592 (31%)	738 (39%)	831 (44%)	810 (43%)	
Excess Alcohol Consumption, (%)					<0.001
Yes	1,463 (88.08%)	1,378 (83.21%)	1,261 (77.08%)	1,073 (68.08%)	
No	198 (11.92%)	278 (16.79%)	375 (22.92%)	503 (31.92%)	
CVD, (%)					<0.001
Yes	61 (3.72%)	153 (8.37%)	203 (10.89%)	345 (18.36%)	
No	1,580 (96.28%)	1,675 (91.63%)	1,661 (89.11%)	1,534 (81.64%)	
Income to poverty ratio	2.40 (1.18, 4.66)	2.64 (1.27, 4.67)	2.31 (1.18, 4.16)	1.91 (1.11, 3.55)	<0.001
Laboratory features					
Total cholesterol (mmol/L)	172 (150, 198)	185 (161, 214)	187 (161, 216)	183 (156, 211)	<0.001
Triglyceride (mmol/L)	0.70 (0.52, 1.05)	0.96 (0.67, 1.49)	1.14 (0.80, 1.61)	1.24 (0.88, 1.70)	<0.001
LDL- cholesterol (mmol/L)	2.56 (2.07, 3.13)	2.79 (2.30, 3.41)	2.90 (2.25, 3.49)	2.69 (2.15, 3.34)	<0.001
HDL- cholesterol (mmol/L)	1.42 (1.19, 1.71)	1.29 (1.09, 1.60)	1.27 (1.06, 1.55)	1.27 (1.06, 1.53)	<0.001
ALT (IU/L)	15 (12, 22)	19 (13, 27)	19 (14, 27)	18 (13, 25)	<0.001
ALP (IU/L)	76 (56, 80)	71 (59, 86)	76 (64, 92)	82 (67, 99)	<0.001
AST (IU/L)	19 (16, 23)	19 (16, 24)	19 (16, 24)	19 (16, 23)	0.013
LSM (kPa)	4.70 (4.00, 5.70)	4.80 (4.00, 5.90)	5.10 (4.10, 6.38)	5.30 (4.30, 6.90)	<0.001
CAP (dB/m)	215 (190, 248)	257 (218, 297)	278 (238, 318)	295 (253, 336)	<0.001
Serum uric acid (μmol/L)	297 (244, 351)	315 (256, 381)	321 (262, 381)	321 (262, 381)	<0.001

Mean and interquartile range for continuous variables: P value was calculated by weighted linear regression model.
% for categorical variables: P value was calculated by weighted chi-square test.
BMI, body mass index; LDL- cholesterol, low-Density Lipoprotein Cholesterol; HDL- cholesterol, high-Density Lipoprotein Cholesterol; ALT, alanine transaminase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; LSM, liver stiffness measure; CAP, controlled attenuation parameter; CVD, cardiovascular disease; WWI, weight-adjusted-waist index.

(see Methods), each unit with a higher WWI score was found to be associated with 7.60 dB/m increased units of CAP [β=7.60, 95% CI (4.42, 10.78), P<0.001]. Sensitivity analysis was conducted after treating the WWI as a categorical variable (quartile). In the fully adjusted model, compared with the lowest WWI quartile (first quartile), the adjusted β for participants in the second quartile, third quartile, and fourth quartile were 6.51, 11.03, and 14.06, respectively (Table 2).

3.3 Association between the weight-adjusted-waist index and liver fibrosis (LSM)

As shown in Table 2, in the unadjusted model, each unit of higher WWI score was found to be associated with 0.72 kPa increased units of LSM [β=0.72, 95% CI (0.61, 0.84), P<0.0001].

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TABLE 2 The association between WWI with CAP and LSM.

Weight-adjusted-waist index	Crude model (Model 1) [a]	Minimally adjusted model (Model 2) [b]	Fully adjusted model (Model 3) [c]
CAP β (95% CI) [d]	31.21 (29.77, 32.65)<0.0001	36.97 (35.26, 38.68)<0.0001	7.60 (4.42, 10.78)<0.0001
WWI group			
Quartile 1	0	0	0
Quartile 2	37.55 (33.98, 41.13)<0.0001	39.48 (35.88, 43.09)<0.0001	6.51 (1.08, 11.94) 0.0188
Quartile 3	56.95 (53.37, 60.53)<0.0001	60.73 (56.92, 64.54)<0.0001	11.03 (4.89, 17.10) 0.0005
Quartile 4	72.75 (69.17, 76.33)<0.0001	81.61 (77.51, 85.72)<0.0001	14.06 (6.95, 21.17) 0.0001
P for trend	35.37 (33.69, 37.05)<0.0001	39.44 (37.49, 41.39)<0.0001	6.88 (3.50, 10.26)<0.0001
LSM β (95% CI)	0.72 (0.61, 0.84)<0.0001	0.90 (0.77, 1.04)<0.0001	0.03 (-0.26, 0.32) 0.8419
WWI group			
Quartile 1	0	0	0
Quartile 2	0.17 (-0.10, 0.45) 0.219	0.24 (-0.05, 0.53) 0.099	-0.44 (-0.94, 0.05) 0.0773
Quartile 3	0.94 (0.66, 1.22)<0.0001	1.06 (0.75, 1.36)<0.0001	-0.25 (-0.81, 0.31) 0.3825
Quartile 4	1.61 (1.33, 1.89)<0.0001	1.87 (1.54, 2.19)<0.0001	0.03 (-0.62, 0.67) 0.9314
P for trend	0.82 (0.69, 0.95)<0.0001	0.94 (0.79, 1.10)<0.0001	0.04 (-0.26, 0.35) 0.7835

In sensitivity analysis, Weight-adjusted-waist index was converted from a continuous variable to a categorical variable (quartile).
[a]Model 1: no covariates were adjusted.
[b]Model 2: adjusted for sex, age, and race.
[c]Model 3: adjusted for sex, age, race, education level, family income to poverty ratio, BMI, Diabetes, hypertension, smoking, drinking, family income to poverty ratio, Total calcium, Total cholesterol, Triglyceride, LDL- cholesterol, HDL- cholesterol, ALT, ALP, AST, Serum uric acid, drinking, and cardiovascular diseases.
[d]95% CI: 95% confidence interval.

However, after adjusting for all covariates, the relationship between WWI and LSM was not significant in Model 3 [β=0.03, 95% CI (-0.26, 0.32), P=0.84].

3.4 Subgroup analysis

We used stratified weighted multivariate regression analysis to investigate the association between WWI and CAP and LSM in different population settings, stratified by gender, age, BMI, T2D, hypertension, excess alcohol consumption, smoking, and CVD.

As displayed in Figure 2, a stronger positive association between WWI and CAP was observed in participants with excess alcohol consumption and hypertension (P< 0.05). However, the correlation between WWI and CAP was similar in the population with different subgroups of gender, age, smoking, BMI, T2D, and CVD. Furthermore, A significant correlation between WWI and LSM was observed in participants with BMI>30 and experience CVD (Figure 3).

3.5 Non-linear relationship between WWI with hepatic steatosis and liver fibrosis

After adjusting for all variables, a non-linear association between WWI and LSM levels was found (Figure 4). We observed a U-shaped relationship between the WWI and LSM (inflection point: 10.92) (Table 3). Specifically, LSM was negatively associated with WWI<10.92 [β=-0.57, 95% CI (-1.06, -0.08), P=0.022], and positively association with WWI >10.92 [β=0.44, 95% CI (0.05, 0.84), P=0.028].

As with the linear association, we also observed a positive correlation between WWI and CAP when conducting the non-linear model. We found consistent positive association between WWI and CAP. Notably, the association was much stronger when WWI>10.75 [WWI>10.75: β=12.98, 95% CI (7.01, 18.95), P<0.0001]; WWI<10.75: [β=4.88, 95% CI (0.80, 8.95), P=0.02] (Figure 5).

4 Discussion

The present study aimed to evaluate the relationship between WWI and hepatic steatosis and liver fibrosis among civilians in the United States. In our cross-sectional study comprising 7,594 participants, we found a significant positive linear association between WWI and hepatic steatosis. Furthermore, we identified a U-shaped relationship between WWI and liver fibrosis (inflection point 10.92). By performing subgroup analyses, a stronger association between WWI and hepatic steatosis was demonstrated in participants with hypertensive disorders and excessive alcohol consumption. Additionally, we saw evidence of a strong correlation between WWI and liver fibrosis in participants with BMI>30 or CVD.

Accumulating evidence has supported the leading role of obesity in the pathogenesis of NAFLD. Given a strong link with dysregulated lipid metabolism, obesity not only contributes to the evolution of hepatic

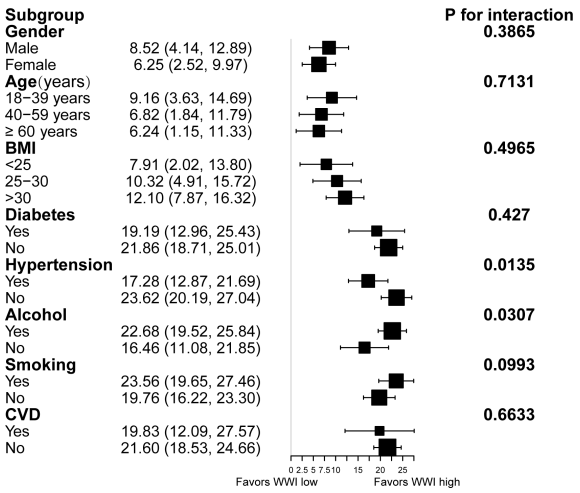

FIGURE 2
Subgroup analysis for the association between WWI and hepatic steatosis.

steatosis and inflammation, but also poses a threat to cardiovascular events and metabolic syndromes (20). Currently, BMI is widely used to determine the severity of obesity. However, obese patients, especially NAFLD patients, tend to demonstrate both fat accumulation and loss of skeletal muscle mass due to physical inactivity, which further increases the risk of hospitalization for NAFLD patients (21, 22). In addition, central obesity, specifically the accumulation of adipose in deep subcutaneous tissue, has been identified as the critical driver for NASH progression, insulin resistance, and cardiovascular events (23). Therefore, BMI, using total weight, may not accurately reflect the health status of obese individuals, particularly NAFLD patients. In contrast, WWI, calculated by normalizing WC with body weight, primarily reflects pure central obesity and can assess high fat mass and low muscle mass (24). For this study, we evaluated the association of WWI with estimated liver histology. We found a significant positive

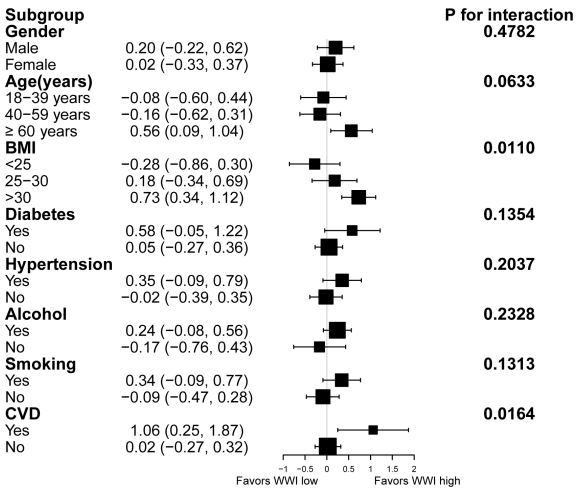

FIGURE 3
Subgroup analysis for the association between WWI and liver fibrosis.

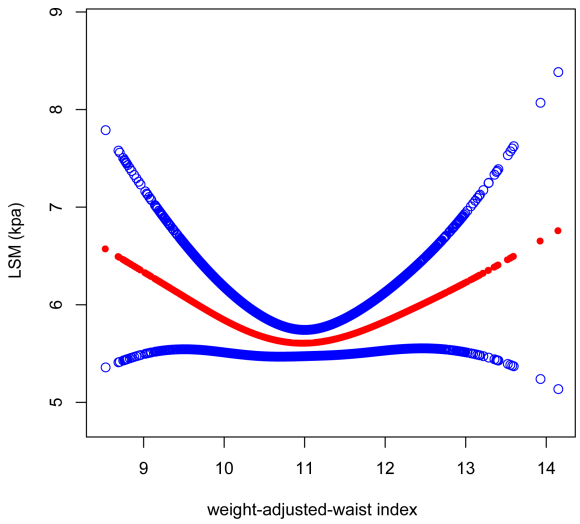

FIGURE 4
Smooth curve fitting for WWI and LSM. The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. WWI, weight-adjusted-waist index; LSM, liver stiffness measurement.

correlation between WWI and CAP. Additionally, we have identified a U-shaped association between WWI and LSM.

WWI was first used to better evaluate the morbidity and mortality of cardiovascular and metabolic diseases in the Korean population (25). Furthermore, trans-ethnic studies have revealed that WWI was positively associated with higher risks of hyperuricemia and multiple kinds of cardiovascular events, including heart failure, abdominal aortic calcification, and left ventricular hypertrophy (14, 26–29).

WWI was thought to represent the severity of central obesity rather than general obesity. As a sign of metabolic syndromes, a series of studies have suggested that central obesity is a risk factor for NAFLD, cardiovascular diseases, and other metabolic diseases for both obese and non-obese populations (30–32). Specifically, central obesity is characterized by the accumulation of abdominal adipose tissue, especially visceral adipose tissue. Mechanically, visceral adipose tissue could constantly secrete proinflammatory stimuli, which could further lead to systematic inflammation, metabolic disorders, and histological progression in the liver of obese patients. Moreover, during the expansion of visceral adipose tissue, proinflammatory macrophages in the adipose further contribute to immune-infiltration in the liver (33). In addition, some subtypes of adipose-derived ceramidases could desensitize insulin activity and contribute to insulin resistance (34). Our study has further highlighted and qualified the critical role central obesity in liver steatosis. In the future, WWI may be a liver fibrosis predictor.

This study has several limitations which should be mentioned. First, the cross-sectional study design did not allow us to determine any causal relationships. Second, the severity of hepatic steatosis and liver fibrosis in this study was determined using VCTE, which needs to be further validated in biopsy-proven cohorts. Furthermore, although the NHANES was conducted in the

TABLE 3 Threshold effect analysis of WWI on LSM and CAP using a two-piecewise linear regression model.

WWI	CAP Adjusted β (95% CI) P value	LSM Adjusted β (95% CI) P value
Fitting by the standard linear model	7.60 (4.42, 10.78)<0.0001	0.03 (-0.26, 0.32) 0.8419
Fitting by the two-piecewise linear model		
Inflection point	10.75	10.92
<K segment effect	12.98 (7.01, 18.95)<0.0001	-0.57 (-1.06, -0.08) 0.0219
>K segment effect	4.88 (0.80, 8.95) 0.0191	0.44 (0.05, 0.84) 0.0280
Log likelihood ratio	0.035	0.003

Adjusted for sex, age, race, education level, BMI, Diabetes, hypertension, smoking, drinking, family income to poverty ratio, Total calcium, Total cholesterol, Triglyceride, LDL- cholesterol, HDL-cholesterol, ALT, ALP, AST, Serum uric acid, drinking, and cardiovascular diseases.

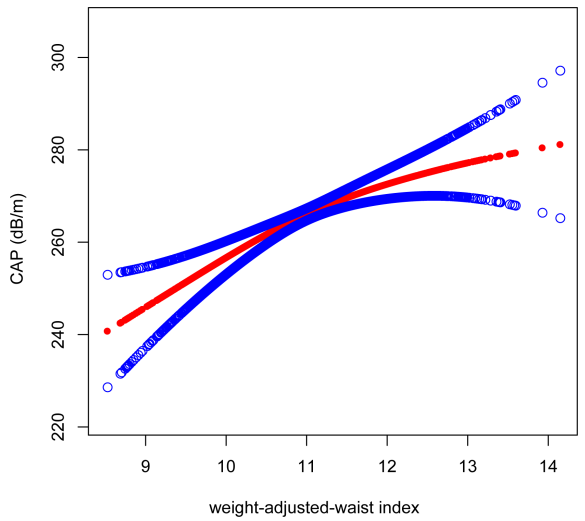

FIGURE 5
Smooth curve fitting for WWI and CAP. The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. WWI, weight-adjusted-waist index; CAP, controlled attenuation parameter.

United States in a multi-ethnic adult population, our results may not reflect other geographic areas or ethnic groups.

4.1 Conclusion

In conclusion, our study found strong link between WWI and hepatic steatosis, and suggested that it may potentially be used as a simple anthropometric index to predict hepatic steatosis.

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

YS and YW contributed equally to the research's conception and design. YS performed the study and wrote the draft. YS contributed to the acquisition and analysis of the data. MF and KZ conducted the statistical analysis. YS wrote the initial drafts of the manuscript. JW reviewed and revised the later drafts of the manuscript. All authors critically reviewed the manuscript, agreed to be fully accountable for ensuring the integrity and accuracy of the work, and 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

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Comparing the effectiveness of long-term use of daily and weekly glucagon-like peptide-1 receptor agonists treatments in patients with nonalcoholic fatty liver disease and type 2 diabetes mellitus: a network meta-analysis

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Objective: In the present network meta-analysis (NMA), we aimed to compare the effectiveness of daily and weekly treatment with glucagon-like peptide-1 receptor agonists for patients with nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM).

Method: We used Stata 17.0 for the NMA. Eligible Randomized controlled trials (RCTs) were searched in PubMed, Cochrane, and Embase databases until December 2022. Two researchers independently screened the available studies. The Cochrane Risk of Bias tool was used to assess the risk of bias in the included studies. We used GRADEprofiler (version3.6) to analyze the evidence certainty. Primary outcomes such as liver fat content (LFC), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels, as well as secondary outcomes such as γ-glutamyltransferase (γGGT) and body weight, were evaluated. Then, each intervention was ranked by the surface under the cumulative ranking curve (SUCRA). As a supplement, we drew forest plots of subgroup using RevMan (version 5.4).

Results: Fourteen RCTs involving 1666 participants were included in the present study. The NMA results showed that exenatide (bid) was the best treatment for improving LFC compared with other agents, liraglutide, dulaglutide, semaglutide (qw) and placebo), and the SUCRA values were 66.8%. Among five interventions (except exenatide (bid) and semaglutide (qw)) evaluated for AST outcome, and six interventions (except exenatide (bid)) evaluated for ALT outcome, semaglutide (qd) was the most effective drug (SUCRA (AST) = 100%, SUCRA (ALT) = 95.6%). The result of LFC in daily group was MD = -3.66, 95% CI [-5.56, -1.76] and in

weekly GLP-1RAs group, it was MD = -3.51, 95% CI [-4, -3.02]. As to AST and ALT, the results in daily group versus weekly group were AST: MD = -7.45, 95% CI [-14.57, -0.32] versus MD= -0.58, 95% CI [-3.18, 2.01] and ALT: MD = -11.12, 95% CI [-24.18, 1.95] versus MD = -5.62, 95% CI [-15.25, 4]. The quality of evidence was assessed as moderate or low.

Conclusion: The daily GLP-1RAs may be more effective in primary outcomes. And the daily semaglutide may be the most effective treatment for NAFLD and T2DM among the six interventions.

KEYWORDS

glucagon-like peptide-1 receptor agonists, nonalcoholic fatty liver disease, type 2 diabetes, liver fat content, alanine aminotransferase, aspartate aminotransferase

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) refers to the excessive accumulation of fats in the liver caused by factors other than alcohol and drug consumption (1). NAFLD is the most common chronic liver disease, ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH) (2). NAFLD is often closely related to metabolic disorders such as obesity and type 2 diabetes mellitus (T2DM) (3). Moreover, NAFLD is highly likely to progress to cirrhosis and cancer without active intervention, thus reducing the quality of life of patients and leading to psychological and physical burdens. Weight loss remains the basic of treatment for NAFLD and NASH (4). Although weight loss can improve NAFLD, the effect cannot last for an extended period, thus NAFLD requires long-term and adequate treatment with some drugs (5). However, specific drugs for NAFLD are scarce.

Recently, some studies have shown the role of glucagon-like peptide-1 receptor agonists (GLP-1RAs) in NAFLD treatment. GLP-1RAs can control energy intake and weight gain by prolonging gastric emptying and suppressing appetite (6, 7). Furthermore, GLP-1RAs can improve liver enzyme functions and liver steatosis and significantly reduce liver fat content (8–12). Many GLP-1RA preparations are available for selection, which can be divided into daily preparations and weekly preparations according to the frequency of administration. Weekly agents include semaglutide (qw), dulaglutide and exenatide (qw), whereas daily agents include liraglutide, semaglutide (qd), and exenatide (bid), which are commonly used preparations. The elimination half-life of weekly preparations is of several weeks, and their structural peculiarity results in a slow release, thus maintaining effective blood concentrations for a long time, delaying the onset. In contrast, the elimination half-life of daily preparations is shorter, thus providing active circulating concentrations, and effective blood concentrations can be reached earlier (13). Therefore, the efficacies of these two preparations differ. Although GLP-1RAs can significantly improve liver enzyme functions and liver fat content, a comparative study on the effect of weekly and daily GLP-1RAs on NAFLD with T2DM is unavailable.

Thus, in the present network meta-analysis (NMA), we aimed to compare the efficacy of the long-term use of weekly and daily GLP-1RAs for NAFLD with T2DM, hoping to provide a basis for selecting appropriate clinical drugs.

2 Material and methods

2.1 Search strategy

A search for all treatments in NAFLD was conducted across the PubMed databases from the date of inception until December 2022 using the following search strategy: (Liraglutide OR Dulaglutide OR Semaglutide OR Albiglutide OR Lixisenatide OR Exenatide OR glucagon-like peptide-1 agonists OR glucagon like peptide OR GLP-1 receptor agonists OR glp-1) AND (Non-Alcoholic Fatty Liver Disease OR Nonalcoholic Fatty Liver Disease OR Nonalcoholic Fatty Liver OR NAFLD OR Nonalcoholic Steatohepatitis OR NASH) AND (liver enzymes OR alanine aminotransferase OR aspartate aminotransferase OR γ-glutamyl transferase OR ALT OR AST OR γGGT OR intrahepatic fat content OR liver fat content OR intrahepatic content of lipid OR hepatic lipid content OR hepatic fat content OR LFC OR IHF OR IHCL OR HFC) in all fields without other limitations.

And search strategies for PubMed, Cochrane and Embase databases were shown in Table 1.

2.2 Inclusion and exclusion criteria

The paper inclusion criteria were as follows: (1) Subjects: clinically diagnosed as NAFLD or NASH with T2DM; (2) Drug interventions: patients in the experimental group were treated with GLP-1RAs; (3) Study type: randomized controlled trials (RCTs);

The paper exclusion criteria were as follows: (1)Animal models; (2)Duplicate articles; (3) Subjects were aged <18 years; (4) Study duration <12weeks. (5) The outcomes: liver fat content (LFC), aspartate aminotransferase (AST), alanine aminotransferase

TABLE 1 Search strategy for each database.

Databases (number of studies)	Search Strategy
PubMed (224)	(Liraglutide OR Dulaglutide OR Semaglutide OR Albiglutide OR Lixisenatide OR Exenatide OR glucagon-like peptide-1 agonists OR glucagon like peptide OR GLP-1 receptor agonists OR glp-1) AND (Non-Alcoholic Fatty Liver Disease OR Nonalcoholic Fatty Liver Disease OR Nonalcoholic Fatty Liver OR NAFLD OR Nonalcoholic Steatohepatitis OR NASH) AND (liver enzymes OR alanine aminotransferase OR aspartate aminotransferase OR γ-glutamyl transferase OR ALT OR AST OR γGGT OR intrahepatic fat content OR liver fat content OR intrahepatic content of lipid OR hepatic lipid content OR hepatic fat content OR LFC OR IHF OR IHCL OR HFC)
Embase (649)	('liraglutide' OR 'dulaglutide' OR 'semaglutide' OR 'albiglutide' OR 'lixisenatide' OR 'exenatide' OR 'glucagon-like peptide-1 agonist' OR 'glucagon like peptide' OR 'glp-1 receptor agonist' OR 'glp-1') AND ('non-alcoholic fatty liver disease' OR 'nonalcoholic fatty liver disease' OR 'nonalcoholic fatty liver' OR 'nafld' OR 'nonalcoholic steatohepatitis' OR 'nash') AND ('liver enzymes' OR 'alanine aminotransferase' OR 'aspartate aminotransferase' OR 'γ-glutamyl transferase' OR 'alt' OR 'ast' OR 'γggt' OR 'intrahepatic fat content' OR 'liver fat content' OR 'intrahepatic content of lipid' OR 'hepatic lipid content' OR 'hepatic fat content' OR 'lfc' OR 'ihf' OR 'ihcl' OR 'hfc')
Cochrane (182)	("Liraglutide" OR "Dulaglutide" OR "Semaglutide" OR "Albiglutide" OR "Lixisenatide" OR "Exenatide" OR "glucagon-like peptide-1 agonist*" OR "glucagon like peptide*" OR "GLP-1 receptor agonist*" OR "glp-1") in All Text AND ("Non-Alcoholic Fatty Liver Disease" OR "Nonalcoholic Fatty Liver Disease" OR "Nonalcoholic Fatty Liver" OR "NAFLD" OR "Nonalcoholic Steatohepatitis" OR "NASH") in All Text AND ("liver enzymes" OR "alanine aminotransferase" OR "aspartate aminotransferase" OR "γ-glutamyl transferase" OR "ALT" OR "AST" OR "γGGT" OR "intrahepatic fat content" OR "liver fat content" OR "intrahepatic content of lipid" OR "hepatic lipid content" OR "hepatic fat content" OR "LFC" OR "IHF" OR "IHCL" OR "HFC")

(ALT), γ-glutamyl transferase (γGGT) and body weight were not clearly reported. (6)The interventions were not GLP-1RAs versus placebo or blank control; (7)Data outcomes could not be extracted.

2.3 Study selection and data extraction

Study selection and data extraction were conducted separately by two individuals. Two reviewers initially selected the relevant studies by reading the title and abstract and then selected the studies for NMA based on the inclusion and exclusion criteria and after reading the full text. Next, any disagreements were resolved by discussion or by a third researcher.

The extracted data included: 1) the baseline information: the last name of the first author, publication year, intervention and control, sample size (female/male), dose (frequency of application), duration, baseline age (mean ± standard deviation [SD]), T2DM, with or without NASH, and the countries of study population, the characteristics of included studies were listed in Table 2; 2) the data used for analysis: mean and SD changes from the baseline to the end of each outcome, and sample size (n); 3) the information for quality assessment; 4) the items of evidence certainty assessment.

2.4 Quality assessment and evidence certainty assessment

The Cochrane Risk of Bias tool (26) was used to assess the risk of bias of the included studies. The following seven items were included: 1) "random sequence generation": describes how the sequence was generated, such as by using a random table of numbers or a computer for generating a random sequence of numbers; 2) "allocation concealment": whether the subjects and researchers were aware of group assignments, such as through assignment hiding *via* telephone and Internet; 3) "blinding of the participants and personnel": whether subjects, researchers, and all participants were blinded; 4) "blinding of outcome assessment": describe whether an outcome assessor was blinded, but objective outcomes, such as serological outcomes, were unlikely to be affected by the lack of blinding; 5) "incomplete outcome data": whether there was any missing data, such as loss to follow-up and exclusion of data from analysis; 6) "selective reporting": whether all outcomes were reported; 7) "other bias": each study was considered to have a "high", "low", or "unclear" risk of bias. The judgment of risk of bias was conducted by two authors separately in Review Manager (Version 5.4).

And then, we used GRADE (Grades of Recommendation, Assessment, Development and Evaluation) model to assess the evidence certainty (27). Since all the included studies were RCTs, we evaluated the following five items: 1) risk of bias: such as allocation concealment, blinding and loss to follow-up, and so on; 2) inconsistency: the results heterogeneity, and whether the authors give a reasonable explanation for its high heterogeneity; 3) indirectness; 4) imprecision: whether the confidence interval (CI) was wide and the sample size was large; 5) publication bias: the number of included studies. This assessment was performed in GRADEprofiler (version 3.6).

2.5 Statistical analysis

First, we constructed network plots of the outcomes to demonstrate all available evidence for each outcomes (Figure 1). Second, the outcomes we selected were all continuous variables, and therefore the mean and standard deviation (SD) changes from the baseline to the end and the sample size (n) were extracted for statistical analysis. The existing evidence only involved indirect comparison; therefore, the network graph had no closed loop and there was no need to examine the inconsistency of the outcomes. We employed SUCRA to evaluate the ranking of each intervention in each outcome (Figure 2). The higher the SUCRA value, the more likely the corresponding intervention to be regarded as the best treatment. "Zero" indicated that the treatment was the worst. The forest plots for each outcome

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TABLE 2 The characteristics of the included RCTs.

reference	Author and publication year	Treatment and sample size (female/male)		Dose (frequency of application)	duration (W)	Baseline age (mean±SD)	T2DM	NASH (Y/N)	Study Country
(12)	Kuchay 2020	Dula(9/23)	blank control (10/22)	0.75mg(4W)→1.5mg (once-weekly)	24	46.6 ± 9.1vs48.1 ± 8.9	Y	–	India
(14)	Cusi 2018	Dula (307/183)	Placebo (155/115)	1.5mg(once-weekly)	24	55.2 ± 9.6vs55.0 ± 9.7	Y	Y	the USA
(15)	Harreiter 2021	Exe(16)	Placebo (14)	2mg(once-weekly)	24	59.4±8.5vs60.9 ±7.4	Y	–	Australia
(16)	Hartman 2020	Dula (30/24)	Placebo (22/29)	1.5mg(once-weekly)	26	58.7±7.8vs56.6 ±8.9	Y	Y	the USA
(17)	Loomba 2022	Sema (31/16)	Placebo (18/6)	0.24mg→2.4mg (once-weekly)	16	59.9±7.1vs58.7 ±9.7	75 %Y	–	Europe and the USA
(18)	Armstrong 2016	Lira (8/18)	Placebo (13/13)	1.8mg(once-daily)	48	50±11vs52±12	Y	Y	England
(19)	Bizino 2019	Lira (9/14)	Placebo (11/15)	1.8mg(once-daily)	26	60±6vs59±7	Y	–	Europe
(9)	Guo 2020	Lira (15/16)	Placebo (10/20)	1.8mg(once-daily)	26	53.1 ± 6.3vs52.6 ± 3.9	Y	–	China
(20)	Matikainen 2018	Lira (2/13)	Placebo (4/3)	1.8mg(once-daily)	16	62±2vs63±2	Y	–	Europe
(21)	Nahra 2021	Lira (60/50)	Placebo (55/57)	1.8mg(once-daily)	54	55.5±9.8vs57.3 ±9.5	Y	Y	8 countries (Europe, Canada and the USA et.)
(22)	Newsome 2021	Sema (47/35)	Placebo (44/36)	0.4mg(once-daily)	72	54.3 ±10.2vs52.4 ±10.8	61 %Y	Y	Europe and the USA
(23)	Smits 2016	Lira(5/12)	Placebo (4/13)	1.8mg(once-daily)	12	60.8±7.4vs65.8 ±5.8	Y	–	Europe
(24)	Samson 2011	Exe(11)	blank control (10)	5ug(2W)→10ug (twice-daily)	48	52±3	Y	–	USA
(25)	Shahinul 2020	Lira(16)	Placebo (16)	0.6mg(1W)→1.2mg (once-daily)	24	–	Y	–	Bangladesh

were depicted in Figure 3, which shown the comparison between each intervention. The forest plots visually demonstrated the 95% confidence interval (CI) of the results of the pairwise comparison of interventions and whether they had any statistical significance. Finally, league plots were drawn based on SUCRA and the forest plots (Figure 3). The league plots ranked the effect of the intervention in each outcome from the best to the worst (Table 3). The results with statistical significance were highlighted in bold. The league plots more intuitively exhibited the effectiveness of each intervention. All of the abovementioned analyses were conducted by Stat17.0.

Then, we divided all studies with included outcomes into two subgroups of daily and weekly preparations, drew forest plots (Figure 4) using a random effects model to compared the mean difference (MD) between the two subgroups, and to observe which one was better in each outcome. The above analysis was performed by RevMan (version 5.4).

3 Results

3.1 Literature selection process and characteristics of studies

According to the search strategy, 1055 studies were searched from the following databases: PubMed, 224 studies; Embase, 649 studies; and Cochrane, 182 studies, and 310 duplicate references were removed. According to the inclusion and exclusion criteria, 14 RCTs were finally included in this NMA. The experimental group included five RCTs (12, 14–17) of weekly GLP-1RAs and nine RCTs (9, 18–25) of daily agents. The detailed literature selection process was shown in Figure 5.

As Table 2 shown, the female to male ratio in the study population was approximately 1.19:1. Subjects from all over the world.

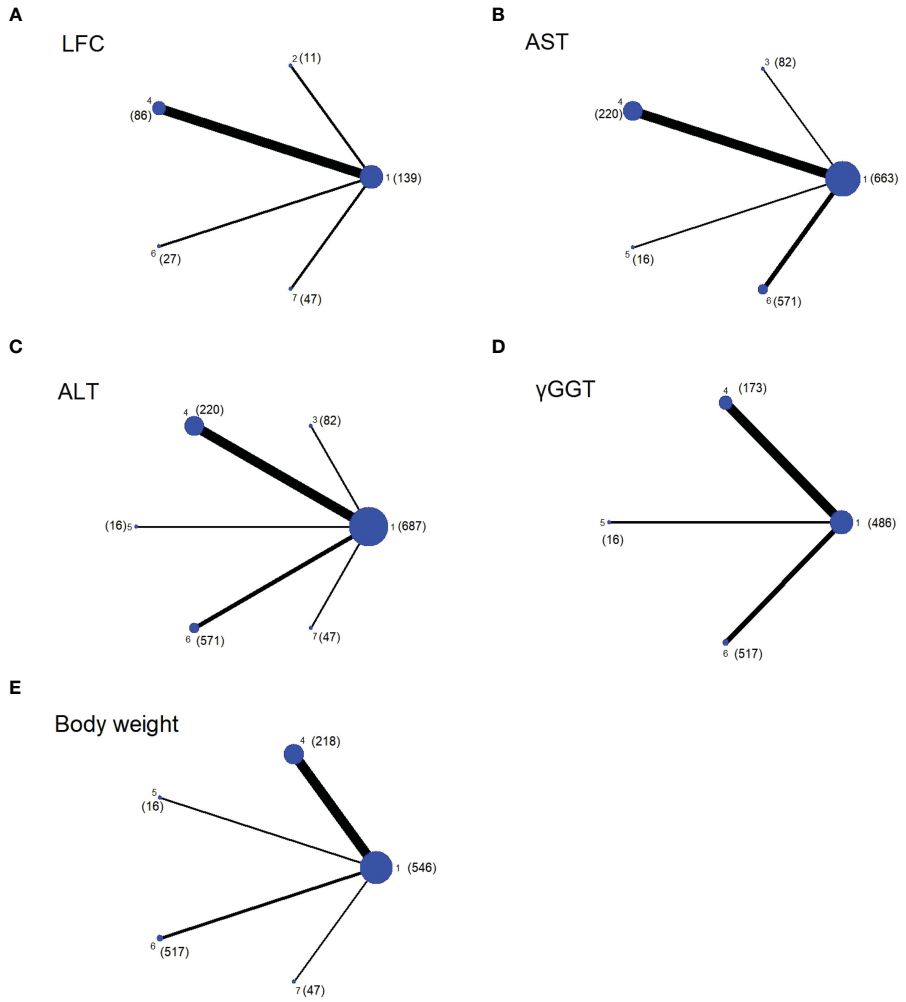

FIGURE 1
Network plots of evidence for each outcome, with the size of the dots representing the sample size (the specific sample size shown in brackets), and the thickness of the lines representing the number of studies comparing the two interventions. The number mean: 1. Placebo; 2. Exenatide (bid); 3. Semaglutide (qd); 4. Liraglutide; 5. Exenatide (qw); 6. Dulaglutide; 7. Semaglutide (qw). **(A)** Network plot of LFC; **(B)** Network plot of AST; **(C)** Network plot of ALT; **(D)** Network plot of γGGT; **(E)** Network plot of Body weight.

3.2 Quality assessment and evidence certainty assessment

The quality of the included studies was assessed by the risk assessment of Cochrane review items. The following aspects were considered during the assessment: random sequence generation, allocation hiding, the blindness of participants and personnel, the blindness of result evaluation, incomplete result data, selective reporting, and other biases. The specific evaluation results were presented in Figure 6.

Using the GRADEprofiler to assess overall quality of evidence. The evaluation results were as follows: two outcomes were assessed as "low", three outcomes were assessed as "moderate". The assessment results were shown in Table 4.

3.3 The outcomes

All experiments were included in this NMA, and the network evidence graphs of each outcome were shown in Figure 1. Among them, weekly GLP-1RA drugs in the treatment of patients with NAFLD mainly include semaglutide (qw), dulaglutide and exenatide (qw) and daily drugs include liraglutide, semaglutide (qd) and exenatide (bid). However, studies on other GLP-1RAs are scarce. The main outcomes we evaluated were LFC, ALT, and AST. Four drugs (except exenatide (bid) and semaglutide (qw)) showed the AST and five drugs (except exenatide (bid)) showed the ALT outcomes, and four drugs (except semaglutide (qd) and exenatide (qw)) showed the LFC outcome. The secondary outcomes were γGGT and body weight, whereas only three drugs (liraglutide, dulaglutide and exenatide (qw)) showed γGGT outcome, and all drugs, except exenatide (bid) and semglutide (qd), showed body weight outcome.

3.4 Network meta-analysis results

The SUCRA curves of interventions for outcomes were shown in Figure 2. Among five interventions (exenatide (bid), liraglutide, dulaglutide, semaglutide (qw) and placebo) evaluated for improving

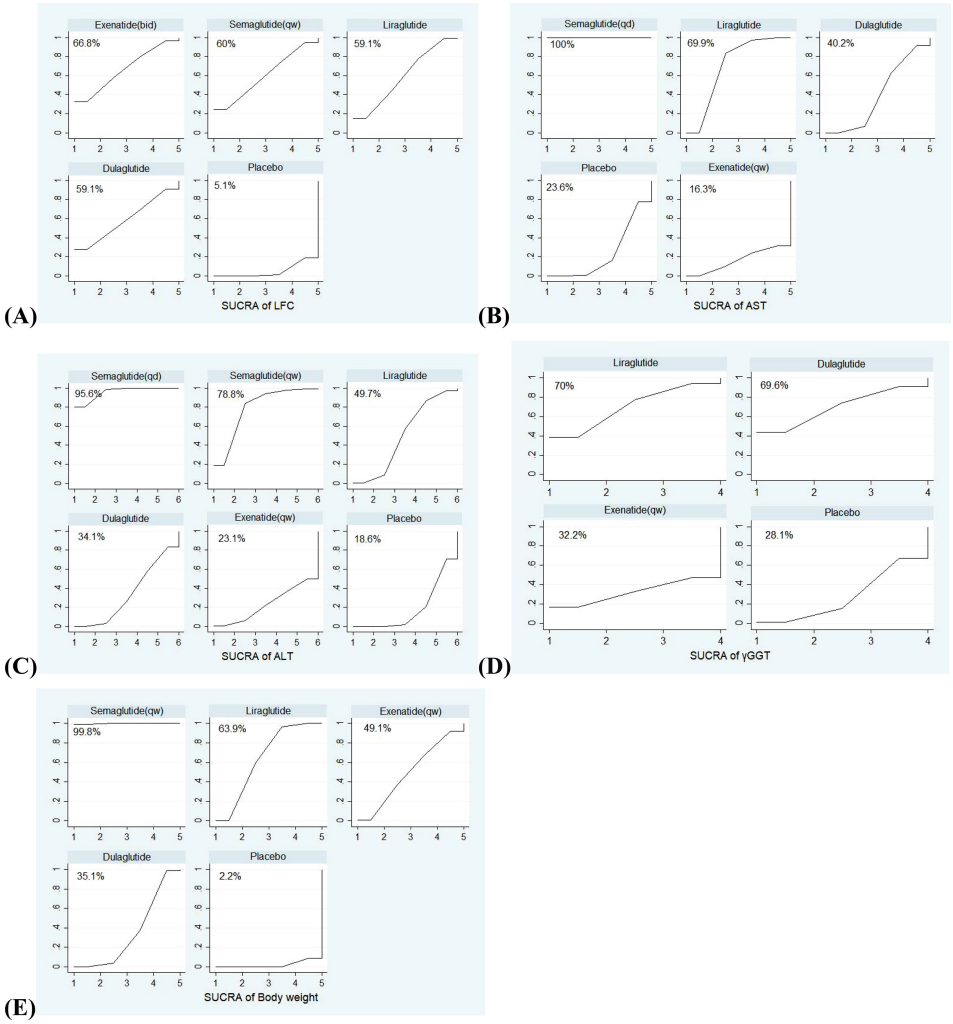

FIGURE 2
The SUCRA (surface under the cumulative ranking curve) of interventions for each outcome. The larger the surface under the curve, the more likely it is to be the best intervention. **(A)** SUCRA of LFC; **(B)** SUCRA of AST; **(C)** SUCRA of ALT; **(D)** SUCRA of γGGT; **(E)** SUCRA of Body weight.

LFC, exenatide (bid) was the best (SUCRA = 66.8%, 59.1%, 59.1%, 60%, and 5.1%, respectively). Among five interventions (semaglutide (qd), liraglutide, dulaglutide, exenatide (qw), and placebo) evaluated for AST outcome, and six interventions (semaglutide (qd), liraglutide, dulaglutide, semaglutide (qw), exenatide (qw) and placebo) evaluated for ALT outcome, semaglutide (qd) was the most effective drug (SUCRA (AST) = 100%, SUCRA (ALT) = 95.6%). For AST, followed by liraglutide and dulaglutide (SUCRA (AST) = 69.9%, and 40.2%, respectively); For ALT, followed by semaglutide (qw) and liraglutide (SUCRA (ALT) = 78.8%, and 49.7%, respectively). Finally, the effects of four interventions (liraglutide, dulaglutide, exenatide (qw), and placebo) on γGGT were compared, and liraglutide was the most effective treatment (SUCRA (γGGT) = 70%), and the effects of five interventions (liraglutide, dulaglutide, semaglutide (qw), exenatide (qw), and placebo) on body weight were compared, semaglutide (qw) seemed better than liraglutide (SUCRA (body weight) = 99.8% *vs* 63.9%).

3.5 Subgroups results

The forest plots were shown that in all outcomes except γGGT, the daily preparations seemed more effective than weekly ones. The result of LFC in daily GLP-1RAs group was MD = -3.66, 95% CI [-5.56, -1.76] and in weekly GLP-1RAs group, it was MD = -3.51, 95% CI [-4, -3.02], p=0.88. As to AST and ALT, the results in daily GLP-1RAs group versus weekly GLP-1RAs group were AST: MD = -7.45, 95% CI [-14.57, -0.32] versus MD = -0.58, 95% CI [-3.18, 2.01], p=0.08 and ALT: MD = -11.12, 95% CI [-24.18, 1.95] versus MD = -5.62, 95% CI [-15.25, 4], p=0.51. The result of Daily GLP-1RAs group also was better than weekly one in body weight (MD = -3.32, 95% CI [-4.61, -2.03] *vs* MD = -1.72, 95% CI [-2.31, -1.13], p=0.03). However, the result of γGGT showed contrary to other outcomes (MD_{daily} = -4.83, 95% CI [-15.5, 5.83] *vs* MD_{weekly} = -6.16, 95% CI [-14.13, 1.81], p=0.85).

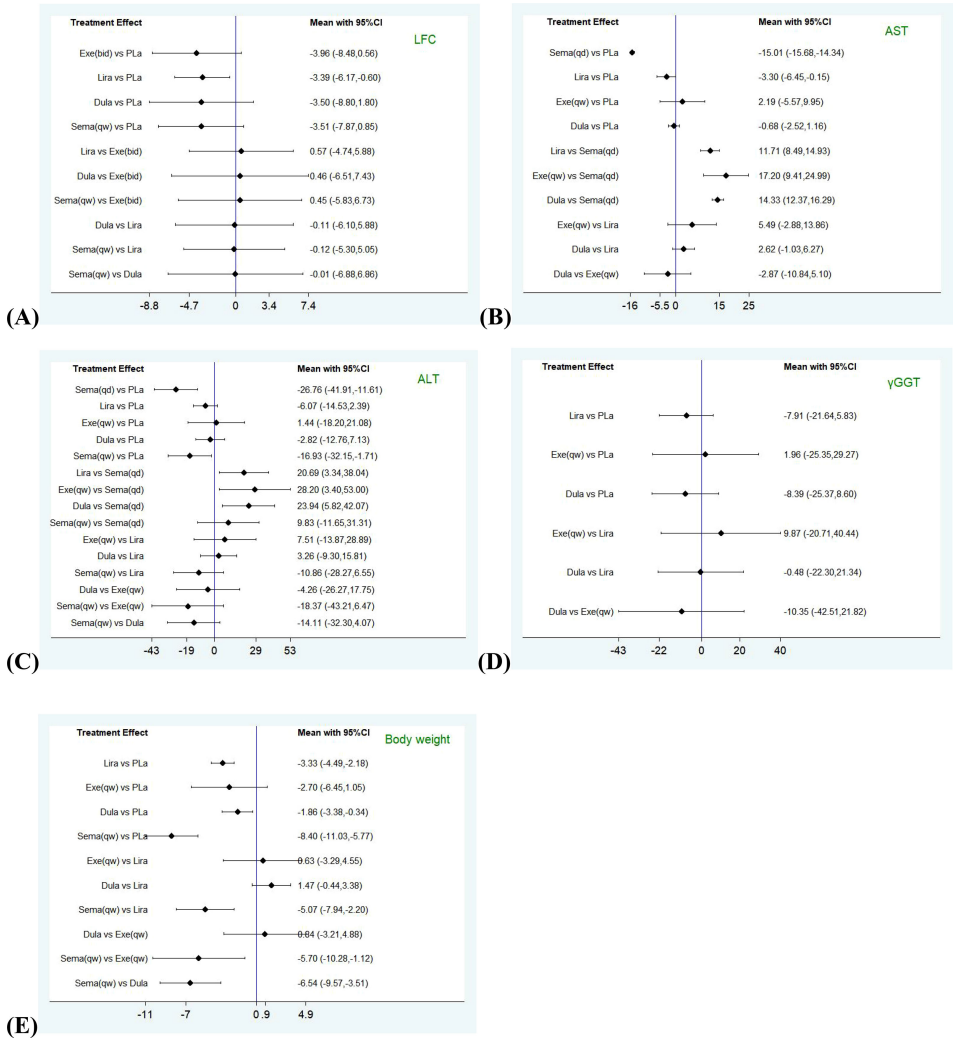

FIGURE 3
Forest plots comparing pairwise interventions for each outcome (LFC, AST, ALT, γGGT, Body weight). LFC, liver fat content; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGGT, γ-glutamyl transferase. **(A)** Forest plot comparing pairwise interventions for LFC; **(B)** Forest plot comparing pairwise interventions for AST; **(C)** Forest plot comparing pairwise interventions for ALT; **(D)** Forest plot comparing pairwise interventions for γGGT; **(E)** Forest plot comparing pairwise interventions for Body weight.

TABLE 3 League plots ranked the effect of the intervention in each outcome from best to worst.

(A) LFC					
Exenatide(bid)					
-0.45 (-6.73, 5.83)	Semaglutide(qw)				
-0.57 (-5.88, 4.74)	-0.12 (-5.3, 5.05)	Liraglutide			
-0.46 (-7.43, 6.51)	-0.01 (-6.88, 6.89)	0.11 (-5.88, 6.1)	Dulaglutide		
-3.96 (-8.48, 0.56)	-3.51 (-7.87, 0.85)	**-3.39** **(-6.17, -0.6)**	-3.5 (-8.8, 1.8)	Placebo	
(B) AST					
Semaglutide(qd)					

(Continued)

TABLE 3 Continued

(B) AST					
-11.71 (-14.93, -8.49)	Liraglutide				
-14.33 (-16.29, -12.37)	-2.62 (-1.03, 6.27)	Dulaglutide			
-15.01 (-15.68, -14.34)	**-3.3 (-6.45, -0.15)**	-0.68 (-2.52, 1.16)	Placebo		
-17.20 (-24.99, -9.41)	-5.49 (-13.86, 2.88)	-2.87 (-10.84, 5.1)	-2.19 (-9.95, 5.57)	Exenatide(qw)	
(C) ALT					
Semaglutide(qd)					
-9.83 (-31.31, 11.65)	Semaglutide(qw)				
-20.69 (-38.04, -3.34)	-10.86 (-28.27, 6.55)	Liraglutide			
-23.94 (-42.07, -5.82)	-14.11 (-32.3, 4.07)	-3.26 (-15.81, 9.3)	Dulaglutide		
-28.2 (-53, -3.4)	-18.37 (-43.21, 6.47)	-7.51 (-28.89, 13.87)	-4.26 (-26.27, 17.75)	Exenatide(qw)	
-26.76 (-41.91, -11.61)	**-16.93 (-32.15, -1.71)**	-6.07 (-14.53, 2.39)	-2.82 (-12.76, 7.13)	1.44 (-18.2, 21.08)	Placebo
(D) γGGT					
Liraglutide					
0.48(-21.34, 22.30)	Dulaglutide				
-9.87(-40.44, 20.71)	-10.35(-42.51, 21.82)	Exenatide(qw)			
-7.91(-21.64, 5.83)	-8.39(-25.37, 8.60)	1.96(-25.35, 29.27)	Placebo		
(E) Body weight					
Semaglutide(qw)					
-5.07 (-7.94, -2.2)	Liraglutide				
-5.7 (-10.28, -1.12)	-0.63 (-4.55, 3.29)	Exenatide(qw)			
-6.54 (-9.57, -3.51)	-1.47 (-3.38, 0.44)	-0.84 (-4.88, 3.21)	Dulaglutide		
-8.4 (-11.03, -5.77)	**-3.33 (-4.49, -2.18)**	-2.7 (-6.45, 1.05)	**-1.86 (-3.38, -0.34)**	Placebo	

Treatments are ranked according to their chances of being the best treatment. From left to right means it's less and less likely to be the best treatment. The leftmost intervention means the highest probability of being the best treatment, The rightmost intervention means the lowest probability of being the best treatment. The data in bold had statistical significance.

4 Discussion

In this NMA, we evaluated GLP-1RAs in the treatment of NAFLD to explore the effectiveness of the long-term use of weekly and daily preparations in improving LFC and liver enzymes involved in NAFLD. In the NMA, the subgroup results and SUCRA showed that the daily agents ranked ahead of the weekly agents with respect to primary outcomes. Though SUCRA showed that semaglutide (qw) was better than other agents on body weight, the subgroup results showed that daily group might be the most effective as a whole. Therefore, we speculate that daily agents show greater promise in NAFLD and T2DM treatment. Furthermore, the daily semaglutide seemed to improve ALT more than the weekly semaglutide, which further validated the conclusion.

Presently, NAFLD is often considered a metabolic disorder associated with liver diseases, and liver steatosis is probably closely related to insulin resistance and T2DM (28). Increased fat content and insulin resistance can lead to liver inflammation and fibrosis (29). A meta-analysis of six RCTs shows that liraglutide can improved liver steatosis (8). Moreover, liraglutide can improve

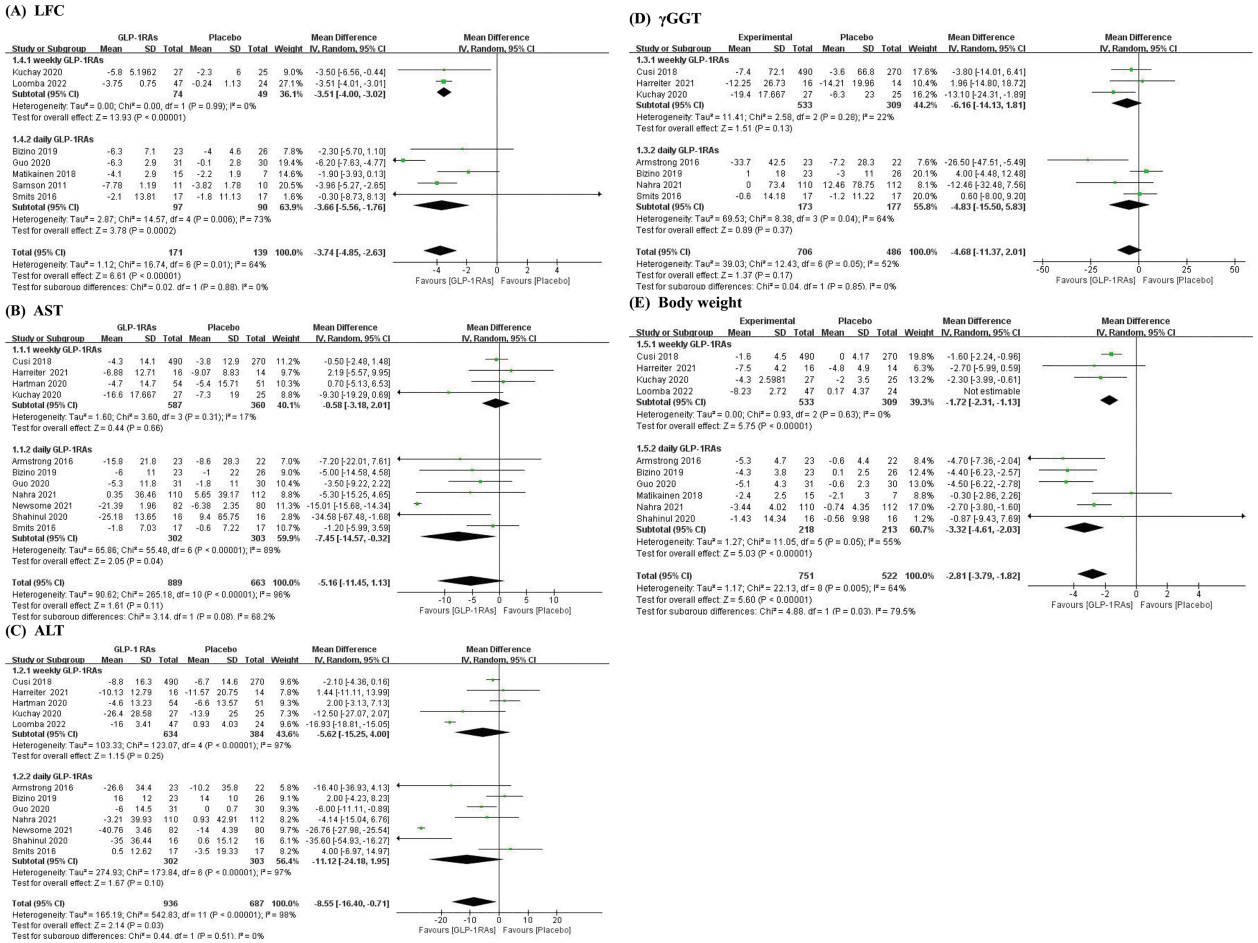

FIGURE 4
Forest plots of subgroup daily and weekly GLP-1RAs. **(A)** Subgroup forest plot of LFC; **(B)** Subgroup forest plot of AST; **(C)** Subgroup forest plot of ALT; **(D)** Subgroup forest plot of γGGT; **(E)** Subgroup forest plot of Body weight.

liver metabolic dysfunction and insulin resistance which play a role in NASH pathogenesis (30). Therefore, we can potentially use GLP-1RAs to treat NAFLD with T2DM.

Although liver biopsy is the gold standard for NAFLD diagnosis, it is not widely used because of its invasiveness. Therefore, researchers have proposed non-invasive examinations instead to diagnose NAFLD and evaluate therapeutic effects. For example, many meta-analyses use LFC to evaluate the improvement of patients with NAFLD (31, 32). Serum biomarkers, such as ALT and AST, are the most common non-invasive tests to assess liver diseases and are commonly used as the clinical indicators of hepatocyte injuries (33). A 6-month, double-blind, and placebo-controlled study shows that lower ALT levels were associated with LFC (34). Therefore, our primary outcomes for assessing GLP-1RA efficacy were LFC and ALT and AST levels. Furthermore, a systematic review included 23 RCTs of the effects of lifestyle interventions on liver steatosis and shows that reduce LFC and lowered liver transaminase levels are strongly associated with weight loss (35). A 5%–10% weight loss resulted in a 40%–80% reduction in liver fats in patients without diabetes and with type 2 diabetes (36). Thus, we used body weight as a secondary outcome in the present NMA.

The subgroup results showed that the daily preparations might be superior to the weekly preparations with respect to primary outcome. And SUCRA showed that semaglutide (qd) might be the best GLP-1RAs among six GLP-1RAs included in our NM. The efficacy of semaglutide (qd) was markedly superior in terms of ALT and AST. A 2019 study shows that semaglutide significantly reduces ALT levels (37), and an RCT by Anne Flint et al. published in 2021 shows that semaglutide significantly improves ALT and AST levels (38). Second, the daily GLP-1RAs significantly reduced LFC and body weight compared with the weekly agents. A 24-week RCTs show that exenatide (bid) can reduce the primary outcome, LFC (10). Although semaglutide (qw) also reduced LFC, the SUCRA values showed that it was slightly less likely to be the optimal treatment than exenatide (bid). However, a NMA compared efficacy and safety of 8 GLP-1RAs show that exenatide (bid) have an increased risk of adverse events withdrawals compared to semaglutide (qw) (39). For body weight, a study including 387 participants found that weight loss with semaglutide (qw) was significantly greater than that with liraglutide (40). And two meta-analyses showed that more significant weight loss was observed after liraglutide intervention than dulaglutide and other GLP-1RA interventions (41, 42). Semaglutide and liraglutide induce

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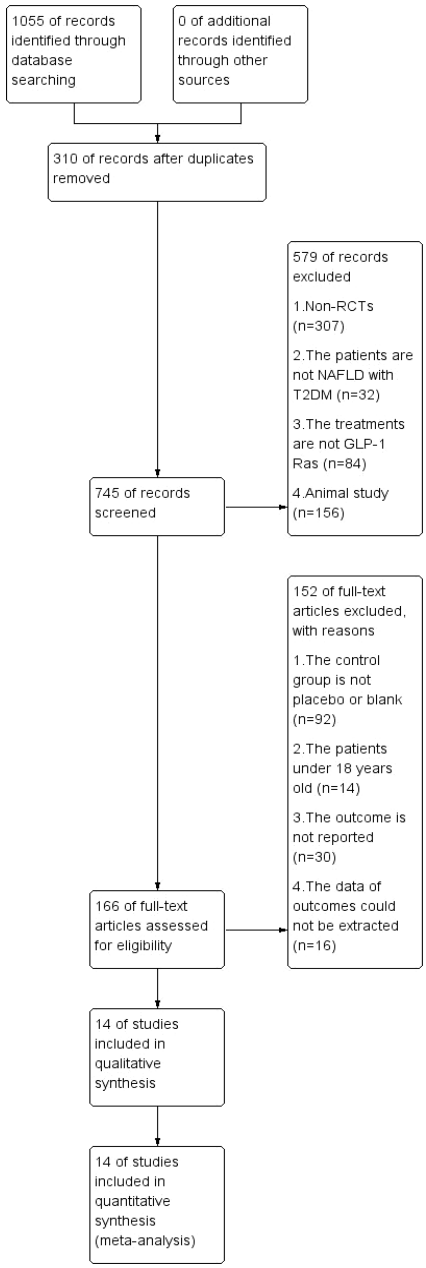

FIGURE 5
Literature selection process.

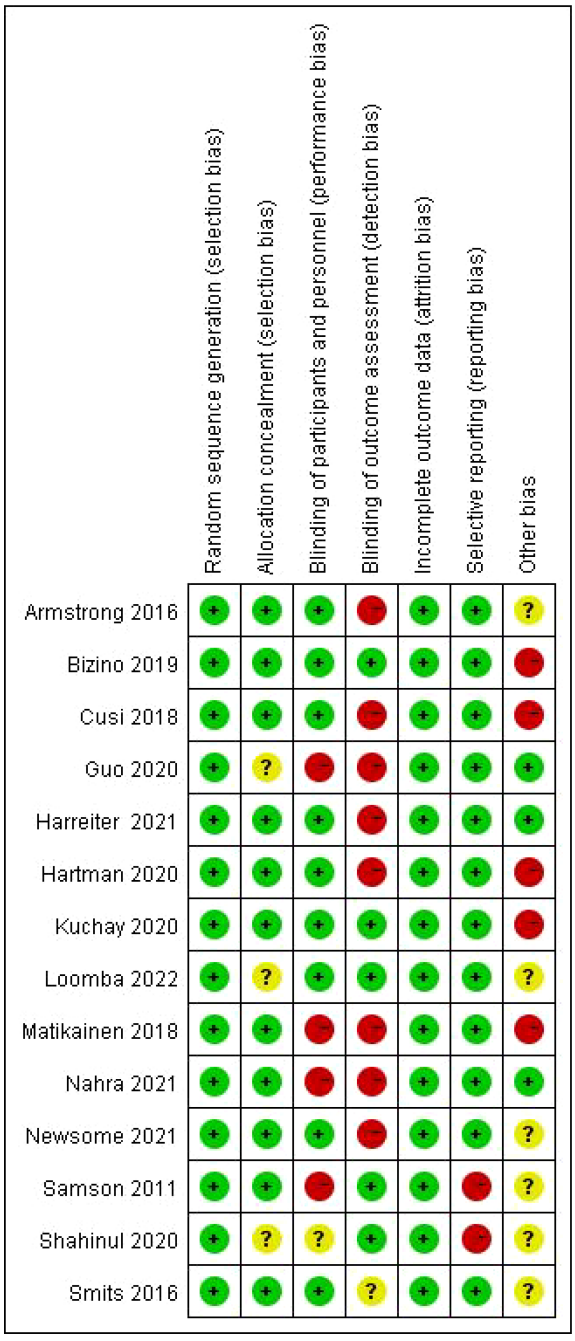

FIGURE 6
Quality assessment using the Cochrane risk assessment tool.

weight loss by lowering energy intake (43, 44), but semaglutide can also reduce weight by reducing appetite (44), which is not obvious in liraglutide (43), this may be the reason why semaglutide is more significant in weight loss. To summarize, daily preparations may be better in the treatment of NAFLD with T2DM. Of course, due to the small number of weekly agents studies included, more weekly agents versus placebo RCTs are needed to validate our results.

4.1 Strengths and limitations

GLP-1RAs have been a popular hypoglycemic drug in recent years. Apart from hypoglycemic and weight loss effects, GLP-1RAs are also of great research value in NAFLD. However, no studies have compared the efficacy of daily and weekly GLP-1RA treatments for NAFLD with T2DM yet. Therefore, we adopted the NMA method to comprehensively analyze the effect of several commonly used GLP-1RAs on the reduction of LFC, liver enzymes, and body weight in patients with NAFLD and T2DM and to obtain an optimal treatment. However, we included only five studies on the weekly agents, which was limited in number and may lead to weak evidence, thus RCTs including more studies on weekly agents *vs.* placebo are needed to validate the present results. Moreover, due to the lack of direct comparative studies of

TABLE 4 The quality of evidence assessment using the GRADE model.

outcome	Outcome important	Number of studies	Sample size	Evidence quality
LFC	critical	7	310	low
AST	critical	11	1552	moderate
ALT	critical	12	1623	moderate
γGGT	moderate	7	1194	low
body weight	important	10	1344	moderate

the two GLP-1RAs, we cannot analyze inconsistent. The league plots showed a comparison between liraglutide and the placebo, showing that the major outcome, LFC, was statistically significant; however, the rest of the results were not statistically significant, which might be because of the small sample size. And there is only one study of semaglutide(qw), thus more studies of weekly semaglutide are needed to compare with daily exenatide.to assess which is superior in LFC. In the future, more large-sample, head-to-head RCTs are required to confirm these findings.

5 Conclusion

We integrated the evidence on GLP-1RAs for NAFLD with T2DM treatment and concluded that the daily preparations were superior to the weekly preparations with respect to primary outcome. We found that the daily GLP-1RAs semaglutide among the six GLP-1RAs ((exenatide (bid), liraglutide, semaglutide (qd), dulaglutide, semaglutide (qw), exenatide (qw)) might be the most effective treatment options for NAFLD. This conclusion may provide a basis for clinicians to treat NAFLD with T2DM.

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

XY and ZG contributed to the conception and design of the study. XY and CY searched the databases and screened the literature. XY and KD participated in data extraction. XY performed the statistical analysis. The first draft of this article was written by XY. 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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A non-linear relationship between triglyceride glucose waist circumference and nonalcoholic fatty liver disease in a Japanese population: a secondary analysis

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Introduction: Nonalcoholic fatty liver disease (NAFLD) is a common metabolic disorder associated with insulin resistance (IR). Triglyceride glucose waist circumference (TyG-WC) is a novel index of IR that reflects both visceral fat and hepatic steatosis. However, it is not known whether TyG-WC and NAFLD exhibit a nonlinear relationship in Japanese subjects with normal plasma glucose level. Thus, we examined the relationship between TyG-WC and NAFLD, in addition to determining the threshold level of TyG-WC associated with NAFLD.

Methods: A secondary analysis was performed based on a previous study that extracted medical examination records from Murakami Memorial Hospital between 2004 and 2015 in order to detect chronic diseases and their risk factors. TyG-WC was determined at baseline. NAFLD is the dependent variable. Univariate and multivariate logistic regression models were used to evaluate the risk of NAFLD incidence. Based on the smoothing plot, a two-piecewise linear regression model was used to examine the threshold effect of TyG-WC on NAFLD. A subgroup analysis was carried out in order to study other factors that may influence the association between TyG-WC and NAFLD.

Results: 14,280 met the criteria for inclusion in the current secondary analysis. The adjusted OR (95% CI) for NAFLD in all subjects was 1.007 (95% CI 1.006–1.009, P < 0.001). The relationship between TyG-WC and NAFLD in Japanese subjects with normal plasma glucose level is nonlinear. TyG-WC is positively associated with NAFLD when TyG-WC is ranged between 480 and 800. In subgroup analyses, there was a significant interaction between BMI and TyG-WC associated NAFLD risk (P for interaction <0.001).

Discussion: The relationship between TyG-WC and NAFLD is nonlinear. TyG-WC is positively associated with NAFLD when TyG-WC is ranged between 480 and 800. There is potential clinical significance for the TyG-WC in identifying groups at high risk for NAFLD in subjects with normal plasma glucose level.

KEYWORDS

nonalcoholic fatty liver disease, insulin resistance, triglyceride, fasting blood glucose, triglyceride glucose waist circumference

1 Introduction

NAFLD is characterized by a variety of histopathologic findings, ranging from steatosis to steatohepatitis, fibrosis, and cirrhosis (1). A number of factors contribute to NAFLD, including metabolic syndrome, obesity, insulin resistance, and hyperlipidaemia (2, 3). Along with the global obesity-related metabolic syndrome epidemic, NAFLD prevalence is increasing (4). Approximately 25% of the population worldwide suffers from NAFLD, ranging from 13% in Africa to 42% in southeast Asia (5, 6). Metabolic dysfunction-associated liver disease (MAFLD) is characterized by metabolic dysregulation and overlaps with other liver diseases, according to an expert panel recently (7). There has, however, been a lack of widespread adoption of the new definition. In order to predict and diagnose NAFLD early, accurate non-invasive methods must be investigated, as it is normally asymptomatic until the advanced stages (8, 9).

Regardless of whether metabolic syndrome is present or absent, there is evidence that IR contributes significantly to the development of NAFLD (10, 11). The triglyceride and glucose index (TyG) has been proposed as an effective alternative to IR. This combines fasting plasma glucose (FPG) with fasting triglyceride (TG) (12). TyG levels have been associated with NAFLD incidence in many studies (13). The combination of TyG-related parameters and obesity indices may be more reliable for identifying patients than TyG alone, according to emerging research (14–17). Evidence indicates TyG-waist circumference (TyG-WC) is a superior predictor of insulin resistance than TyG alone (14, 18, 19). TyG-WC has been shown to be related to NAFLD among Iranians in a cross-sectional study (20).

In Japanese people, however, it is not known whether TyG-WC is associated with NAFLD. Further, it is not clear whether TyG-WC and NAFLD exhibit a nonlinear relationship. In this study, we examined the relationship between TyG-WC and NAFLD in Japanese subjects with normal plasma glucose level, in addition to determining the threshold level of TyG-WC associated with NAFLD.

2 Materials and methods

2.1 Data source

A public database called Datadryad.org, where investigators can reanalyse data from previous studies, was used for this study. The research cites Okamura et al.'s data packets (21). A second analysis was performed based on previous research that aims to detect chronic diseases and their risk factors. Previous study extracted cases from Murakami Memorial Hospital's medical examination program between 2004 and 2015, then, a follow-up study was carried out on incident type 2 diabetes and fatty liver.

This study adopted a cross-sectional design and its exclusion criteria are as follows: 1) viral hepatitis (determined by hepatitis B antigen and hepatitis C antibody measurements)(N=416); 2) drinking excessively: males 210 grams per week or females 140 grams per week (N=1923) (22); 3) data with incomplete covariables (N = 873); 4) T2DM or fasting plasma glucose over 6.1 mmol/L at baseline-examination (N = 1131); 5) any medication usage (N = 2,321). Informed consent was not required because the data had been de-identified. Murakami Memorial Hospital's ethics committee approved the previous study (21). As a result, there was no need for an additional ethical approval for this study. It followed the Declaration of Helsinki.

2.2 Collection of data

As mentioned previously, a self-administered questionnaire was adopted to collect clinical baseline information (21). A comprehensive list of demographic information, anthropometric and clinical measurements or lifestyle characteristics, including sex, age, height, weight, WC, systolic blood pressure (SBP), diastolic blood pressure (DBP), drinking habit, and smoking habit. Exercise habit is defined as exercising more than once a week; Smoking status was divided into nonsmokers, former smokers, and current smokers based on smoking history. Drinking status was classified into three groups based on alcohol consumption: minimal or no consumption, 40 grams or less per week; light, 40-140 grams per week; moderate, 140-210 grams per week. Haematological indicators were tested after fasting, including haemoglobin A1c (HBA1c), total cholesterol (TC), triglyceride (TG), FPG, gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and high-density lipoprotein cholesterol (HDL-C). According to previous studies (14, 23), TyG = Ln [TG (mg/dL) × FPG (mg/dL)/ 2], TyG-WC = TyG × WC (cm).

2.3 NAFLD diagnosis by abdominal ultrasound

A trained technician performed abdominal ultrasonography to diagnose fatty liver. Without consulting any other information about the participants, the gastroenterologist diagnosed fatty liver based on the images. Among the four known criteria (vascular blurring, hepatorenal echo contrast, deep attenuation and liver brightness), participants with hepatorenal echo contrast and liver brightness were diagnosed with fatty liver (24).

2.4 Statistical analysis

For all statistical analyses, EmpowerStats (www.empowerstats.com , X&Y Solutions, Inc., Boston, MA) and R (http://www.R-project.org, The R Foundation) platforms were used. Categorical variables were expressed as the frequency (percentage).

Normal distribution continuous variables were expressed as mean ± standard deviation (SD), and unnormal distribution continuous variables as median (quartile 1-quartile 3). By using Kruskal Wallis H (variables with non-normal distribution) or one-way ANOVA (variables with normal distribution) or chi-squared (categorical) tests, differences among TyG-WC groups were

evaluated. A two-tailed *P* value of 0.05 was used to determine statistical significance. NAFLD risk factors were determined using multivariate and univariate logistic regression models. We also explored the relationship between TyG-WC and NAFLD according to gender. Initially, all variables were analysed using univariate analysis.

Afterwards, variables with clinical significance and variables with statistical significance in univariate analysis ($P < 0.05$) were included in the multivariate analyses. In order to evaluate the collinearity of all explanatory variables, a correlation matrix was used. Variance inflation factor (VIF) based multiple regression model was used to assess collinearity (25). The Additional File Table S1 illustrates the presence of collinearity when the VIF is greater than 5. Four different models were built: Model 1, without covariate adjustment; Model 2, adjusted for age, smoking status, habit of exercise, SBP; Model 3, adjusted for Model 2+HbA1C, FPG, TG, TC, HDL, GGT, ALT, AST, and Model 4, adjusted for Model 3 +BMI. We selected these confounders on the basis of their associations with the NAFLD or a change in effect estimate of more than 10% (26).

The data analysis results were verified by converting TyG-WC according to triquantiles and examining the possibility of nonlinearity, and calculation of the *P* value for the trend was carried out. The threshold effect of TyG-WC on NAFLD was examined using a two-piecewise linear regression model based on the smoothing plot. In order to determine the threshold level of TyG-WC at which the association between TyG-WC and NAFLD changed and became noticeable, a recurrence method was employed. Detecting the maximum model likelihood was based on moving the inflection point along a predefined interval. Logistic regression model was used for subgroup analysis. To test the subgroup effect modification, interaction terms were used between subgroup indicators, followed by likelihood ratio tests.

3 Results

3.1 Subjects description

In the previous study, 20,944 subjects were recruited. Only 14,280 met the criteria for inclusion in the current secondary analysis (Figure 1). The average age of the subjects was 44 ± 9 years, and 54.51% were men. Subject baseline characteristics are listed in Table 1. TyG-WC group (T3) individuals were usually older and had higher BMI, WC, SBP, DBP, ALT, AST, GGT, TG, TC, HbA1C, TyG values than TyG-WC group (T1). Comparatively, HDL-C values for T3 groups were lower than those for T1 groups. Additionally, the prevalence of NAFLD gradually increases as the value of TyG-WC increases (T1: 0.626% vs. T2: 9.226% vs. T3: 44.864%).

3.2 Association between TyG-WC and NAFLD

Male, age, BMI, weight, WC, SBP, DBP, ALT, AST, GGT, TC, TG, HbA1C, TyG, were found to be risk factors for NAFLD in the univariate analysis (Table 2). A comparison of the effect sizes of TyG-WC on NAFLD in among male, female and all participants is shown in Table 3. The Model 1 shows TyG-WC to be positively associated with NAFLD in total participants. According to Model 2, NAFLD risk increased by 1.7% for every unit increase in TyG-WC (OR = 1.017, 95% CI 1.016-1.017, $P<0.001$) in total participants after accounting for age, habit of exercise, smoking status, and SBP. In Model 3, after adjusting for Model 2+ALT, AST, GGT, HDL, TC, TG, FPG, HbA1C, for each unit increase in TyG-WC, the risk of NAFLD increased 1.4% (OR = 1.014, 95% CI 1.013-1.015, $P < 0.001$) in total participants. The fully adjusted OR (95% CI) for

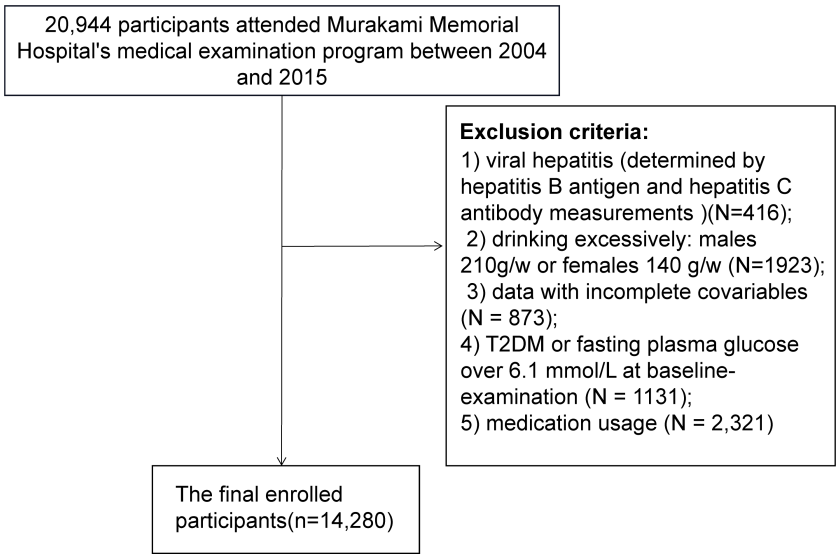

FIGURE 1
Flow chart.

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TABLE 1 Characteristics of the participants by triquartile grouping of TyG-WC.

TyG-WC	T1 (339.450-559.133)	T2 (559.145-660.110)	T3 (660.112-1097.184)	*P*-value
N	4955	4769	4556	
Age, years	41.055 ± 8.467	44.260 ± 8.877	45.466 ± 8.739	<0.001
BMI, kg/m2	19.550 ± 1.786	21.845 ± 1.897	25.040 ± 2.797	<0.001
Weight, kg	50.467 ± 6.185	59.828 ± 7.252	71.436 ± 9.854	<0.001
WC, cm	67.329 ± 4.645	76.223 ± 4.138	85.811 ± 6.340	<0.001
SBP, mmHg	106.176 ± 12.313	114.288 ± 13.169	122.084 ± 14.534	<0.001
DBP, mmHg	65.717 ± 8.559	71.145 ± 9.357	77.037 ± 10.020	<0.001
ALT, IU/L	13.000 (11.000-17.000)	16.000 (13.000-21.000)	23.000 (17.000-32.000)	<0.001
AST, IU/L	16.000 (13.000-19.000)	17.000 (14.000-20.000)	19.000 (16.000-23.250)	<0.001
GGT, IU/L	12.000 (10.000-14.000)	15.000 (11.000-20.000)	21.000 (15.000-31.000)	<0.001
TG, mg/dL	40.000 (30.500-54.000)	64.000 (50.000-82.000)	112.000 (84.000-154.000)	<0.001
TC, mg/dL	187.155 ± 31.071	197.971 ± 32.589	210.234 ± 33.052	<0.001
HDL, mg/dL	65.758 ± 14.560	56.789 ± 13.751	45.959 ± 10.837	<0.001
HbA1C, %	5.118 ± 0.302	5.175 ± 0.310	5.246 ± 0.338	<0.001
TyG-WC	502.080 ± 38.700	608.815 ± 28.657	739.364 ± 64.803	<0.001
TyG	7.466 ± 0.448	7.999 ± 0.382	8.620 ± 0.472	<0.001
Gender				<0.001
Female	4004 (80.807%)	2019 (42.336%)	817 (17.932%)	
Male	951 (19.193%)	2750 (57.664%)	3739 (82.068%)	
Habit of exercise				<0.001
No	4091 (82.563%)	3849 (80.709%)	3864 (84.811%)	
Yes	864 (17.437%)	920 (19.291%)	692 (15.189%)	
Drinking status				<0.001
Non or small	4239 (85.550%)	3493 (73.244%)	3043 (66.791%)	
Light	638 (12.876%)	1040 (21.808%)	1110 (24.363%)	
Moderate	78 (1.574%)	236 (4.949%)	403 (8.845%)	
Smoking status				<0.001
Non	3974 (80.202%)	2839 (59.530%)	1938 (42.537%)	
Former	451 (9.102%)	908 (19.040%)	1213 (26.624%)	
Current	530 (10.696%)	1022 (21.430%)	1405 (30.838%)	
NAFLD				<0.001
No	4924 (99.374%)	4329 (90.774%)	2512 (55.136%)	
Yes	31 (0.626%)	440 (9.226%)	2044 (44.864%)	

Values are expressed as n (%) or mean ± SD or median (quartile1-3).
BMI, body mass index; WC, waist circumference; DBP, diastolic blood pressure; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; GGT gamma-glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP, diastolic blood pressure; SBP, systolic blood pressure; NAFLD, nonalcoholic fatty liver disease.

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TABLE 2 Univariate logistics regression model showing variables associated with NAFLD.

	Statistics	OR (95%CI)	*P* value
Gender			
Female	6840 (47.899%)	1.0	
Male	7440 (52.101%)	5.018 (4.513, 5.579)	<0.00001
Age, years	43.533 ± 8.891	1.019 (1.014, 1.024)	<0.00001
BMI, kg/m2	22.068 ± 3.137	1.647 (1.614, 1.681)	<0.00001
Weight, kg	60.283 ± 11.619	1.130 (1.124, 1.136)	<0.00001
WC, cm	76.196 ± 9.100	1.204 (1.195, 1.213)	<0.00001
SBP, mmHg	113.961 ± 14.833	1.053 (1.050, 1.057)	<0.00001
DBP, mmHg	71.141 ± 10.391	1.079 (1.074, 1.084)	<0.00001
ALT, IU/L	19.770 ± 14.459	1.103 (1.098, 1.109)	<0.00001
AST, IU/L	18.227 ± 8.662	1.090 (1.083, 1.097)	<0.00001
GGT, IU/L	19.154 ± 16.165	1.040 (1.037, 1.043)	<0.00001
HDL, mg/dL	56.449 ± 15.472	0.927 (0.923, 0.931)	<0.00001
TC, mg/dL	198.131 ± 33.565	1.013 (1.012, 1.014)	<0.00001
TG, mg/dL	79.030 ± 56.073	1.017 (1.017, 1.018)	<0.00001
HbA1C, %	5.178 ± 0.321	4.417 (3.841, 5.078)	<0.00001
Habit of exercise			
No	11804 (82.661%)	1.0	
Yes	2476 (17.339%)	0.815 (0.724, 0.918)	0.00076
Drinking Status			
Non or small	10775 (75.455%)	1.0	
Light	2788 (19.524%)	0.994 (0.890, 1.109)	0.91106
Moderate	717 (5.021%)	1.152 (0.952, 1.394)	0.14530
Smoking status			
Non	8751 (61.282%)	1.0	
Former	2572 (18.011%)	2.122 (1.904, 2.364)	<0.00001
Current	2957 (20.707%)	1.930 (1.738, 2.144)	<0.00001
TyG-WC	613.431 ± 107.254	1.018 (1.017, 1.018)	<0.00001
TyG	8.012 ± 0.641	7.616 (6.953, 8.344)	<0.00001

NAFLD in total participants was 1.007 (95% CI 1.006–1.009, $P < 0.001$) for every 1-unit increase in TyG-WC.

Female and male had fully adjusted ORs (95% CI) of 1.008 (1.005, 1.011) and 1.007 (1.005, 1.009), respectively. We also conducted the sensitivity analysis using TyG-WC as a categorical variable (triquantile), and the same trend was observed (*P* for trend was $P < 0.001$).

3.3 Results of two-piecewise linear regression model

TyG-WC ranging between 480 and 800 showed a significant association between TyG-WC and NAFLD (OR1.009, 95% CI:1.007, 1.010, $P< 0.001$). The risk of NAFLD increased 9% for each additional unit of TyG-WC (Table 4; Figure 2).

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TABLE 3 Relationship between TyG-WC and NAFLD risk in different models.

	Model 1 Odds ratios (95%CI)	*P* value	Model 2 Odds ratios (95%CI)	*P* value	Model 3 Odds ratios (95%CI)	*P* value	Model 4 Odds ratios (95%CI)	*P* value
Female								
TyG-WC	1.019 (1.018, 1.021)	<0.001	1.019 (1.017, 1.020)	<0.001	1.016 (1.014, 1.018)	<0.001	1.008 (1.005, 1.011)	<0.001
TyG-WC (trisection)								
T1	1.0		1.0		1.0		1.0	
T2	19.189 (11.753, 31.329)	<0.001	16.388 (9.984, 26.898)	<0.001	10.966 (6.580, 18.274)	<0.001	5.475 (3.243, 9.244)	<0.001
T3	127.822 (78.746, 207.482)	<0.001	94.839 (57.561, 156.260)	<0.001	36.777 (21.339, 63.385)	<0.001	7.162 (3.865, 13.273)	<0.001
P for trend	<0.001		<0.001		<0.001			
Male								
TyG-WC	1.016 (1.015, 1.017)	<0.001	1.016 (1.015, 1.017)	<0.001	1.013 (1.012, 1.014)	<0.001	1.007 (1.005, 1.009)	<0.001
TyG-WC (trisection)								
T1	1.0		1.0		1.0		1.0	
T2	8.147 (4.650, 14.274)	<0.001	7.456 (4.250, 13.080)	<0.001	4.467 (2.479, 8.048)	<0.001	2.598 (1.442, 4.679)	0.001
T3	63.144 (36.403, 109.528)	<0.001	52.458 (30.153, 91.262)	<0.001	14.577 (8.070, 26.332)	<0.001	4.292 (2.339, 7.876)	<0.001
P for trend	<0.001		<0.001		<0.001		<0.001	
Total								
TyG-WC	1.017 (1.016, 1.018)	<0.001	1.017 (1.016, 1.017)	<0.001	1.014 (1.013, 1.015)	<0.001	1.007 (1.006, 1.009)	<0.001
TyG-WC (trisection)								
T1	1.0		1.0		1.0		1.0	
T2	13.914 (9.614, 20.137)	<0.001	12.147 (8.378, 17.611)	<0.001	7.842 (5.335, 11.527)	<0.001	4.202 (2.845, 6.204)	<0.001
T3	102.810 (71.388, 148.060)	<0.001	80.084 (55.378, 115.812)	<0.001	25.576 (17.236, 37.953)	<0.001	6.534 (4.303, 9.921)	<0.001
P for trend	<0.001		<0.001		<0.001			

Model 1, without covariate adjustment; Model 2, adjusted for age, smoking status, habit of exercise, SBP; Model 3, adjusted for Model 2+HbA1C, FPG, TG, TC, HDL, GGT, ALT, AST, and Model 4, adjusted for Model 3+BMI.

3.4 Subgroup analysis

In subgroup analyses, there was a significant interaction between BMI and TyG-WC associated NAFLD risk (Table 5) (*P* for interaction< 0.001), while the interaction between age, gender, smoking status, drinking status, habit of exercise and TyG-WC was not significant. The results of BMI stratification showed that non-obese people had a higher risk of NAFLD than overweight and obese people.

TABLE 4 The results of the two-piecewise linear regression model.

Inflection point of TyG-WC	Odds ratio (OR)	95%CI	*P* value
<480	1.022	0.984-1.062	0.2539
480-800	1.009	1.007, 1.010	<0.0001
>800	1.001	0.996, 1.006	0.7112

Adjusted for age, smoking status, habit of exercise, SBP, HbA1C, FPG, TG, TC, HDL, GGT, ALT, AST, BMI.

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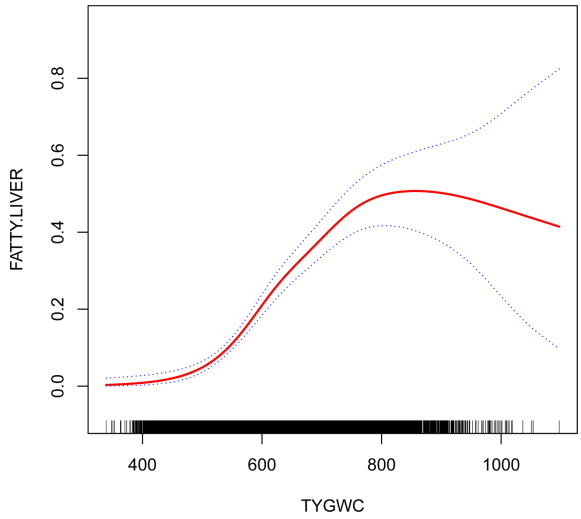

FIGURE 2
The relationship between triglyceride glucose waist circumference and nonalcoholic fatty liver disease. The graph shows the relationship between TyG-WC and NAFLD. The 95%CI is represented by the area between two dotted lines. When the TyG-WC value is ≤480 or ≥800, there is no correlation with NAFLD. However, when the TyG-WC value is between 480 and 800, there is a significant correlation with NAFLD. As the TyG-WC value increases, so does the risk of NAFLD.

TABLE 5 Subgroup analysis of the association between TyG-WC on NAFLD risk.

Subgroup	No. of participants	adjusted OR (95% CI)	*P* for interaction
Gender			0.3561
Female	6840	1.008 (1.005, 1.011)	
Male	7440	1.007 (1.005, 1.009)	
Age			0.5639
<45	8319	1.008 (1.006, 1.010)	
>=45, <60	5325	1.008 (1.005, 1.010)	
>=60	636	1.007 (1.000, 1.013)	
BMI			<0.0001
<24	10894	1.010 (1.008, 1.013)	
>=24, <28	2742	1.005 (1.003, 1.007)	
>=28	644	1.004 (0.999, 1.009)	
Drinking status			0.0990
Non or small	10775	1.010 (1.008, 1.012)	
Light	2788	1.004 (1.001, 1.007)	
Moderate	717	1.009 (1.003, 1.015)	
Smoking Status			0.1861
Non	8751	1.009 (1.007, 1.011)	
Former	2572	1.006 (1.003, 1.009)	
Current	2957	1.007 (1.004, 1.010)	
Habit of exercise			0.9967
No	11804	1.007 (1.006, 1.009)	
Yes	2476	1.011 (1.007, 1.015)	

4 Discussion

The present study assessed the relationship between TyG-WC and NAFLD among Japanese subjects with normal plasma glucose level. Despite adjusting for other covariates, TyG-WC remained associated with NAFLD in the Japanese population (OR = 1.007, 95% CI 1.006, 1.009). Furthermore, we revealed a threshold effect of TyG-WC and NAFLD, that both low and high levels of TyG-WC had no significant association with NAFLD, but in the range of 480 to 800, TyG-WC was strongly associated with NAFLD. In addition, TyG-WC and BMI interacted to affect NAFLD in subgroup analysis (*P* value for interaction <0.001).

In spite of the complexity of NAFLD's mechanism, IR plays a crucial role in its progression. The identification of IR, however, is not straightforward. In the current state of IR detection, the gold standard is still the HEC test (27). Due to its complexity and time-consuming nature, HEC is limited in clinical applications. In consequence, the homeostasis model assessment of IR (HOMA-IR) has become a globally recognized alternative indicator of IR. Studies have found an independent relationship between HOMA-IR and NAFLD (28). It is, however, difficult in many laboratories to detect insulin concentrations, which is needed for HOMA-IR to be calculated. As a result, new indicators are needed to identify IR and NAFLD.

There is a growing body of research confirming that TyG can be used for IR assessment, and it has the advantage that it requires just two simple haematological indices (FPG and TG) to calculate. TyG can be used to identify IR through its association with HOMA-IR and HEC (29). It was concluded by Lim et al. that TyG had superior prediction ability over insulin resistance when it came to NAFLD (30). More and more evidence show that obesity is closely related to insulin resistance, and because TyG is universally accepted as a promising surrogate marker of IR, the combination of obesity and TyG may be more powerful than other surrogate markers in identifying IR (14). It has been claimed by Cho et al. that the TyG-WC is an indicator of coronary artery disease that can be used to predict the progression of coronary atherosclerosis better than other indices (31). Khamseh et al. performed a cross sectional study to analyse the association between TyG-WC and NAFLD in individuals with overweight/obesity (20). They concluded that TyG-WC was significant associated with NAFLD in individuals with overweight/obesity. The current study results are similar to theirs. Nevertheless, their study limited its participants to obese and overweight people. Furthermore, the non-linear relationship between TyG-WC and NAFLD was not considered. It was clearly observed in the current study that TyG-WC and NAFLD are not linearly related. NAFLD and TyG-WC ranging between 480 and 800 showed a statistically significant association. The magnitude of the TyG-WC was not associated with NAFLD when it was ≤ 480 or ≥ 800.

Interestingly, our current study found that people with lower BMI had a higher OR value.(BMI <24kg/m^2,OR:1.010, 95% CI:1.008, 1.013)than those with higher BMI(24kg/m^2≤BMI<28kg/m^2,OR:1.005,95%CI:1.003,1.007;BMI≥28,OR:1.004,95%CI:0.999, 1.009). Some studies suggest that the relationship between TyG index and NAFLD risk is significantly stronger in non-obese subjects than in obese subjects (13, 32). It is unclear how BMI influences TyG-WC and NAFLD, but it may be related to relatively lean people's significantly reduced skeletal muscle mass. Relevant studies have shown that when body mass index decreased, skeletal muscle weight, skeletal muscle index and limb fat decreased significantly, and low muscle mass was positively correlated with NAFLD (33, 34). Further research is needed on the specific mechanism.

4.1 Strengths and limitation

The following are some of the strengths of this study (1): A strict adjustment was made for confounding factors in this study (2). Sensitivity analysis was conducted, the continuous independent variables were converted to categorical variables for analysis to improve the reliability of the results (3). It was investigated whether TyG-WC and NAFLD have a nonlinear relationship (4). Different populations were considered when calculating effect sizes.

In spite of this, there are a few limitations to consider (1): The diagnosis of NAFLD in this study was made by ultrasonography rather than liver biopsy. A further limitation of ultrasonography is its inability to distinguish between steatohepatitis and steatosis. Nevertheless, ultrasound examinations have been widely applied in epidemiological studies to diagnose NAFLD (35) (2). Raw data did not include HOMA-IR and waist-to-hip ratio that were associated with NAFLD and IR (3). In this study, nutritional habits and energy intake were not recorded, but covariates associated with dietary habits, such as TG and HDL-C were adjusted (4). The conclusion cannot be generalized to other races since only Japanese subjects were included in the study.

In short, TyG-WC value between 480 and 800 was positively correlated with NAFLD. The magnitude of the TyG-WC was not associated with NAFLD when it was ≤ 480 or ≥ 800. In addition, the effect size was higher in people with lower BMI(BMI <24 kg/m^2) than those with higher BMI (BMI≥24 kg/m^2). Therefore, when TyG-WC≥480 is worthy of attention, especially when TyG-WC is in the range of 480-800, TyG-WC has a strong positive association with NAFLD.

5 Conclusions

The relationship between TyG-WC and NAFLD in Japanese subjects with normal plasma glucose level is nonlinear. TyG-WC is positively associated with NAFLD when TyG-WC is ranged between 480 and 800. The magnitude of the TyG-WC is not associated with NAFLD when it is ≤ 480 or ≥ 800. There is potential clinical significance for the TyG-WC in identifying groups at high risk for NAFLD. It is a low-cost and simple and

biochemical measurement that can be used to screen and assess NAFLD risk in large populations. Furthermore, these findings could be useful for establishing diagnostic or predictive models of incident NAFLD in the future.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

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

Author contributions

Conceptualization: XJH and XYH. Methodology: XJH. Software: XYH. Validation: YQ. Formal analysis: XYH. Writing—original draft preparation: XYH. Writing—review and editing: YQ and TS. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article.

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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.2023.1188214/full#supplementary-material

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Causal effects of nonalcoholic fatty liver disease on cerebral cortical structure: a Mendelian randomization analysis

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Background: Previous studies have highlighted changes in the cerebral cortical structure and cognitive function among nonalcoholic fatty liver disease (NAFLD) patients. However, the impact of NAFLD on cerebral cortical structure and specific affected brain regions remains unclear. Therefore, we aimed to explore the potential causal relationship between NAFLD and cerebral cortical structure.

Methods: We conducted a Mendelian randomization (MR) study using genetic predictors of alanine aminotransferase (ALT), NAFLD, and percent liver fat (PLF) and combined them with genome-wide association study (GWAS) summary statistics from the ENIGMA Consortium. Several methods were used to assess the effect of NAFLD on full cortex and specific brain regions, along with sensitivity analyses.

Results: At the global level, PLF nominally decreased SA of full cortex; at the functional level, ALT presented a nominal association with reduced SA of parahippocampal gyrus, TH of pars opercularis, TH of pars orbitalis, and TH of pericalcarine cortex. Besides, NAFLD presented a nominal association with reduced SA of parahippocampal gyrus, TH of pars opercularis, TH of pars triangularis and TH of pericalcarine cortex, but increased TH of entorhinal cortex, lateral orbitofrontal cortex and temporal pole. Furthermore, PLF presented a nominal association with reduced SA of parahippocampal gyrus, TH of pars opercularis, TH of cuneus and lingual gyrus, but increased TH of entorhinal cortex.

Conclusion: NAFLD is suggestively associated with atrophy in specific functional regions of the human brain.

KEYWORDS

brain regions, surface area, thickness, mendelian randomization analysis, NAFLD

1 Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as one of the most common chronic liver diseases, affecting 32.4% of the world population (1). It is generally defined as a spectrum of diseases, ranging from nonalcoholic fatty liver, nonalcoholic steatohepatitis (NASH), and liver cirrhosis, which can increase the risk of hepatocellular carcinoma (2). In addition, recent studies indicated that NAFLD is a multi-system disease that can affect various organs and systems, including kidney dysfunction (3), cardiovascular diseases (4), and extrahepatic tumors (5).

Neuropsychiatric diseases are considered as one of the manifestations of NAFLD, such as dementia, depression, and impaired brain health. To be more specific, a system review revealed that patients with NAFLD had an overall 1.44-fold increased risk of cognitive impairment compared with healthy controls (6). Moreover, a study demonstrated that NAFLD constitutes an independent risk factor for anxiety and depression. Besides, previous works have demonstrated close interplays between NAFLD and brain health, including smaller total brain volume, decreased cerebral blood blow and greater arterial stiffness (7–9). These findings suggest there are associations between NAFLD and the whole brain. However, it is also important to identify the link between specific brain region and NAFLD, which may know more about the mechanism of liver-brain axis, and help pave the way to the treatment target of dementia.

The human cerebral cortex, the outer gray matter layer of the brain, plays an important role in cognitive function. Surface area (SA) and thickness (TH) are regarded as important indicators of the human cerebral cortex to study the associations between the brain and the neuropsychiatric diseases (10). Given the uncertainty about the effect of NAFLD on the specific brain regions, further studies that explore the potential impact of NAFLD on the health of specific brain region are warranted.

Mendelian randomization (MR) is an analytic method that uses genetic variants as instruments to estimate the causal effect of risk factors on outcomes (11). MR has become an important method in the recent medical literature because it can overcome the limitations of observational analyses, which are often biased by confounding factors. To date, the use of MR has succeeded in assessing causal relationships in the studies of NAFLD, including several risk factors of NAFLD (12, 13) and relationship between NAFLD and other diseases (14, 15). However, to the best of our knowledge, the causal relation between NAFLD and cerebral cortical structure has not been demonstrated yet.

Hence, the present study used human genetic data within the MR framework to reveal the effect of NAFLD on the SA and TH of full cortex. We also carried out subgroup analyses based on specific brain regions. Considering NAFLD is closely associated with alanine transaminase (ALT) and percent liver fat (PLF), we also selected ALT and PLF as exposures. In the end, three sets of parameters: ALT, NAFLD, and PLF, were used to conduct the MR estimates. Our results shed light on the patterns and mechanisms of brain damage caused by NAFLD and provided new insights into the possible existence of a liver-brain axis.

2 Materials and methods

2.1 Exposure data

2.1.1 Alanine transaminase

We obtained the summary statistics of ALT from a recent genome-wide association study (GWAS) by Pazoki Raha et al. (16), which included 437,267 individuals of European ancestry. Of note, the ALT levels were log 10 transformed to approximate normal distribution (corresponding to per 10 times of ALT) in the original article.

2.1.2 Non-alcoholic fatty liver disease

Genetic associations with NAFLD were extracted from the largest GWAS meta-analysis to date, which consisted of 8,434 NAFLD cases and 770,180 controls of European ancestry, comprising data from 4 cohorts: Electronic Medical Records and Genomics (eMERGE), UK Biobank, FinnGen and Estonian Biobank (17). In the eMERGE cohort, NAFLD was defined by the use of electronic health record (EHR) codes (ICD-9: 571.5, ICD9: 571.8, ICD-9: 571.9, ICD-10: K75.81, ICD-10: K76.0 and ICD-10: K76.9). In the UK Biobank and Estonian Biobank, NAFLD diagnosis was established from hospital records (ICD-10: K74.0 and K74.2 [hepatic fibrosis], K75.8 [non-alcoholic steatohepatitis], K76.0 [NAFLD] and K76.9 [other specified diseases of the liver]). In the FinnGen Consortium, NAFLD was defined by EHR code K76.0.

2.1.3 Percent liver fat

Genetic associations with PLF were extracted from a GWAS from a cohort (18) that consisted of 32,858 European participants from the UK Biobank. The cohort used deep learning to process over 38,000 abdominal MRI scans to quantify volume, fat, and iron in seven organs and tissues, including the liver. The GWAS of PLF adjusted for several covariates, including age at imaging visit, age squared, sex, imaging center, scan date, scan time, genotyping batch, and genetic relatedness.

2.2 Outcome data

We obtained the GWAS data for SA and TH from the ENIGMA Consortium (19). The ENIGMA Consortium conducted a genome-wide association meta-analysis study on cortical structures, which included the SA and TH of the full cortex, as well as SA and TH for thirty-four brain cortical regions with known functional specializations. The thirty-four brain regions were defined using the Desikan-Killiany cortical atlas, and established coarse partitions of the cortex. The SA and TH were measured using MRI in 51,665 individuals from 60 cohorts around the world, with approximately 94% of European descent. Both SA and TH of brain regions were weighted by the entire brain, indicating the SA and TH of specific regions across the SA and TH of the entire brain. These data can be accessed at https://enigma.ini.usc.edu/research/download-enigma-gwas-results/. All GWASs data used in the study are shown in Table S1.

2.3 Instrumental variable selection

To identify the causal relationship between NAFLD and the cerebral cortical structure, we used three sets of genetic instruments, including: i) index Single Nucleotide Polymorphisms (SNP) representing ALT, ii) index SNPs representing NAFLD, and iii) index SNPs representing PLF. Genetic instruments were selected via the following criteria: i) a GWAS-correlated P-value of 5×10^{-8}, ii) the minor allele frequency (MAF) threshold of the variants of interest was 0.01, iii) a linkage disequilibrium (LD) r^2 of < 0.001, and < 10 MB from the index variant, iv) an F statistic of 10 was regarded as sufficiently robust to counteract weak instrument bias. Finally, when no SNP in the outcome dataset met this criterion, proxy SNPs with LD set at $r^2 > 0.8$ were used. The study flow chart is presented in Figure 1.

2.4 Ethics

This study used publicly available data from participant studies that were approved by an ethical standards committee with respect to human experimentation. No separate ethical approval was required in this study.

2.5 MR analysis

Three different methods of MR [inverse-variance weighted (IVW), MR Egger, and weighted median] were performed to address variant heterogeneity and the pleiotropy effect. IVW was used as the main analysis, because it is reported to be slightly more powerful than other methods under certain conditions (20). However, IVW assumes that all genetic variants are valid instruments (21), which may not be true in practice. Therefore, MR-Egger and weighted median were used as complements to improve the IVW estimates as they could provide more robust estimates in a broader set of scenarios. MR-Egger method allows all genetic variants to have pleiotropic effect but requires that the pleiotropic effects be independent of the variant-exposure association (22). Weighted median allows for the use of invalid instruments when less than half of the instruments used in the MR analysis are valid (22). We only performed MR-Egger and weighted median when $P_{IVW} < 0.05$. When all methods had consistent β directions, the effect estimates were considered significant (23). For significant estimates, we further assessed horizontal pleiotropy using the MR-PRESSO global test (24, 25). If the SNP was identified by MR-PRESSO outlier test as outliers, it would be removed and then the MR analysis was re-performed. Additionally, as the MR estimate may be biased in the present of invalid instruments, several sensitivity analyses were performed. MR-Egger regression test was used to obtain the intercept, which was an indicator for directional pleiotropy ($P < 0.05$ was considered as the presence of directional pleiotropy) (26). Funnel plots were used to assess the probable pleiotropy and heterogeneity. The Cochran's Q test was also used to evaluate heterogeneity (27).

Additionally, we established a multiple testing significance threshold at different outcome (full cortex, specific brain regions), defined as $P < 0.05/(3\times n)$ (where n is the number of outcomes). Therefore, a P value less than 8.3×10^{-3} (0.05/6) was considered statistically significant in the estimation of SA and TH of full cortex, while a P value less than 2.5×10^{-4} (0.05/204) was considered statistically significant in the estimation of SA and TH of certain brain region. A P value less than 0.05 was considered nominally significant evidence for a potential causal association (23, 28, 29). All analyses were performed using the package TwoSampleMR (30) (version 0.5.6) and package MRPRESSO (25) (version 1.0) in R (version 4.1.3).

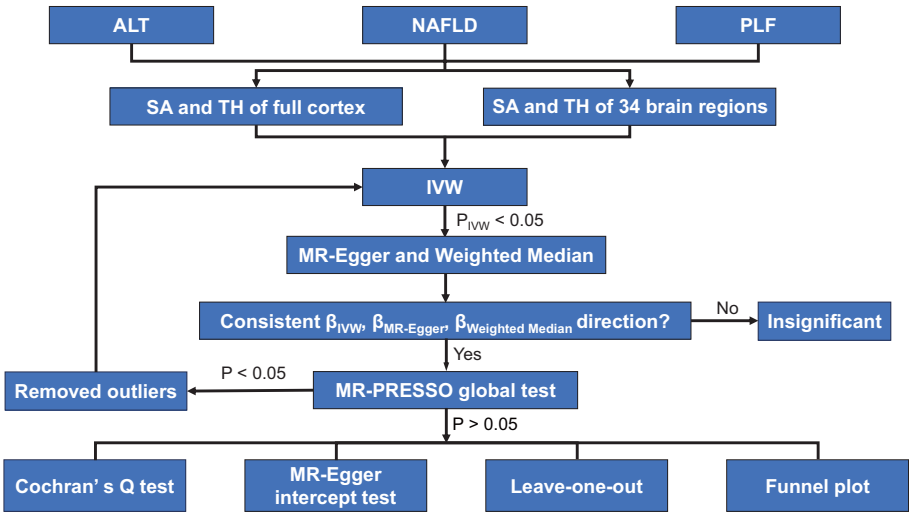

FIGURE 1
Study flame chart of the Mendelian randomization study revealing the causal relationship between alanine transaminase, non-alcoholic fatty liver disease, and percent liver fat and the cerebral cortical structure. ALT, alanine transaminase; IVW, inverse-variance weighted; MR-PRESSO, Mendelian randomization-pleiotropy residual sum and outlier; NAFLD, non-alcoholic fatty liver disease; PLF, percent liver fat; SA, surface area; TH, thickness.

3 Results

In total, 10 index SNPs were selected to genetically predict ALT, 10 index SNPs were used to genetically predict PLF and 4 SNPs predict NAFLD. F statistics for these genetic instruments were all larger than the normally selected value of 10, ranging from 10.6 to 23,115.9, indicating no evidence of no weak instruments (31). SNP rs429358 was overlapped in PLF and NAFLD. SNP rs58542926 was overlapped in ALT and PLF. There was no overlapping between ALT and NAFLD. All the details about the SNPs were shown in the Table S2.

MR analysis was performed to evaluate the causal relationships of NAFLD with SA and TH of brain region and full cortex (Figure 1). Table 1 showed the NAFLD's causal effect on the full cortex. All the results about the main analysis were presented in Table S3 and Figure 2. Table 2 and Figure 3 showed the nominally significant brain regions affected by NAFLD.

As is shown in the Table 1, PLF was found to decrease SA of full cortex (β = -900.7396 mm^2, 95% CI: -1625.1751 mm^2 to -176.3041 mm^2, p = 0.01481) but had no causal relationship with TH (β = -0.0047 mm, 95% CI: -0.0105 mm to 0.0011 mm, p = 0.11007). Heterogeneity was not observed by Cochran's Q test (p = 0.93, Table 3). The P value for MR-Egger intercept is 0.32, indicating there is no pleiotropy (Table 3). ALT had no causal relationship with the SA and TH of full cortex (β_{SA} = -6700.0683 mm^2, 95% CI: -15685.3599 mm^2 to 2285.2232 mm^2, p_{SA} = 0.14387; β_{TH} = -0.0338 mm, 95% CI: -0.0921 mm to 0.0244 mm, p_{TH} = 0.25534). Genetic predicted NAFLD had no causal relationship with the SA and TH of full cortex (β_{SA} = -880.5200 mm^2, 95% CI: -1775.4473 mm^2 to 14.4072 mm^2, p_{SA} = 0.05380; β_{TH} = -0.0019 mm, 95% CI: -0.0076 mm to 0.0038 mm, p_{TH} = 0.51819).

3.1 Causal estimates of genetically predicted ALT on the brain regions

The genetically predicted ALT was nominally associated with reduced SA in the parahippocampal gyrus (β = -60.4594 mm^2, 95% CI: -104.9948 mm^2 to -15.9239 mm^2, p = 0.0078). A similar result was obtained when analyzing index SNPs predicted ALT and TH of several brain regions, including pars opercularis (β = -0.0861 mm, 95% CI: -0.1402 mm to -0.0319 mm, p = 0.00185), pars orbitalis (β = -0.1023 mm, 95% CI: -0.1874 mm to -0.0172 mm, p = 0.01849) and pericalcarine cortex (β = -0.0913 mm, 95% CI: -0.1767 mm to -0.006 mm, p = 0.03603).

3.2 Causal estimates of genetically predicted NAFLD on the brain regions

The genetically predicted NAFLD was nominally associated with reduced SA in the parahippocampal gyrus (β = -5.3315 mm^2, 95% CI: -9.2083 mm^2 to -1.4547 mm^2, p = 0.00703). NAFLD was also found to be nominally associated with reduced TH of cuneus (β = -0.0075 mm, 95% CI: -0.0130 mm to -0.0020 mm, p = 0.00719), lingual gyrus (β = -0.0063 mm, 95% CI: -0.0112 mm to -0.0015 mm, p = 0.01093), pars opercularis (β = -0.0072 mm, 95% CI: -0.0117 mm to -0.0027 mm, p = 0.00171), pars triangularis (β = -0.0058 mm, 95% CI: -0.0109 mm to -0.0008 mm, p = 0.02205), and pericalcarine cortex (β = -0.0086 mm, 95% CI: -0.0141 mm to -0.0031 mm, p = 0.00208). However, there was nominally significant evidence that the NAFLD was associated with increased TH of the entorhinal cortex (β = 0.0251 mm, 95% CI: 0.0093 mm to 0.0410 mm, p = 0.00191), lateral orbitofrontal cortex (β = 0.0062 mm, 95% CI: 0.0003 mm to 0.0121 mm, p = 0.04105) and temporal pole (β = 0.0143 mm, 95% CI: 0.0005 mm to 0.0281 mm, p = 0.04204).

TABLE 1 Mendelian randomization estimates from alanine transaminase, non-alcoholic fatty liver disease and percent liver fat on genetically predicted full cortex.

Exposures Outcomes	Method	β (95%CI)	SE	P value
ALT				
Surface area of full cortex	IVW	-6700.0683 (-15685.3599, 2285.2232)	4584.3320	0.14387
Thickness of full cortex	IVW	-0.0338 (-0.0921, 0.0244)	0.0297	0.25534
NAFLD				
Surface area of full cortex	IVW	-880.5200 (-1775.4473, 14.4072)	456.5955	0.05380
Thickness of full cortex	IVW	-0.0019 (-0.0076, 0.0038)	0.0029	0.51819
PLF				
Surface area of full cortex	IVW	-900.7396 (-1625.1751, -176.3041)	369.6100	0.01481
Thickness of full cortex	IVW	-0.0047 (-0.0105, 0.0011)	0.0030	0.11007

ALT, alanine transaminase; IVW, inverse-variance weighted; NAFLD, non-alcoholic fatty liver disease; PLF, percent liver fat; SE, Standard error.

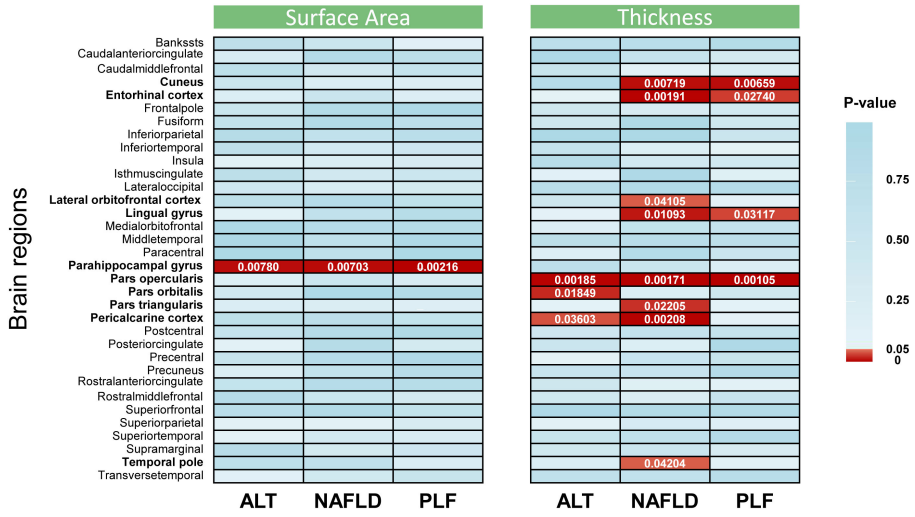

FIGURE 2
Inverse-variance weighted estimates from alanine transaminase, non-alcoholic fatty liver disease, and percent liver fat on cerebral cortical structure. The color of each block represented the p value of each analysis: red blocks indicated $p < 0.05$ and blue blocks indicated $p \geq 0.05$. A p value $< 2.5 \times 10^{-4}$ was considered statistically significant, while a p value < 0.05 was considered nominally significant. The brain regions whose p values were less than 0.05 are highlighted in bold in the left of the figure. ALT, alanine transaminase; NAFLD, non-alcoholic fatty liver disease; PLF, percent liver fat.

TABLE 2 Mendelian randomization estimates from alanine transaminase, non-alcoholic fatty liver disease and percent liver fat on genetically predicted specific brain regions.

Exposures Outcomes	Method	β (95%CI)	SE	P value
ALT				
Surface area of parahippocampal gyrus	IVW	-60.4594 (-104.9948, -15.9239)	22.72216	0.00780
Thickness of pars opercularis	IVW	-0.0861 (-0.1402, -0.0319)	0.02764	0.00185
Thickness of pars orbitalis	IVW	-0.1023 (-0.1874, -0.0172)	0.04343	0.01849
Thickness of pericalcarine cortex	IVW	-0.0913 (-0.1767, -0.006)	0.04356	0.03603
NAFLD				
Surface area of parahippocampal gyrus	IVW	-5.3315 (-9.2083, -1.4547)	1.97796	0.00703
Thickness of cuneus	IVW	-0.0075 (-0.0130, -0.0020)	0.00280	0.00719
Thickness of entorhinal cortex	IVW	0.0251 (0.0093,0.0410)	0.00810	0.00191
Thickness of lateral orbitofrontal cortex	IVW	0.0062 (0.0003,0.0121)	0.00303	0.04105
Thickness of lingual gyrus	IVW	-0.0063 (-0.0112, -0.0015)	0.00249	0.01093
Thickness of pars opercularis	IVW	-0.0072 (-0.0117, -0.0027)	0.00230	0.00171
Thickness of pars triangularis	IVW	-0.0058 (-0.0109, -0.0008)	0.00255	0.02205
Thickness of pericalcarine cortex	IVW	-0.0086 (-0.0141, -0.0031)	0.00280	0.00208
Thickness of temporal pole	IVW	0.0143 (0.0005, 0.0281)	0.00703	0.04204
PLF				
Surface area of parahippocampal gyrus	IVW	-6.0644 (-9.9393, -2.1895)	1.97698	0.00216
Thickness of cuneus	IVW	-0.0077 (-0.0132, -0.0021)	0.00282	0.00659
Thickness of entorhinal cortex	IVW	0.0246 (0.0027, 0.0465)	0.01115	0.02740
Thickness of lingual gyrus	IVW	-0.0063 (-0.0119, -0.0006)	0.00290	0.03117
Thickness of pars opercularis	IVW	-0.0077 (-0.0123, -0.0031)	0.00235	0.00105

ALT, alanine transaminase; IVW, inverse-variance weighted; NAFLD, non-alcoholic fatty liver disease; PLF, percent liver fat; SE, Standard error.

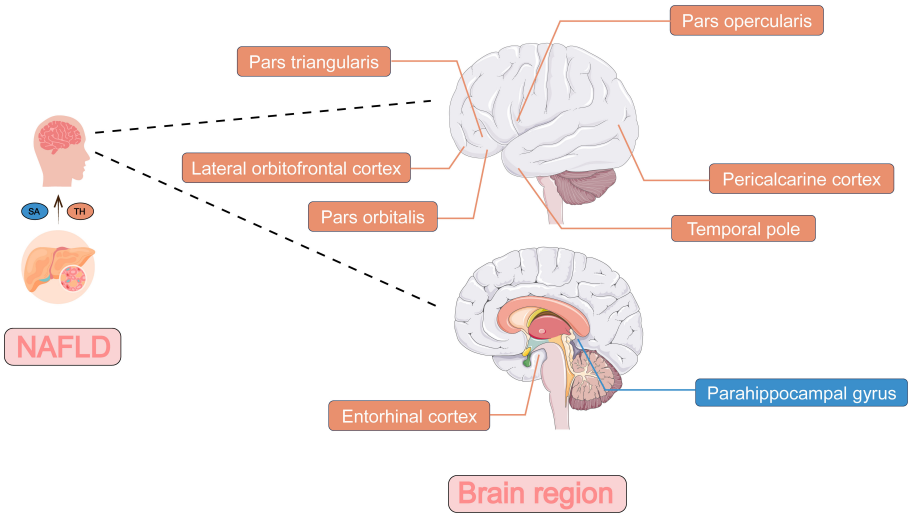

FIGURE 3
The two-sample Mendelian randomization framework showed that NAFLD potentially influenced cerebral cortical structure. The influence on the surface area of brain regions was shown in blue and the influence on the thickness of brain regions was shown in orange. NAFLD, non-alcoholic fatty liver disease; SA, surface area; TH, thickness.

TABLE 3 Heterogeneity and pleiotropy tests of the significant and nominally significant Mendelian randomization estimates.

Exposures Outcomes	Cochrane's Q test		MR–Egger intercept test	
	Q-value	P_Q	Intercept	$P_{Intercept}$
ALT				
Surface area of parahippocampal gyrus	1.88701	0.93	0.38531	0.64
Thickness of pars opercularis	6.1745	0.4	0.00079	0.45
Thickness of pars orbitalis	1.67879	0.95	-0.00064	0.69
Thickness of pericalcarine cortex	11.38044	0.08	0.00295	0.04
NAFLD				
Surface area of parahippocampal gyrus	0.65043	0.72	-1.02464	0.65
Thickness of entorhinal cortex	0.48841	0.78	-0.00475	0.61
Thickness of lateral orbitofrontal cortex	0.1001	0.95	0.00077	0.81
Thickness of pars opercularis	0.18859	0.91	-0.00068	0.79
Thickness of pars triangularis	0.32784	0.85	-0.00088	0.76
Thickness of pericalcarine cortex	0.04032	0.98	0.00039	0.9
Thickness of temporal pole	0.76527	0.68	0.00015	0.98
PLF				
Surface area of full cortex	3.09366	0.93	76.57317	0.32
Surface area of parahippocampal gyrus	2.17483	0.98	-0.41264	0.32
Thickness of cuneus	6.49498	0.59	0.0002	0.73
Thickness of entorhinal cortex	15.07717	0.06	0.00026	0.91
Thickness of lingual gyrus	10.98328	0.2	0.00005	0.93
Thickness of pars opercularis	3.93683	0.86	0.00008	0.86

ALT, alanine transaminase; NAFLD, non-alcoholic fatty liver disease; PLF, percent liver fat.

3.3 Causal estimates of genetically predicted PLF on the brain regions

The PLF was nominally associated with reduced SA of the parahippocampal gyrus (β = -6.0644 mm^2, 95% CI: -9.9393 mm^2 to -2.1895 mm^2, p = 0.00216). In addition, genetic predisposition to PLF was nominally associated with decreased TH in several regions, including the cuneus (β = -0.0077 mm, 95% CI: -0.0132 mm to -0.0021 mm, p = 0.00659), lingual gyrus (β = -0.0063 mm, 95% CI: -0.0119 mm to -0.0006 mm, p = 0.03117), and pars opercularis (β = -0.0077 mm, 95% CI: -0.0123 mm to -0.0031 mm, p = 0.00105). However, a positive association was obtained when analyzing index SNPs predicted PLF and TH of entorhinal cortex (β = 0.0246 mm, 95% CI: 0.0027 mm to 0.0465 mm, p = 0.02740).

3.4 Sensitivity analysis

For both significant and nominally significant estimates, we next performed MR-Egger and weighted median analyses. All of these results were directionally consistent with the IVW analyses except for estimates of NAFLD on the TH of cuneus and lingual gyrus (Table S4), which were considered as insignificant. For the remaining significant and nominally significant estimates, we performed MR-PRESSO global tests, but no horizontal pleiotropy was detected (Table S5). Cochran's Q test, MR-Egger intercept test, leave-one-out analyses, and funnel plot were also performed. Table 3 showed that no heterogeneity was detected (all p_Q > 0.05). Besides, all P-values of MR Egger intercept tests were > 0.05. Scatter plots, leave-one-out analyses and funnel plots were shown in Supplementarys Figure S1–S9. The estimates were not biased by single SNP, indicating that estimates were not violated.

4 Discussion

To the best of our knowledge, our study is the first to determine the causal relationship between NAFLD and the cerebral cortical structure. Our results showed that ALT, PLF, and NAFLD could affect the cerebral cortical structure, and supported the findings of earlier observational studies indicating the pathophysiologic interactions between NAFLD and brain functions, thereby highlighting the existence of the liver-brain axis.

At the global level, we found that genetically predicted PLF was nominally associated with decreased SA of full cortex. To the best of our knowledge, a limited number of studies have published the evaluation of the association between PLF and full cortex. A previous study (8) showed that higher liver fat was associated with decreased total-cerebral blood flow and gray matter- cerebral blood flow, which could be an explanation for our findings. Also, the study revealed that NAFLD was linked with lower total brain volume. However, our findings showed that SNPs predicted NAFLD have no relationship with the SA and TH of full cortex. This could be because only 4 SNPs were used for MR analysis. At the brain region level analysis, the suggestive relationships were mostly about TH of brain regions. This suggested that measuring the TH of brain regions can be a measure to evaluate the extent of damage caused by NAFLD to the brain. Besides, most of the differences in cortical structure observed in intelligence, cognitive function and neuropsychiatric diseases have been reported for TH (32–34), perhaps suggesting that NAFLD causes neuropsychiatric diseases by mediating the destruction of the TH of specific brain regions.

The present study provided evidence that ALT, NAFLD and PLF were all nominally associated with decreased SA of parahippocampal gyrus. The parahippocampal gyrus is an essential site that coordinates with hippocampus (35) to be responsible for memory encoding, storage and retrieval. It has been proved to be vital in the mechanism of several brain diseases and psychiatric condition, such as Posttraumatic stress disorder (36), Alzheimer's disease (37) and schizophrenia (38). Besides, some studies indicated that liver diseases could have impact on parahippocampal gyrus. Jiang et al. (39) showed that people with advanced liver fibrosis had worse cognitive functioning and decreased grey matter in the hippocampus and parahippocampal gyrus. Chen et al. (40) found that patients with cirrhosis tended to damage parahippocampal gyrus and other gray matter regions, and decrease brain microstructural complexity, which may contribute to the cognitive impairment. The underlying mechanism of alterations of parahippocampal gyrus in patients with liver diseases warrants further investigations. Whether NAFLD will lead to changes of parahippocampal gyrus and thus lead to neuropsychiatric disorders could also be expected in the future studies.

Besides, our study found that the TH of pars opercularis is nominally influenced by ALT, NAFLD and PLF. The nominally causal effect of ALT on pars orbitalis and NAFLD on the TH of pars triangularis were also observed. These parts make up the inferior frontal gyrus, which is a key region in language processing and speech production, along with various cognitive functions, such as motor inhibition (41), response inhibition (42), and social cognitive processes (43). Our findings were consistent with previous studies. Chen et al. (44) suggested there is aberrant spontaneous activity of inferior frontal gyrus in the patients with low-grade hepatic encephalopathy. Yang et al. (45) also found a decreased functional connectivity between right dorsolateral prefrontal cortex and inferior frontal gyrus in the patients with cirrhosis. These studies illustrate the connection between inferior frontal gyrus and liver. However, whether NAFLD will lead to these functional changes or neuropsychiatric disorders mediating the alteration of TH of the three parts could also be expected in the future studies.

NAFLD may have an influence on the morbidity of complications in patients with diabetes, involving diabetic retinopathy (46, 47), which indicated the relationship between the liver and the eyes. Our study also indicates a suggestively significant association between ALT and TH of pericalcarine, as well as association between PLF and TH of cuneus. The pericalcarine, the primary visual cortex, processes the visual signals. Also, the pericalcarine can activate the cuneus, which responds to the visual stimuli (48). Considering the NAFLD may have an influence on the pericalcarine and cuneus, the connection between liver and eyes could probably be explained.

Several mechanisms that NAFLD affects brain health are considered and constantly evolving. (1) liver fat may activate microglial cells in the brain by inducing inflammation, and thus resulting in elevated expression of inflammatory cytokines (49); (2) similar with obesity, patients with NAFLD may also increase brain insulin resistance, thereby causing oxidative stress, excessive free fatty acids, and brain mitochondrial disorders (50); (3) impaired liver function can cause insufficient detoxification and allows neurotoxins to enter the cerebral circulation, which can increase permeability of blood–brain barrier and neuroinflammation (51, 52).

Notably, some of our estimates deviated from logical expectation. NAFLD should lead to a smaller TH of the brain regions. However, in our study, genetically predicted NAFLD leads to increased TH of lateral orbitofrontal cortex, temporal pole and entorhinal cortex. Similarly, PLF correlated with larger TH of entorhinal cortex. The possible explanation may be a compensatory hypertrophy or encephaledema. Further studies are needed to investigate the underlying mechanism.

The primary strength of our study is a comprehensive MR study, which can overcome the shortcomings of observational studies. Our study assessed the associations between NAFLD and specific brain regions, and may pave the way to understand the mechanisms that link NAFLD to dementia and other neuropsychiatric diseases. This is essential to achieving more optimized surveillance and providing treatments for patients with NAFLD. However, this study has several limitations. First, the groups in our study were all European, and the conclusions in other populations should be interpreted with caution. Second, the present study did not investigate severity of the changes of brain region. Third, the underlying mechanisms of the change of brain regions warrant further investigation. Future studies should investigate the mechanism underlying the association between NAFLD and neuropsychiatric diseases to explore novel treatments for neuropsychiatric disorders in patients with NAFLD.

5 Conclusion

This is the first comprehensive MR analysis that reveals associations between NAFLD and the cerebral cortical structure. Our estimates illustrate that NAFLD suggestively decreases specific functional regions of the human brain. For patients with NAFLD, a brain MRI could potentially be used for early diagnosis of neuropsychiatric disorders. The mechanisms of the association between NAFLD and brain function alterations should be studied further.

Data availability statement

All GWAS data are available and can be freely downloaded from the IEU OpenGWAS project (https://gwas.mrcieu.ac.uk/), GWAS Catalog project (https://www.ebi.ac.uk/gwas/), and ENIGMA Consortium (https://enigma.ini.usc.edu/).

Ethics statement

This study used publicly available data from participant studies that were approved by an ethical standards committee with respect to human experimentation. No separate ethical approval was required in this study.

Author contributions

HM and ZM proposed the idea and elaborated the research. ZM performed the main data analysis and wrote the draft of the manuscript. HM supervised the whole research and is responsible for the integrity of data analysis. All authors have given consent to the publication of this study.

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Conflict of interest

All authors declare that no potential conflicts of interest are disclosed for this study. All authors have given consent for publication.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1276576/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Scatter plots of nominal significant estimates from genetically predicted alanine transaminase on **(A)** surface area of parahippocampal gyrus; **(B)**

thickness of pars opercularis; **(C)** thickness of pars orbitalis; **(D)** thickness of pericalcarine cortex. The scatter plots represented the instrument variable effects on the exposure and the outcome variables (black point), with the confidence intervals for both estimates denoted by the horizontal and vertical lines, respectively. Each colored slope was indicative of the causal effect of a unit increase in the exposure on the outcome, estimated by the method in the legend utilized to shade the trendline that was, inverse-variance weighted (light blue), weighted median (light green) and MR-Egger (dark blue).

SUPPLEMENTARY FIGURE 2
Leave-one-out plots of nominal significant estimates from genetically predicted alanine transaminase on **(A)** surface area of parahippocampal gyrus; **(B)** thickness of pars opercularis; **(C)** thickness of pars orbitalis; **(D)** thickness of pericalcarine cortex. The leave-one-out analysis was performed by recalculating the Mendelian randomization estimates using the Inverse-variance weighted estimates method, by sequentially dropping one SNP at a time to examine whether a single SNP that might have a large horizontal pleiotropic effect and influence the estimates.

SUPPLEMENTARY FIGURE 3
Funnel plots of significant and nominal significant estimates from genetically predicted alanine transaminase on **(A)** surface area of parahippocampal gyrus; **(B)** thickness of pars opercularis; **(C)** thickness of pars orbitalis; **(D)** thickness of pericalcarine cortex. Funnel plots was used to visualize overall heterogeneity of Mendelian randomization estimates for the effect of exposure on the outcomes before elimination.

SUPPLEMENTARY FIGURE 4
Scatter plots of nominal significant estimates from genetically predicted non-alcoholic fatty liver disease on **(A)** surface area of parahippocampal gyrus; **(B)** thickness of entorhinal cortex; **(C)** thickness of lateral orbitofrontal cortex; (d) thickness of pars opercularis; **(E)** thickness of pars triangularis; **(F)** thickness of pericalcarine cortex; **(G)** thickness of temporal pole. The scatter plots represented the instrument variable effects on the exposure and the outcome variables (black point), with the confidence intervals for both estimates denoted by the horizontal and vertical lines, respectively. Each colored slope was indicative of the causal effect of a unit increase in the exposure on the outcome, estimated by the method in the legend utilized to shade the trendline that was, inverse-variance weighted (light blue), weighted median (light green) and MR-Egger (dark blue).

SUPPLEMENTARY FIGURE 5
Leave-one-out plots of significant and nominal significant estimates from genetically predicted non-alcoholic fatty liver disease on **(A)** surface area of parahippocampal gyrus; **(B)** thickness of entorhinal cortex; **(C)** thickness of lateral orbitofrontal cortex; **(D)** thickness of pars opercularis; **(E)** thickness of pars triangularis; **(f)** thickness of pericalcarine cortex; **(G)** thickness of temporal pole. The leave-one-out analysis was performed by recalculating the Mendelian randomization estimates using the Inverse-variance weighted estimates method, by sequentially dropping one SNP at a time to examine whether a single SNP that might have a large horizontal pleiotropic effect and influence the estimates.

SUPPLEMENTARY FIGURE 6
Funnel plots of significant and nominal significant estimates from genetically predicted non-alcoholic fatty liver disease on **(A)** surface area of parahippocampal gyrus; **(B)** thickness of entorhinal cortex; **(C)** thickness of lateral orbitofrontal cortex; **(D)** thickness of pars opercularis; **(E)** thickness of pars triangularis; **(F)** thickness of pericalcarine cortex; **(G)** thickness of temporal pole. Funnel plots was used to visualize overall heterogeneity of Mendelian randomization estimates for the effect of exposure on the outcomes before elimination.

SUPPLEMENTARY FIGURE 7
Scatter plots of significant estimates from genetically predicted percent liver fat on **(A)** surface area of full cortex; **(B)** surface area of parahippocampal gyrus; **(C)** thickness of cuneus; **(D)** thickness of entorhinal cortex; **(E)** thickness of lingual gyrus; **(F)** thickness of pars opercularis. The scatter plots represented the instrument variable effects on the exposure and the outcome variables (black point), with the confidence intervals for both estimates denoted by the horizontal and vertical lines, respectively. Each colored slope was indicative of the causal effect of a unit increase in the exposure on the outcome, estimated by the method in the legend utilized to shade the trendline that was, inverse-variance weighted (light blue), weighted median (light green) and MR-Egger (dark blue).

SUPPLEMENTARY FIGURE 8
Leave-one-out plots of significant and nominal significant estimates from genetically predicted percent liver fat on **(A)** surface area of full cortex; **(B)** surface area of parahippocampal gyrus; **(C)** thickness of cuneus; **(D)** thickness of entorhinal cortex; **(E)** thickness of lingual gyrus; **(F)** thickness of pars opercularis. The leave-one-out analysis was performed by recalculating the Mendelian randomization estimates using the Inverse-variance weighted estimates method, by sequentially dropping one SNP at a time to examine whether a single SNP that might have a large horizontal pleiotropic effect and influence the estimates.

SUPPLEMENTARY FIGURE 9
Funnel plots of significant and nominal significant estimates from genetically predicted percent liver fat on **(A)** surface area of full cortex; **(B)** surface area of parahippocampal gyrus; **(C)** thickness of cuneus; **(D)** thickness of entorhinal cortex; **(E)** thickness of lingual gyrus; **(F)** thickness of pars opercularis. Funnel plots was used to visualize overall heterogeneity of Mendelian randomization estimates for the effect of exposure on the outcomes before elimination.

SUPPLEMENTARY TABLE 1
Case definition and exclusion criteria in the GWASs used in the present study.

SUPPLEMENTARY TABLE 2
Details about Single Nucleotide Polymorphisms used as exposures.

SUPPLEMENTARY TABLE 3
Inverse-variance weighted estimates of the effect of alanine transaminase, non-alcoholic fatty liver disease and percent liver fat on brain.

SUPPLEMENTARY TABLE 4
Inverse-variance weighted, MR-Egger and weighted median estimates of the significant and nominally significant Mendelian randomization estimates.

SUPPLEMENTARY TABLE 5
MR-PRESSO estimates of the significant and nominally significant Mendelian randomization estimates.

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Analysis of the mediating role of BMI in associations of different folate forms with hepatic steatosis and liver fibrosis in adolescents in the USA: results from the NHANES 2017-2018

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Background: Most studies have explored the relationship between serum total folate and nonalcoholic fatty liver disease (NAFLD) in adults, but there has been no study on the relationship between different folate forms and hepatic steatosis or liver stiffness in adolescents.

Objective: To investigate the association of different folate forms with hepatic steatosis or liver stiffness in adolescents, and further explore the intermediary role of BMI in this relationship.

Methods: The cross-sectional study included 549 participants from the 2017-2018 National Health and Nutrition Inspection Survey (NHANES) survey cycle who had complete data. Four folate data (red blood cell folate, serum total folate, 5-methyl-tetrahydrofolate and folic acid) were included in our study. Controlled attenuation parameters (CAP) and liver stiffness came from the results of liver ultrasound transient elastography. We used linear regression to analyze the relationship between different forms of folate and CAP or liver stiffness, and logistic regression to analyze the relationship between different forms of folate and NAFLD or significant fibrosis. We also used restricted cubic splines to analyze the nonlinear relationship between different forms of folate and NAFLD or significant fibrosis. Finally, we used regression-based intermediary analysis to distinguish the direct and BMI-mediated effects of folate on CAP or liver stiffness. All the analyses adjusted the relevant covariates.

Results: The means of CAP and liver hardness in this study were 223.02dB/m and 5.03kPa, respectively. We found that in model 2, there was a negative correlation between serum total folate (β: -18.53; 95%CI: -29.32 to -7.73) or 5-methyltetrahydrofolate (β: -14.13; 95%CI: -28.98 to -7.86) and CAP. However,

when the BMI was further adjusted in model 3, this negative correlation no longer existed (serum total folate: β: -8.36; 95%CI: -17.69 to 0.97; 5-methyltetrahydrofolate: β: -8.05; 95%CI: -17.19 to 1.09). Similarly, we found a negative correlation between serum total folate or 5-Methyl-tetrahydrofolate and liver stiffness in model 2. There was no significant correlation between red blood cell folate or folic acid and CAP or liver stiffness in either model 2 or model 3. The nonlinear relationship between different folate forms and NAFLD or significant fibrosis was not significant. It is estimated that 76% of the total association between serum total folate and CAP is mediated by BMI. The mediating proportion of BMI in the total correlation between serum total folate and liver stiffness was 50%. Similarly, we found that BMI significantly mediated the relationship between 5-Methyl-tetrahydrofolate and CAP or liver stiffness, with a mediating ratio of 77% and 49%, respectively.

Conclusion: Our results show that serum total folate or 5-Methyl-tetrahydrofolate are negatively correlated with hepatic steatosis or liver stiffness in adolescents, and BMI plays major mediating role in this relationship. Our findings emphasize the importance of monitoring the concentration of serum folate, not just the serum total folate concentration.

KEYWORDS

folate, hepatic steatosis, liver fibrosis, BMI, NAFLD, significant fibrosis

Introduction

Obesity is a major public health problem, with an obesity rate of 20.9% among adolescents aged 12-19 years in the United States (1). The prevalence of obesity has increased among all young people over the past few decades, leading to fatty liver disease becoming the most common liver disease in the U.S. adolescent population (2). Fatty liver disease is known to affect the long-term health of the liver and can lead to liver fibrosis and cirrhosis in adolescents (3). Despite the emergence of fatty liver as a public health problem, adolescent fatty liver has not been fully diagnosed due to lack of screening and awareness of potential long-term complications among health care providers (4, 5). Instantaneous elastography may be a useful tool for assessing adolescents at high risk for fatty liver disease due to obesity or other components of metabolic syndrome. Instantaneous elastography can simultaneously measure liver stiffness and control attenuation parameters (CAP), which has a good diagnostic accuracy for early detection of liver diseases in high-risk adolescents (6, 7).

At present, weight loss is still an important way to prevent nonalcoholic fatty liver disease (NAFLD). But losing weight is challenging to achieve and even more challenging to maintain, with only 20% of obese people able to do so (8). Therefore, drug approaches are being actively sought to reverse liver steatosis. Folate is a water-soluble vitamin B9 that plays an important role in single-carbon metabolism and methylation reactions (9). Some animal studies have shown that folate supplementation can reduce liver steatosis (10, 11). The blood contains various forms of folate, including 5-methyl-tetrahydrofolate, folic acid, 5-formyl-tetrahydrofolate, tetrahydrofolate and 5,10-methenyl-tetrahydrofolate. A case-control study showed that severe NAFLD in obese subjects was associated with lower serum folate concentrations (12). Two cross-sectional studies have shown that elevated serum folate may be negatively correlated with NAFLD in adults (13, 14). Only one cross-sectional study explored the relationship between different forms of folate and NAFLD in adults (15). To our knowledge, no current studies have investigated the relationship between different folate forms and CAP or liver stiffness in the adolescent population. Furthermore, several studies have shown that lower serum folate concentrations are associated with higher body mass index (BMI) (16–18).

Therefore, the aim of this study was to explore the relationship between the concentration of different forms of folate and CAP or liver stiffness in adolescents and the mediating role of BMI in this relationship.

Abbreviations: BMI, Body mass index; CAP, controlled attenuation parameters; CI, confidence interval; NHANES, the National Health and Nutrition Examination Survey; OR, odds ratio; nonalcoholic fatty liver disease (NAFLD); VCTE, vibration-controlled transient elastography; UMFA, unmetabolized folic acid.

Methods

Study population

The study analyzed the National Health and Nutrition Examination Survey (NHANES) data from the survey cycle of

2017 to 2018. The data was analyzed from December 2022 to May 2023. The NHANES is a nationally representative cross-sectional study. The NHANES has been approved by the Institutional Review Board of the National Institute for Nutrition and Health, and all data are accessible on https://www.cdc.gov/nchs/nhanes/index.htm. Among the 9254 participants in the 2017-2018 survey cycle, a total of 5494 participants have complete vibration-controlled transient elastography (VCTE) data. After excluding 4510 adults (≥ 20 years old), 314 participants with missing folate data and 121 participants with missing covariant data, the final cohort consists of 549 participants with complete data (Figure 1).

Measurement of liver stiffness and hepatic steatosis

The NHANES examined all participants aged 12 and over with VCTE. If the participant (1) cannot lie on the examination table, (2) is pregnant, (3) has an implanted electronic medical device, (4) wears a bandage or a lesion near the ribs in the right abdomen or (5) refuses the examination or experiences a limited period of time during the examination, it is considered ineligible to undergo liver elastography. Participants were examined to evaluate CAP scores and liver stiffness measurements using the FibroScan model 502 V2 Touch equipped with a medium (M) or extra large (XL) wand (probe). If they have < 10 complete liver stiffness measurements or a liver stiffness interquartile (IQR) range/median ≥ 30%, or a fasting time < 3 h, transient elastography results are considered incomplete. The NHANES reported that the reliability of inter-observer CAP score was 0.94 and the reliability of liver stiffness test was 0.861 (19).

Firstly, the CAP and liver stiffness were analyzed as continuous variables. Then we defined NAFLD as the CAP score greater than or equal to 263dB/m (≥ S1) (20). Liver stiffness greater than or equal to 8kPa is considered to have significant fibrosis (≥F2) (21–23). We excluded participants with hepatitis B or hepatitis C and heavy drinkers(n=6) when defining NAFLD.

Measurements of different folate forms

Serum and whole blood samples were collected by venipuncture and analyzed in the nutrition biomarker laboratory of the Centers for Disease Control and Prevention. In NHANES, five serum folate forms (5-methyl-tetrahydrofolate, folic acid, 5-formyl-tetrahydrofolate, tetrahydrofolate and 5,10-methenyl-tetrahydrofolate) were tested by liquid chromatography tandem mass spectrometry. The above five forms of folate were added together to calculate the total serum folate. The concentrations of serum total folate and whole blood folate were determined by microbiological method, and red blood cell folate was calculated. 5-methyl-tetrahydrofolate is the main bioactive form of serum total folate. The folic acid in our study refers to unmetabolized folic acid (UMFA) in the serum.The presence of UMFA in circulation may increase pro-inflammatory markers (24), reduce the cytotoxicity of natural killer cells, and damage DNA hydroxymethylation (25). Therefore, we speculate that the potential side effects of UMFA may mask the benefit of 5-methyltetrahydrofolic acid to NAFLD, and the relationship between serum folate and NAFLD may be different according to the form of serum folate. Therefore, we included red blood cell folate, serum total folate, 5-methyltetrahydrofolate and

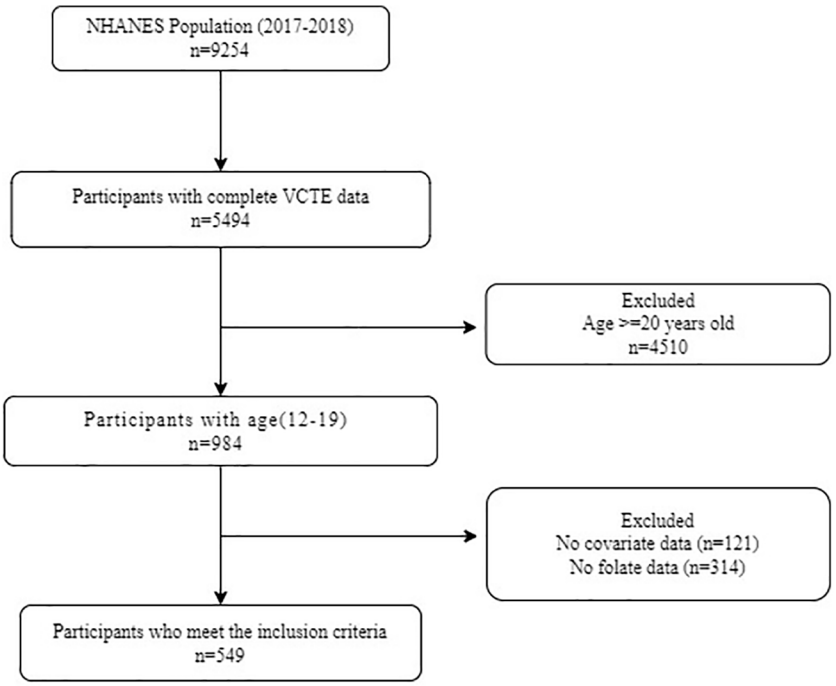

FIGURE 1
Flow diagram of study participants. NHANES, National Health and Nutrition Examination Survey; VCTE, vibration controlled transient elastography,.

folic acid in the analysis. Except in restricted cubic splines, different forms of folate concentrations were naturally logtransformed to minimize the effect of outliers and to improve the interpretation of associational results.

Covariates

Demographic covariates are obtained through self-reported questionnaires, including age, gender, race/ethnicity and income-to-poverty ratios. The income-to-poverty ratios is the ratio of household income to poverty. Trained health technicians measured the height and weight of the participants. Overweight is defined as BMI between the 85th and 95th percentile, and obesity is defined as BMI ≥ 95th percentile. Set the normal level as the reference level. In NHANES, participants' smoking status (now, ever and never) was self-reported. The participants' blood samples were sent to the NHANES laboratory for analysis to get the value of total cholesterol. Data on total dietary energy intake were obtained from a 24-hour dietary survey.

Statistical analysis

The baseline feature is shown as mean ± SE of continuous variables or the percentage of classified variables. The baseline characteristics were compared according to the quartile of serum total folate, and the chi-square test of category variables or analysis of variance of continuous variables were used to analyze the differences.

We used linear regression models to evaluate the relationship between different folate forms and CAP or liver stiffness. The regression model is not adjusted at first, and then gradually adjusted according to the following factors: (1) age, sex, race/ethnicity and income-poverty ratio; (2) smoking status, total cholesterol and total energy intake; (3) BMI. Confounding factors were selected based on known CAP risk factors and folate-related risk factors in our data set. Then, we divided folate levels into four equal parts, and used the first quarter group as a reference category in the regression model to reanalyze the relationship between different folate forms and CAP or liver stiffness. We also used logistic regression to analyze the relationship between different folate forms and NAFLD or significant fibrosis including the above covariables in the adjusted models, and calculated the adjusted odds ratio (OR) and 95% confidence interval (CI).

To evaluate the potential nonlinear relationship between different folate forms and NAFLD or significant fibrosis, we performed a restricted cubic curve analysis (26). Three nodes located in the 5th, 50th and 95th percentiles of different folate forms are used in restricted cubic spline. Through the test of the spline, it is concluded whether there is a significant non-linear correlation.

We used regression-based intermediary analysis to distinguish between direct and BMI-mediated effects of folate on CAP or liver stiffness. Three estimates are as follows: (1) overall effects, that is, the overall association between folate and CAP or liver stiffness, including those mediated by BMI; (3) direct effects, that is, the association between folate and CAP or liver stiffness, adjusted according to BMI; and (3) indirect effects, that is, the association between folate and CAP or liver stiffness, mediated by BMI. In this study, the regression model of intermediary analysis is adjusted for all covariables.

We have done the sensitivity analysis in the following aspect. In order to test the independent correlation between 5-methyl-tetrahydrofolate or folic acid and NAFLD or significant fibrosis, we further mutually adjusted 5-methyl-tetrahydrofolate and folic acid. Use R (version 4.0.4) for analysis. The significance threshold was 0.05 and the bilateral P value was reported.

Results

Baseline characteristic of participants

A total of 549 participants were included in the final analysis, with an average age of 15.6 years old, of whom 34.4% were male. The means of CAP and liver stiffness were 223.02dB/m and 5.03kPa, respectively. The characteristics of participants classified by serum total folate quartile are shown in Table 1. We found that participants with higher serum total folate concentrations included younger, non-Hispanic white people, people who never smoked and had a low incidence of obesity. Similarly, we found that participants with higher serum total folate concentrations had lower CAP and liver stiffness (Table 1).

Relationship between different folate forms and CAP

We first analyzed the relationship between different folate forms and CAP. In model 2, there was a negative correlation between serum total folate (β: -18.53; 95%CI: -29.32 to -7.73) or 5-methyltetrahydrofolate (β: -14.13; 95%CI: -28.98 to -7.86) and CAP. However, when the BMI was further adjusted in model 3, this negative correlation no longer existed (serum total folate: β: -8.36; 95%CI: -17.69 to 0.97; 5-methyltetrahydrofolate: β: -8.05; 95%CI: -17.19 to 1.09). There was no significant correlation between red blood cell folate or folic acid and CAP in either model 2 or model 3. After changing folate from a continuous variable to a classified variable (quartile), we get the same results (Table 2).

We also discussed the relationship between folate and the prevalence of NAFLD in adolescents. In model 2, higher serum total folate (OR: 0.49; 95%CI: 0.29 to 0.81) or 5-methyltetrahydrofolate (OR: 0.48; 95%CI: 0.29 to 0.78) was associated with less NAFLD disease, regardless of whether serum total folate was a continuous variable or a classification variable (quartile). After adding BMI to the model, this correlation disappeared (serum total folate: OR: 0.71; 95%CI: 0.38 to 1.31; 5-methyltetrahydrofolate: OR: 0.69; 95%CI: 0.37 to 1.26). Similarly, we did not find this relationship in the analysis of red blood cell folate and folic acid (eTable 1).

We also used restricted cubic splines to analyze the nonlinear relationship between different folate forms and the prevalence of NAFLD, but did not find a significant nonlinear correlation between them (eFigures 1–4).

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TABLE 1 Basic characteristics of the study participants.

Characteristics	Overall	Serum total folate, nmol/L				*P*
		Q1 [6.15, 27.70)	Q2 [27.70, 37.60)	Q3 [37.60, 50.70)	Q4 [50.70, 113.00]	
Participants	549	138	137	137	137	
Age, year						<0.001
Mean ± SE	15.6 ± 1.5	16.1 ± 1.4	15.9 ± 1.5	15.7 ± 1.5	14.7± 1.5	
Gender (%)						0.893
Male	189 (34.4)	47 (34.1)	48 (35.0)	50 (36.5)	44 (32.1)	
Female	360 (65.6)	91 (65.9)	89 (65.0)	87 (63.5)	93 (67.9)	
Race/ethnicity (%)						0.060
Non-Hispanic White	190 (34.6)	44 (31.9)	35 (25.5)	55 (40.1)	56 (40.9)	
Non-Hispanic Black	102 (18.6)	31 (22.5)	31 (22.6)	23 (16.8)	17 (12.4)	
Mexican American	115 (20.9)	30 (21.7)	30 (21.9)	25 (18.2)	30 (21.9)	
Other Hispanic	33 (6.0)	7 (5.1)	11 (8.0)	3 (2.2)	12 (8.8)	
Other Race	109 (19.9)	26 (18.8)	30 (21.9)	31 (22.6)	22 (16.1)	
Income-poverty ratio (%)						0.280
≤ 2	322 (58.7)	82 (59.4)	83 (60.6)	71 (51.8)	86 (62.8)	
> 2	227 (41.3)	56 (40.6)	54 (39.4)	66 (48.2)	51 (37.2)	
Total cholesterol, mg/dL						0.268
Mean ± SE	156 ± 5	154 ± 6	153 ± 5	160 ± 5	156 ± 6	
Total energy, kcal/day						0.479
Mean ± SE	1846 ± 27	1828 ± 27	1838 ± 27	1821 ± 27	1898 ± 26	
Smoking (%)						0.040
never	535 (97.4)	129 (93.5)	134 (97.8)	136(99.3)	136 (99.3)	
ever	5 (0.9)	3 (2.2)	1 (0.7)	1 (0.7)	0 (0)	
now	9 (1.6)	6 (4.3)	2 (1.5)	0 (0)	1 (0.7)	
BMI (%)						0.005
normal	310 (56.6)	72 (52.2)	77 (56.2)	67 (48.9)	94 (68.6)	
overweight	96 (17.5)	24 (17.4)	18 (13.1)	31 (22.6)	23 (16.8)	
obesity	143 (26.0)	42 (30.4)	42 (30.7)	39 (28.5)	20 (14.6)	
CAP, dB/m						0.002
Mean ± SE	223.02 ± 7.50	229.59 ± 7.75	227.88 ± 7.35	225.91 ± 7.35	208.66 ± 7.41	
Liver Stiffness, kPa						0.006
Mean ± SE	5.03 ± 1.83	5.66 ± 2.42	5.13 ± 1.58	4.71 ± 1.08	4.62 ± 1.26	

BMI, body mass index; CAP, Controlled Attenuation Parameter.

Relationship between different folate forms and liver stiffness

Except for the edge significance of model 2, the relationship between different folate forms and liver stiffness was similar to that between different folate forms and CAP (Table 3). Similarly, we found a negative correlation between serum total folate or 5-Methyl-tetrahydrofolate and significant fibrosis (eTable 2). The nonlinear relationship between different folate forms and significant fibrosis was not significant (eFigures 5–8).

Mediating role of BMI

Because the adjustment of BMI in model 3 masked the correlation between serum total folate or 5-methyl-

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TABLE 2 Associations of different folate forms with CAP among teenagers aged 12-19 years(n=549).

Folate	Unadjusted model			Adjusted model								
				Model 1 [a]			Model 2 [b]			Model 3 [c]		
	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*
Red blood cell folate (continuous), nmol/L	3.75	-11.31 to 18.82	0.626	2.61	-12.49 to 17.71	0.735	1.81	-13.30 to 16.91	0.815	3.28	-9.53 to 16.09	0.616
Red blood cell folate (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	0.29	-12.98 to 13.56	0.966	-1.46	-14.54 to 11.63	0.828	-2.50	-15.66 to 10.66	0.710	-2.03	-13.20 to 9.13	0.721
Q3	13.73	0.47 to 27.01	0.043	9.57	-3.70 to 22.85	0.158	8.44	-4.86 to 21.73	0.214	7.91	-3.36 to 19.19	0.169
Q4	2.26	-11.01 to 15.53	0.738	1.23	-12.00 to 14.46	0.855	-0.11	-13.37 to 13.15	0.987	0.94	-10.31 to 12.20	0.870
P for trend	0.346			0.507			0.639			0.498		
Serum total folate (continuous), nmol/L	-18.81	-29.29 to -8.33	<.001	-16.94	-27.64 to -6.24	0.002	-18.53	-29.32 to -7.73	0.001	-8.36	-17.69 to 0.97	0.080
Serum total folate (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	-1.72	-14.90 to 11.47	0.799	-1.97	-14.93 to 10.98	0.765	-2.63	-15.59 to 10.34	0.691	-1.01	-12.12 to 10.09	0.858
Q3	-3.68	-16.87 to 9.51	0.584	-1.99	-15.01 to 11.03	0.765	-4.09	-17.20 to 9.02	0.541	-2.97	-14.20 to 8.27	0.605
Q4	-20.94	-34.12 to -7.75	0.002	-19.85	-33.23 to -6.47	0.004	-21.09	-34.50 to -7.68	0.002	-7.48	-19.12 to 4.16	0.208
P for trend	0.003			0.007			0.003			0.199		
5-Methyl-tetrahydrofolate (continuous), nmol/L	-18.56	-28.80 to -8.32	<.001	-13.69	-27.26 to -6.34	0.002	-14.13	-28.98 to -7.86	0.001	-8.05	-17.19 to 1.09	0.085
5-Methyl-tetrahydrofolate (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	-1.71	-14.91 to 11.48	0.799	-1.69	-14.63 to 11.26	0.798	-2.07	-15.03 to 10.90	0.755	-1.27	-12.38 to 9.83	0.822
Q3	-2.43	-15.62 to 10.76	0.718	-1.29	-14.34 to 11.75	0.846	-3.49	-16.64 to 9.66	0.603	-2.54	-13.79 to 8.72	0.659
Q4	-20.28	-33.47 to -7.08	0.003	-19.34	-32.70 to -5.99	0.005	-20.19	-33.57 to -6.80	0.003	-7.30	-18.89 to 4.29	0.218
P for trend	0.004			0.009			0.005			0.222		
Folic acid (continuous), nmol/L	-2.44	-10.48 to 5.61	0.553	0.298	-7.71 to 8.30	0.942	0.745	-7.26 to 8.75	0.855	1.44	-5.36 to 8.23	0.679
Folic acid (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	-1.39	-14.71 to 11.93	0.838	-0.216	-13.38 to 12.95	0.974	0.18	-12.97 to 13.34	0.978	-1.19	-12.34 to 9.96	0.834

(Continued)

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TABLE 2 Continued

Folate	Unadjusted model			Adjusted model								
				Model 1 [a]			Model 2 [b]			Model 3 [c]		
	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*
Q3	-5.36	-18.68 to 7.96	0.430	-1.82	-15.08 to 11.43	0.788	-1.11	-14.36 to 12.14	0.870	-3.49	-14.73 to 7.75	0.543
Q4	-6.87	-20.19 to 6.45	0.313	-2.55	-15.84 to 10.75	0.707	-1.74	-15.06 to 11.58	0.798	2.43	-8.87 to 13.73	0.674
P for trend	0.253			0.668			0.762			0.789		

BMI, body mass index; CAP, Controlled Attenuation Parameter; CI, confidence interval.
[a]Adjusted for gender, age, race/ethnicity, income-poverty ratio.
[b]Adjusted for gender, age, race/ethnicity, income-poverty ratio, total energy, total cholesterol, smoking status.
[c]Adjusted for gender, age, race/ethnicity, income-poverty ratio, total energy, total cholesterol, smoking status and BMI.

TABLE 3 Associations of different folate forms with liver stiffness among teenagers aged 12-19 years (n=549).

Folate	Unadjusted model			Adjusted model								
				Model 1 [a]			Model 2 [b]			Model 3 [c]		
	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*
Red blood cell folate (continuous), nmol/L	-0.67	-1.57 to 0.23	0.144	-0.59	-1.51 to 0.32	0.203	-0.34	-1.22 to 0.54	0.455	-0.31	-1.18 to 0.57	0.492
Red blood cell folate (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	-0.46	-1.25 to 0.34	0.259	-0.45	-1.24 to 0.34	0.264	-0.41	-1.18 to 0.36	0.296	-0.40	-1.16 to 0.37	0.310
Q3	-0.15	-0.95 to 0.64	0.706	-0.07	-0.88 to 0.73	0.858	0.15	-0.63 to 0.92	0.712	0.14	-0.63 to 0.91	0.719
Q4	-0.53	-1.32 to 0.27	0.196	-0.44	-1.25 to 0.36	0.280	-0.27	-1.05 to 0.50	0.487	-0.25	-1.01 to 0.52	0.528
P for trend	0.322			0.457			0.818			0.858		
Serum total folate (continuous), nmol/L	-1.13	-1.76 to -0.51	<.001	-1.06	-1.71 to -0.41	0.001	-0.72	-1.35 to -0.09	0.026	-0.57	-1.21 to 0.07	0.079
Serum total folate (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	-0.53	-1.32 to 0.27	0.193	-0.57	-1.36 to 0.22	0.160	-0.37	-1.14 to 0.37	0.335	-0.36	-1.12 to 0.40	0.351
Q3	-0.95	-1.74 to -0.16	0.019	-0.87	-1.66 to -0.07	0.033	-0.53	-1.30 to 0.24	0.177	-0.50	-1.27 to 0.26	0.197
Q4	-1.04	-1.83 to -0.25	0.010	-0.94	-1.76 to -0.13	0.024	-0.69	-1.48 to 0.10	0.087	-0.49	-1.28 to 0.31	0.230
P for trend	0.006			0.017			0.079			0.201		
5-Methyl-tetrahydrofolate (continuous), nmol/L	-1.14	-1.75 to -0.53	<.001	-1.07	-1.70 to -0.44	0.001	-0.73	-1.35 to -0.11	0.021	-0.58	-1.20 to 0.04	0.068

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TABLE 3 Continued

Folate	Unadjusted model			Adjusted model								
				Model 1 [a]			Model 2 [b]			Model 3 [c]		
	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*	β	CI (95%)	*P*
5-Methyl-tetrahydrofolate (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	-0.47	-1.26 to 0.32	0.248	-0.50	-1.29 to 0.29	0.216	-0.33	-1.09 to 0.43	0.394	-0.33	-1.09 to 0.42	0.389
Q3	-0.97	-1.76 to -0.18	0.017	-0.89	-1.68 to -0.09	0.029	-0.56	-1.33 to 0.21	0.154	-0.54	-1.31 to 0.23	0.168
Q4	-1.02	-1.81 to -0.23	0.012	-0.91	-1.72 to -0.09	0.029	-0.67	-1.45 to 0.12	0.097	-0.47	-1.26 to 0.32	0.241
P for trend	0.005			0.017			0.078			0.194		
Folic acid (continuous), nmol/L	-0.10	-0.59 to 0.38	0.673	-0.06	-0.55 to 0.42	0.799	-0.06	-0.52 to 0.41	0.816	-0.05	-0.51 to 0.41	0.828
Folic acid (categorical), nmol/L												
Q1	Ref			Ref			Ref			Ref		
Q2	0.80	0.01 to 1.59	0.049	0.76	-0.04 to 1.55	0.063	0.76	-0.01 to 1.52	0.052	0.73	-0.03 to 1.49	0.059
Q3	0.16	-0.63 to 0.95	0.686	0.18	-0.62 to 0.98	0.654	0.20	-0.56 to 0.98	0.596	0.18	-0.58 to 0.94	0.644
Q4	0.05	-0.74 to 0.84	0.901	0.14	-0.67 to 0.94	0.739	0.14	-0.63 to 0.91	0.722	0.20	-0.57 to 0.97	0.610
P for trend	0.710			0.915			0.927			0.960		

BMI, body mass index; CI, confidence interval; OR, Odds Ratio.
[a]Adjusted for gender, age, race/ethnicity, income-poverty ratio.
[b]Adjusted for gender, age, race/ethnicity, income-poverty ratio, total energy, total cholesterol, smoking status.
[c]Adjusted for gender, age, race/ethnicity, income-poverty ratio, total energy, total cholesterol, smoking status and BMI.

tetrahydrofolate and CAP or liver stiffness, we further analyzed the mediating effect of BMI. All mediating analyses are adjusted based on gender, age, race/ethnicity, income-poverty ratio, total energy, total cholesterol and smoking status. It is estimated that 76% of the total association between serum total folate and CAP is mediated by BMI. BMI also significantly regulated the relationship between serum total folate and liver stiffness. The mediating proportion of BMI in the total correlation between serum total folate and liver stiffness was 50%. Similarly, we found that BMI significantly mediated the relationship between 5-methyl-tetrahydrofolate and CAP or liver stiffness, with a mediating ratio of 77% and 49%, respectively (Tables 4, 5).

Sensitivity analyses

After the mutual adjustment of 5-methyl-tetrahydrofolate and folic acid, the negative correlation between 5-methyl-tetrahydrofolate and CAP was still significant, but the negative correlation between 5-methyl-tetrahydrofolate and liver stiffness was weakened. After further adjustment, there was still no significant correlation between folic acid and CAP or liver stiffness (eTable 3).

Discussion

Our data showed that without further adjustment for BMI in the model, serum total folate and 5-methyl-tetrahydrofolate were independently associated with CAP and liver stiffness, regardless of whether folate form was a continuous or categorical variable. Serum total folate and 5-methyl-tetrahydrofolate were also independently associated with NAFLD and significant fibrosis. However, after further adjustment for BMI, this significance disappeared. We also found that there was no significant correlation between red blood cell folate or folic acid and CAP or liver stiffness, regardless of BMI adjustment.The nonlinear relationships between different forms of folate and NAFLD and significant fibrosis were also not significant. The results of sensitivity analysis also support these conclusions. In mediation analysis, we found that BMI fully mediated the correlation between serum total folate or 5-methyl-tetrahydrofolate and CAP, and

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TABLE 4 Estimated proportion of different folate forms with CAP mediated by BMI.

Measure	Red blood cell folate, nmol/L		Serum total folate, nmol/L		5-Methyl-tetrahydrofolate, nmol/L		Folic acid, nmol/L	
	β (95% CI)	*P* Value	β (95% CI)	*P* Value	β (95% CI)	*P* Value	β (95% CI)	*P* Value
Exposure to mediator	-0.15 (-1.96 to 1.66)	0.874	-2.86 (-4.14 to -1.58)	<.001	-2.87 (-4.13 to -1.63)	<.001	-0.28 (-0.29 to 0.68)	0.569
Mediator to outcome	4.76 (4.19 to 5.34)	<.001	4.76 (4.19 to 5.34)	<.001	4.76 (4.19 to 5.34)	<.001	4.76 (4.19 to 5.34)	<.001
Direct effect	2.83 (-10.22 to 15.42)	0.647	-4.23 (-13.89 to 5.84)	0.412	-4.06 (-13.53 to 5.96)	0.416	2.09 (-3.29 to 7.91)	0.449
Indirect effect	-0.70 (-10.45 to 8.35)	0.882	-13.50 (-20.27 to -6.96)	<.001	-13.57 (-20.17 to -7.14)	<.001	-1.34 (-5.58 to 2.64)	0.553
Total effect	2.13 (-12.30 to 17.30)	0.773	-17.74 (-29.62 to -6.65)	0.002	-17.64 (-29.16 to -6.75)	0.002	0.75 (-6.37 to 7.87)	0.794
Proportion mediated, %	NA		76	0.002	77	0.002	NA	

BMI, body mass index; CI, confidence interval; CAP, Controlled Attenuation Parameter.
Adjusted for Adjusted for gender, age, race/ethnicity, income-poverty ratio, total energy, total cholesterol and smoking status.

partially mediated the correlation between serum total folate or 5-methyl-tetrahydrofolate and liver stiffness.

Numerous studies have shown that serum folate levels are associated with liver steatosis and liver stiffness in adults (13–15, 27), but to date, to our knowledge, no studies have explored the relationship between serum folate and liver steatosis or liver stiffness in adolescents. Our study fills this gap. Folate may also serve as a biomarker or potential treatment for hepatic steatosis and liver stiffness, not only in adults but also in adolescents.

One study conducted in adults showed that red blood cell folate was independently associated with an increased risk of NAFLD (28). Another study showed that higher UMFA concentrations in adults were significantly associated with a higher prevalence of NAFLD (15). We found no such correlation in our study, and we speculate that there are two reasons for this. First, the age of the population we studied was different. Previous studies have linked red blood cell folate to insulin resistance and metabolic syndrome (29). However, insulin resistance and metabolic syndrome are uncommon in adolescents. Also because of younger age, folic acid accumulation is less. Therefore, in our study, red blood cell folate and folic acid were not found to be associated with the prevalence of NAFLD. Second, we define nonalcoholic fatty liver disease differently. The above studies mainly defined NAFLD by the United States fatty liver index or the fatty liver index, whereas our study defined NAFLD by CAP of transient elastography.

The underlying mechanism by which serum folate levels are negatively correlated with hepatic steatosis and liver stiffness can be

TABLE 5 Estimated proportion of different folate forms with liver stiffness mediated by BMI.

Measure	Red blood cell folate, nmol/L		Serum total folate, nmol/L		5-Methyl-tetrahydrofolate, nmol/L		Folic acid, nmol/L	
	β (95% CI)	*P* Value	β (95% CI)	*P* Value	β (95% CI)	*P* Value	β (95% CI)	*P* Value
Exposure to mediator	-0.15 (-1.96 to 1.66)	0.874	-2.86 (-4.14 to -1.58)	<.001	-2.87 (-4.13 to -1.63)	<.001	-0.28 (-0.29 to 0.68)	0.569
Mediator to outcome	0.17 (0.13 to 0.21)	<.001	0.17 (0.13 to 0.21)	<.001	0.17 (0.13 to 0.21)	<.001	0.17 (0.13 to 0.21)	<.001
Direct effect	-0.51 (-1.83 to 0.42)	0.482	-0.48 (-1.08 to -0.06)	0.020	-0.50 (-1.08 to -0.08)	0.014	0.02 (-0.25 to 0.31)	0.890
Indirect effect	-0.03 (-0.68 to 0.27)	0.881	-0.47 (-1.53 to -0.05)	<.001	-0.48 (-1.54 to -0.05)	<.001	-0.04 (-0.30 to 0.12)	0.834
Total effect	-0.53 (-2.22 to 0.46)	0.478	-0.97 (-2.33 to -0.23)	0.004	-0.98 (-2.35 to -0.24)	0.004	-0.03 (-0.25 to 0.22)	0.827
Proportion mediated, %	NA		50	0.004	49	0.004	NA	

BMI, body mass index; CI, confidence interval.
Adjusted for Adjusted for gender, age, race/ethnicity, income-poverty ratio, total energy, total cholesterol and smoking status.

explained in different ways. First, the risk of hepatic steatosis is elevated in the context of folate deficiency, possibly because folate deficiency is associated with increased expression of lipid biosynthesis genes, resulting in disruption of liver lipid metabolism (30). Secondly, some studies have shown that lipid transport is blocked in the liver of folate-deficient animals, thus promoting liver fat accumulation (31–33). Furthermore, the deficiency of one carbon unit of folate binding interferes with purine signaling and accelerates the progression of liver fibrosis (34). In addition, folate regulates microRNA expression in the liver, reduces blood glucose and lipid concentrations, increases insulin sensitivity, and improves liver function (35). Finally, folate deficiency interferes with the fibroblast growth factor path (36).

Folate has a powerful antioxidant function for human health and is able to directly remove reactive oxygen species (37). Oxidative stress may be involved in the pathogenesis of NAFLD by promoting inflammation (38, 39). In addition, several clinical studies have shown that reduced serum folate levels are associated with increased BMI and are associated with insulin resistance (24, 40), which is considered a risk factor for NAFLD. Some studies have also shown that a higher BMI predicts more inflammatory cytokines (41). The above may be the reason why BMI plays a major mediating role in the negative correlation between serum folate concentration and hepatic steatosis and liver stiffness.

Our research has the following advantages. First of all, the population we studied came from a nationally representative cross-sectional survey. Secondly, we use transient elastography to define NAFLD and measure liver stiffness. As a simple, non-invasive and accurate technique, transient elastography is considered as a non-invasive standard tool for the evaluation of liver fibrosis. Transient elastography also included a control attenuation parameter (CAP) score, which measures ultrasonic attenuation associated with hepatic steatosis. Third, we use multiple models and multiple dimensions (continuous variables and classified variables, linear and nonlinear relations and various sensitivity analysis) to prove our conclusion.

However, this study also has some limitations. First of all, due to the limitations of the cross-sectional design of the study, the temporal causality may not be cautious. Therefore, reverse causality and unmeasured residue confusion may prevent causal inferences from the association between different forms of folate levels and hepatic steatosis and liver stiffness. A prospective study is needed to confirm or refute our observations. Second, there are currently no general cutoff guidelines for CAP score and liver stiffness among adolescents. However, the cutoff points for CAP score and liver stiffness we used came from several good studies (3–5). Finally, because there are fewer smokers among teenagers, as a covariable, this will affect our results to some extent. These differences may lead to selection bias. Therefore, our results are carefully interpreted.

Conclusion

Our results show that serum total folate or 5-Methyl-tetrahydrofolate are negatively correlated with hepatic steatosis or liver stiffness in adolescents, and BMI plays major mediating role in this relationship. Our findings emphasize the importance of monitoring the concentration of serum folate, not just the serum total folate concentration. The results of this study can provide guidance for the biomarker effect of serum folate level and drug therapy, so as to prevent hepatic steatosis and liver sclerosis more effectively. Since this study is the first attempt to investigate the relationship between different folate forms and hepatic steatosis or liver stiffness in adolescents, more longitudinal and intervention studies are needed in the future.

Data availability statement

Data described in the article and code book are publicly accessible online via the NHANES website (https://wwwn.cdc.gov/nchs/nhanes/). Analytic code will be made available upon request.

Ethics statement

The studies involving humans were approved by the Institutional Review Board of the National Institute for Nutrition and Health. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

JW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. YF: Data curation, Investigation, Methodology, Writing – original draft. LY: Data curation, Investigation, Methodology, Writing – original draft. KL: Data curation, Investigation, Methodology, Software, Writing – original draft. QX: Data curation, Investigation, Methodology, Writing – original draft. XC: Data curation, Investigation, Methodology, Writing – original draft. JS: Data curation, Investigation, Methodology, Writing – original draft. YZ: Data curation, Investigation, Methodology, Writing – original draft. ZZ: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1273580/full#supplementary-material

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Anti-osteoporotic treatments in the era of non-alcoholic fatty liver disease: friend or foe

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Over the last years non-alcoholic fatty liver disease (NAFLD) has grown into the most common chronic liver disease globally, affecting 17-38% of the general population and 50-75% of patients with obesity and/or type 2 diabetes mellitus (T2DM). NAFLD encompasses a spectrum of chronic liver diseases, ranging from simple steatosis (non-alcoholic fatty liver, NAFL) and non-alcoholic steatohepatitis (NASH; or metabolic dysfunction-associated steatohepatitis, MASH) to fibrosis and cirrhosis with liver failure or/and hepatocellular carcinoma. Due to its increasing prevalence and associated morbidity and mortality, the disease-related and broader socioeconomic burden of NAFLD is substantial. Of note, currently there is no globally approved pharmacotherapy for NAFLD. Similar to NAFLD, osteoporosis constitutes also a silent disease, until an osteoporotic fracture occurs, which poses a markedly significant disease and socioeconomic burden. Increasing emerging data have recently highlighted links between NAFLD and osteoporosis, linking the pathogenesis of NAFLD with the process of bone remodeling. However, clinical studies are still limited demonstrating this associative relationship, while more evidence is needed towards discovering potential causative links. Since these two chronic diseases frequently co-exist, there are data suggesting that anti-osteoporosis treatments may affect NAFLD progression by impacting on its pathogenetic mechanisms. In

the present review, we present on overview of the current understanding of the liver-bone cross talk and summarize the experimental and clinical evidence correlating NAFLD and osteoporosis, focusing on the possible effects of anti-osteoporotic drugs on NAFLD.

KEYWORDS

anti-osteoporotic drugs, non-alcoholic fatty liver disease, denosumab, romosozumab, bisphosphonates, calcitonin, selective estrogen receptor modulators, PTH

1 Introduction

Non-alcoholic fatty liver disease (NAFLD; or metabolic dysfunction-associated fatty liver disease, MAFLD; or metabolic dysfunction-associated steatotic liver disease, MASLD) is one of the most common causes of chronic liver disease worldwide. Indeed, over the past couple of decades NAFLD has grown into the most common chronic liver disease, with prevalence of 17-38% in the general population (1), and 50-75% in patients with obesity and/or type 2 diabetes mellitus (T2DM) (2, 3). NAFLD is determined as steatosis affecting 5% of the liver volume or weight (accumulation of fat in more than 5% of hepatocytes) (4), and encompasses a spectrum of liver diseases, ranging from simple steatosis (non-alcoholic fatty liver, NAFL) and non-alcoholic steatohepatitis (NASH or metabolic dysfunction-associated steatohepatitis, MASH) to fibrosis and cirrhosis with liver failure or/and hepatocellular carcinoma. Notably, based on recent estimates, cirrhosis due to NAFLD is expected to be the leading cause of liver transplantation in the US by 2030 (5). Additionally, the economic burden of NAFLD/NASH on health systems is enormous, while that there is currently no globally approved treatment specifically for NAFLD/NASH (6).

Currently, NAFLD is diagnosed by detecting steatosis (either by imaging or histologically) and by excluding other causes of liver disease, including exclusion of alcoholic liver disease (ALD) which has similar pathologic spectra with NAFLD (in NAFLD the daily alcohol consumption should not exceed 20 g in women or 30 g in men) (7). For the diagnosis, as well as its staging, of NASH and cirrhosis, liver biopsy remains the 'gold standard', which, however, also has limitations since it is an invasive method with possible complications, sampling errors and high cost (8, 9). Of note the renaming of NAFLD as MAFLD or MASLD has recently been proposed, with new proposed diagnostic criteria, namely hepatic steatosis based on histological (biopsy), imaging or biochemical confirmation along with one of the following: (a) overweight/obesity; (b) T2DM; or (c) metabolic dysfunction as indicated by 2 of the following: increased waist circumference, hypertension, elevated triglycerides, low HDL, prediabetes (IGT, IFG), HOMA index>2.5, elevated CRP, which rather put a positive diagnosis and not an exclusion of other causes of liver disease (10).

Osteoporosis is also a chronic disease characterized by decreased bone density and a disruption of the bone's architectural structure, resulting in bone fragility and increased fracture risk (11). Osteoporosis is particularly prevalent in postmenopausal women, where the noted estrogen decrease leads to an increase of the activity of the osteoclasts, increasing their responsiveness to RANKL which binds to RANK on the osteoclast membrane, and resulting in the differentiation of osteoclast precursors into mature osteoclasts (12). The drugs used for the treatment of osteoporosis are antiresorptives (e.g. bisphosphonates, denosumab and raloxifene), bone anabolic agents (e.g. teriparatide and romosozumab) and calcitonin (13). Similar to NAFLD, osteoporosis progresses as a silent disease until an osteoporotic fracture occurs, which also poses a very significant disease-related and socio-economic burden (6).

Recent data have been highlighting potential links between NAFLD and osteoporosis, linking the pathogenesis of NAFLD to the process of bone remodeling. In this context, it is considered that chronic low-grade inflammation plays a crucial role in the pathogenesis of both diseases (14, 15). However, this field is still open and more evidence is needed towards understanding the potential common pathogenetic mechanism(s) and the correlations/links between NAFLD and osteoporosis.

In the present review, we present an overview of the current understanding of the liver-bone cross talk and summarize the experimental and clinical evidence correlating NAFLD and osteoporosis. Since these two diseases frequently co-exist, medications for the treatment of osteoporosis may affect NAFLD progression by impacting on underlying pathogenetic mechanisms/links. As such, herein, we also focus on insights into the possible effects of anti-osteoporotic drugs on NAFLD.

2 Pathogenetic mechanisms linking NAFLD and osteoporosis

Potential common pathogenetic mechanisms linking NAFLD and osteoporosis have been recently described. The pathogenesis of NAFLD was initially described with the 'two-hit' hypothesis, with steatosis, i.e. the accumulation of triglycerides (TAGs) in the liver,

representing the 'first hit' and triggering the expression of pro-inflammatory cytokines (e.g. NF-a, IL-6) which was described as the 'second' hit (16). The latter results to the activation of pro-inflammatory pathways and potential fibrogenesis in the liver. However, the spectrum of mechanisms implicated in NAFLD pathogenesis appears to be much more complex, and, thus, has been more recently described with the "multiple-hit hypothesis" which includes multiple genetic and environmental factors that may result to obesity, insulin resistance, gut microbiome alterations, adipose tissue dysfunction and liver fat accumulation with or without hepatic inflammation (17). For example, hepatic mitochondrial dysfunction leads to endoplasmic reticulum (ER) stress, oxidative stress and the production of reactive oxygen species (ROS), while autophagy and apoptosis also play crucial role in NAFLD (18–20). Of note, even certain gut microbiome modifications appear to trigger the expression of pro-inflammatory cytokines (e.g. IL-6, TNF-α), whilst all the aforementioned mechanisms combined with genetic factors and epigenetic alterations may lead to chronic liver inflammation (17).

In this context, focus has been placed not only on traditional cytokines (e.g. IL-6, TNF-α), but also on additional factors implicated in these pathways such as adipokines (21, 22). Among these, adiponectin is the most abundant and is secreted predominantly by the white adipose tissue. Notably, adiponectin has anti-inflammatory and anti-atherogenic effects, and plays an important role in lipid and glucose metabolism by increasing insulin sensitivity, promoting the oxidation of free fatty acids, decreasing *de novo* synthesis/accumulation of lipids and protecting hepatic cells from apoptosis (23, 24). The effects of adiponectin on the liver are mediated by its receptors (AdipoR1, AdipoR2), interacting with the adaptor protein phosphotyrosine interaction (APPL1). Indeed, AdipoR1 activates the AMP-activated protein kinase (AMPK) and AdipoR2 the peroxisome proliferator–activated receptor-alpha (PPAR-a) signaling, and, thus, through these pathways adiponectin acts against hepatic lipid accumulation and regulates glucose homeostasis. Moreover, through the blockage of nuclear factor kappa (NF-κβ), adiponectin reduces inflammation. Notably, a diet rich in saturated and trans-fats, which directly induces significant hepatic fatty infiltration, has been shown to also reduce the circulating levels of adiponectin (25, 26). Furthermore, data show that adiponectin impedes hepatic fibrosis, by inhibiting platelet-derived growth factor (PDGF) stimulation and downregulating the transforming growth factor beta 1 (TGF-β1) (26), whilst as NAFLD progresses, adiponectin levels appear to decline (27). Interestingly, expression of adiponectin receptors has also been found in bone cells, both osteoblasts and osteoclasts (28). In addition, data from *in vitro* experiments and animal studies support an osteogenic role for adiponectin, by promoting osteoblastogenesis and limiting osteoclastogenesis (29). Accordingly, since NAFLD is associated with decreased adiponectin levels, the adiponectin downstream signaling pathways may favor osteoclast function and bone loss in patients with NAFLD. However, it should be noted that human data do not consistently point towards a favorable effect of adiponectin on bone biology (28, 29). Bacchetta et al. showed a negative association between bone mineral density (BMD) and adiponectin in patients with chronic kidney disease (30), whilst Jürimäe et al. demonstrated a negative correlation between adiponectin and BMD in a group of middle-aged premenopausal women (31). Moreover, a recent case-control study, including 210 postmenopausal women, showed an inverse relationship between serum adiponectin levels and T-score in women with osteoporosis and osteopenia (32). Finally a prospective study by Barbour et al., showed that high adiponectin levels were correlated with a higher fracture risk in men, but not in women (33).

Recent research focus has also been placed on osteocalcin (OC) which is secreted by osteoblasts and constitutes the most abundant non-collagen protein in bone (34). In its uncarboxylated form, OC exerts effects on bone by binding calcium (35), whilst it also plays a role on the pancreas-liver crosstalk and metabolism by promoting directly insulin expression in the pancreas and by increasing GLP-1 and adiponectin expression in adipocytes (36). Conversely, OC expression in osteoblasts is promoted by insulin and adiponectin (37). Several studies have demonstrated an inverse association between NAFLD and serum OC levels, with Yilmaz et al. showing that patients with NAFLD and increased hepatocyte ballooning degree had lower OC levels (38). Furthermore, Yang et al. demonstrated that Korean men with NAFLD had lower BMD and OC levels compared to those without (39), whilst Luo et al. showed that among postmenopausal Chinese women with normal blood glucose levels those with NAFLD had lower OC levels (40). Finally, Fang et al. also revealed that lower serum OC levels was an independent risk factor for NAFLD and progression to NASH (41). Interestingly, the hepatic inflammation observed during the progression of NAFLD due to lipotoxicity and the production of pro-inflammatory (e.g., TNF-α, IL-1, IL-6, IL-17) and prothrombotic factors appears to affect both the pathogenesis of NAFLD and bone tissue metabolism (14). RANKL, RANK and osteoprotegerin (OPG) are osteokines which are expressed in bone cells and regulate bone remodeling (42). The OPG/RANKL balance is highly important for the maintenance of bone health, with denosumab, a RANKL-binding monoclonal antibody, being approved as an anti-osteoporotic treatment (42). Of note, upregulation of the RANK/RANKL pathway induces the expression of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, which, in turn, promote osteoclast activation and bone resorption (43). RANKL binds to RANK on the osteoclast membrane, resulting in the differentiation of osteoclast precursors into mature osteoclasts (12), whilst OPG diminishes osteoclastogenesis and, thus, bone loss by binding to RANKL, preventing the RANK-RANKL activation of osteoclasts. Potential associations between RANKL, OPG and NAFLD have been investigated, with experimental data showing that the hepatic expression of RANKL may be elevated in patients with NAFLD (44). Furthermore, a study by Amrousy et al. in children with obesity and NAFLD showed that these children had both higher TNF-a and IL-6 levels and lower OC, OPG and adiponectin levels compared to the study controls (45). Moreover, Mantovani et al., trying to access bone turnover markers in postmenopausal T2DM patients with and without NAFLD (10 patients with NAFLD and fibrosis, 52 with NAFLD and without fibrosis, and 15 without NAFLD), found that RANKL levels gradually diminished from the study patients without

NAFLD, to those with steatosis and then to those with steatosis and fibrosis, while sclerostin levels were higher in patients with NAFLD (46). Another study by Niksersht et al. in a sample of 57 men with NAFLD and 25 controls demonstrated that patients with NAFLD had lower levels of RANKL and OPG compared to controls, whilst OPG and RANKL gene expression was also reduced, suggesting a potential role in NAFLD pathogenesis (47). Similarly, Hadinia et al. showed that patients with NAFLD exhibited lower plasma RANK levels compared to controls, with diminished mRNA RANK levels (48). A previous study by Yilmaz et al. had also demonstrated that serum OPG levels were lower in patients with NASH compared to controls, suggesting that OPG could be used as a biomarker for NASH (49). A case-control study Niu et al. including T2DM patients with NAFLD (N=367) and without (N=379) NAFLD showed that OPG levels were lower in those with NAFLD (50). Interestingly, Erol et al., trying to investigate whether there is a correlation between OPG and insulin resistance in children with obesity, found that OPG levels were lower in such children, but failed to detect a difference in OPG concentrations between children with both obesity and NAFLD compared to those with obesity without NAFLD (51). Finally, Ayaz et al. have shown that serum OPG levels and carotid intima media thickness (CIMT) were higher in patients with NAFLD, with a positive association between OPG and CIMT in these patients (52). It appears that most of the studies point towards a positive correlation of serum RANKL with NAFLD, while serum OPG decreases with disease severity. Though, high serum OPG and low serum RANKL levels have also been reported in patients with advanced NAFLD-related fibrosis (46). It is difficult to explain this discrepancy, however, it should be noted that the elevation of circulating OPG levels and the decreased RANKL levels could represent a compensatory mechanism to limit the liver damage during the progress of NAFLD to fibrosis.

Of note, OPG and other molecules involved in bone metabolism, such as osteopontin (OPN) have also been associated with the progression of hepatic fatty infiltration to fibrosis (14). Indeed, focus is now placed on OPG which is a member of the TNF-a receptor family and acts as a cytokine by preventing RANK from binding to RANKL (53). In this context, Yang et al., in an attempt to suggest OPG as a noninvasive biomarker for NASH diagnosis and NAFLD progression, found that serum OPG was lower in NASH patients compared to normal controls (54). Similarly, OPN is a glycoprotein that plays a role in bone remodeling, bone matrix mineralization, bone remodeling, cell chemotaxis and cell survival and apoptosis (55). Bertola et al. studied the hepatic expression of OPN and its surface receptor CD44, in patients with obesity, showing that hepatic OPN levels were higher in those with severe steatosis and insulin resistance, suggesting their local implication in the hepatic injury progression (56). Moreover, Gómez-Santos et al. showed that OPN levels are higher in older patients, whilst this finding did not apply to patients with NAFLD where higher OPN levels were noted in younger patients. By also studying OPN deficient mice during aging, this study also showed that in older mice decreased OPN levels resulted in augmented senescence, ER stress, hepatic steatosis, and inflammation (57).

Insulin-like growth factor-1 (IGF-1) is mainly secreted by the liver while is also locally produced in small amounts by bones, affecting positively bone remodeling (58). Yao et al. in their metanalysis demonstrated that IGF-1 levels were decreased in patients with NAFLD compared to healthy controls (59). Moreover, Dichtel et al. showed that decreased IGF-1 levels were correlated with higher histological severity of NAFLD (60). Decreased IGF-1 have been reported in both patients with osteoporosis and NAFLD, indicating the important role of IGF-1 in the liver-bone axis (58).

To this end, Wang et al. investigated the role of IGF-1 in the progression of both NAFLD and osteoporosis (61). Using 48 female mice divided into two groups, WT and fed with high fat diet, they showed that bone loss, deterioration of bone microarchitecture and NAFLD were progressing in parallel. They demonstrated that changes of the TNF-α, IL-6, as well as IGF- 1 and IGFBP-1 levels appear to play crucial roles in the different stages of NAFLD in HFD-fed mice. In particular, they showed that in 24 weeks the levels of TNF-α and IL-6 were higher in mice fed with HFD compared to controls leading to changes in the OPG/RANK/RANKL pathway. They concluded that changes in bone microstructure and BMD regarding the 'second hit' were due to higher levels of TNF-a and IL-6. They also showed that, in 32 weeks, IGF-1 was lower in mice fed with HFD resulting to reduced osteoblast activity, justifying bone changes in the progressive stage of NAFLD (61).In addition to the aforementioned factors, vitamin D deficiency, which has known effects on bone metabolism, seems to also influence NAFLD progression by inducing pro-inflammatory processes and oxidative stress, as well as stimulating the proliferation of stellate cells and the production of pro-fibrotic factors (e.g. PDGF and TGFβ) (14). Indeed, The role of vitamin D deficiency in the progression of NAFLD has been demonstrated in animal models (14), whilst clinical data, such as those from Wang et al., also suggest that 25(OH)-vitamin D levels are lower in patients with NAFLD (62).

Advanced glycation end-products (AGEs), molecules deriving from the glycation of proteins or lipids, seem to play a role in both the pathogenesis of NAFLD and osteoporosis (63, 64). Asadipooya et al. in their review provided a thorough description of how AGEs, through their receptors (RAGE), provoke inflammation, cellular proliferation, and increased oxidative stress that lead to the progression of steatosis to NASH and fibrosis, while vice versa oxidative stress and inflammation trigger the AGEs production (63). Of note, AGEs are also involved in bone metabolism. At low concentrations, AGEs promote osteoblastic activity, but at higher concentrations impair mineralization, induce osteoclastogenesis by upregulating RANKL, restrain osteoblasts' growth, inhibit their differentiation and promote their apoptosis (64). Thus, targeting AGE-RAGE signaling appear to be very promising in preventing the progression of both NAFLD and bone loss.

Finally, emerging data also suggest that the Wnt signaling pathway may contribute to the liver-bone crosstalk. The role of the Wnt signaling pathway in osteogenesis is well-described, with Wnt-derived proteins diminishing apoptosis in osteoblast precursor cells and promoting osteoblast differentiation (65). Similarly, the role of Wnt/beta catenin signaling - where the binding of a Wnt ligand with a surface receptor (Fzd) and a co-receptor (LRP5/6) is responsible for the stabilization of beta/catenin, its nuclear

translocation and Wnt target gene expression - in NAFLD development has been described, as recently reviewed in detail by Harini et al. (66). In this context, the role of the canonical and non-canonical Wnt pathway is considered crucial in the development of NAFLD, with the latter being promoted or suppressed according to Wnt5a binding, whilst NAFLD can induced by the inhibition of the canonical pathway. As such, it is noteworthy that mutations in the Wnt co-receptor low density lipoprotein (LDL) receptor-related protein 6 (LRP6) can provoke NAFLD (66), with Liu et al. demonstrating that LRP6$^{+/-}$ mice were protected against insulin resistance and obesity (67).

3 Studies on NAFLD and osteoporosis links

A number of mostly cross-sectional studies have examined the interrelation between NAFLD and BMD, as presented in Table 1. Of these, several demonstrated that patients with NAFLD had higher risk of osteoporosis. For example, Chen et al. have shown that there is a decrease in the rate of bone production and an increase in the rate of bone resorption in elderly patients with NAFLD relative to individuals without NAFLD (78). Similarly, a recent study by Lee et al. in men older than 50 years showed a higher 10-year probability of a major osteoporotic fracture in those with NAFLD compared to those without, while this association was more pronounced in those with sarcopenia (82). Contrary, there are also studies demonstrating either no correlation or a positive correlation between NAFLD and BMD (Table 1). However, as summarized in Table 1, several limitations make the findings of these studies questionable, particularly since no liver biopsies were performed to conclusively diagnose/stage NAFLD and fractures were self-reported, whilst various confounding factors (e.g. vitamin D levels, metabolic bone markers, other medications) were not accessed/included in the analysis. Meta-analysis data correlating NAFLD and osteoporosis have been presented by Su et al. which showed that NAFLD is associated with decreased BMD and higher risk of osteoporosis or osteoporotic fractures, with male sex potentially being a risk factor for decreased BMD in adults with NAFLD, whilst ethnic disparities appear to be also present between non-Asian and Asian populations regarding both BMD and osteoporotic fractures (87). Moreover, the systematic review and meta-analysis by Pan et al. which included seven eligible studies showed a significant association between NAFLD and the prevalence and risk of osteoporosis or osteoporotic fractures in both men and women (88).

4 Anti-osteoporotic treatments and NAFLD

Several pharmacotherapies, including denosumab, bisphosphonates, teriparatide, raloxifene, calcitonin, and romosozumab, have well-established efficacy in the treatment of osteoporosis, reducing the risk of osteoporotic fractures (89). Given that osteoporosis and NAFLD frequently co-exist, particularly in older adults, such medications against osteoporosis may affect NAFLD progression by impacting on pathogenetic mechanisms/pathways shared by both these chronic diseases (Figure 1).

4.1 Bisphosphonates and NAFLD

Bisphosphonates are a class of anti-osteoclastic drugs which are widely used as a pharmaceutical treatment for osteoporosis, constituting first-line therapeutic choices for osteoporosis. These have a structure like pyrophosphate and act by inhibiting bone resorption and remaining on the bone surface (90). Bisphosphonates are divided in nitrogen-containing and non-nitrogen-containing agents (91), and can be used continuously for 3-5 years. However, the long use of bisphosphonates may have side effects, such as atypical femoral fractures and jaw osteonecrosis (92). To date, no experimental or clinical study has showed that the nitrogen bisphosphonates may impact on NAFLD. However, there are experimental data from Hasuzawa et al. in a NASH mouse model induced by a methionine and choline deficient diet which show that clodronate (a non-nitrogen bisphosphonate which acts as a potent and selective inhibitor of the vesicular nucleotide transporter, VNUT) may improve NASH and diminish hepatic inflammation, steatosis, and fibrosis (93). *In vitro* experiments also showed that clodronate reduced hepatic neutrophil infiltration, hepatocyte apoptosis, and cytokine production, suggesting that VNUT-dependent vesicular ATP release plays a role in aggravating hepatic steatosis (93).

4.2 Selective estrogen receptor modulators and NAFLD

Selective estrogen receptor modulators (SERMS) act as estrogen agonists in the bone tissue, inhibiting the osteoclast activity, and as estrogen antagonists in breast and uterine tissues, thus exerting anti-osteoporotic effects without increasing the risk of breast cancer, as estrogen replacement therapy does; although, they increase the risk for thrombosis and pulmonary emboli (92). Raloxifene hydrochloride is the first SERM used for the treatment of osteoporosis (94), with data from the MORE study showing that it increases BMD in the spine and the femoral neck, whilst decreasing the vertebral fracture risk (95). Bazedoxifene is another SERM which has been approved for the treatment of osteoporosis in post-menopausal women (96); although, it is considered inferior compared to other anti-osteoporotic drugs since it has been shown to augment the lumbar spine BMD, but not the hip BMD. Furthermore, bazedoxifene is combined with estrogens forming a tissue selective estrogen complex (TSEC), which is used to moderate vasomotor symptoms (97). Interestingly, Takamura et al. presented a case report regarding a 53-year-old woman with liver impairment and histologically confirmed NASH after the initiation of raloxifene treatment (98). Matsumura et al. reported a similar case regarding a 70-year-old woman, whose NAFLD deteriorated within three months after starting raloxifene (99). Both these clinical cases suggested that

TABLE 1 Selected studies (in chronological order) on the association between non-alcoholic fatty liver disease (NAFLD) and osteoporosis/bone mineral density (BMD) in humans.

	Study design	Origin	Study population	Methods	Outcome	Limitations
Li et al., 2012 (68)	Cross-sectional	China	7797 participants over 40 years old, (2441 men and 5356 women), 2352 with NAFLD	Questionnaire	Association between NAFLD and osteoporotic fractures in men but not in women	1. no causal inference due to cross-sectional design; 2. self-report, thus asymptomatic fractures could not be reported; 3. no biopsy for the diagnosis of NAFLD; 4. confounding factors, such as dietary calcium intake or serum 25-hydroxyvitamin D that could not be ruled out
Moon et al., 2012 (69)	Cross-sectional	South Korea	481 adult women (216 premenopausal and 265 postmenopausal)	DEXA lumbar BMD	Postmenopausal women without NAFLD had higher lumbar BMD compering to those with NAFLD, therefore there was no difference found in premenopausal women	1. no causal inference due to cross-sectional design; 2. waist circumference measurement to define metabolic syndrome was not available in all patients; 3. no biopsy for the diagnosis of NAFLD
Purnak et al., 2012 (70)	Cross-sectional	Turkey	102 adults patients with NAFLD and 54 healthy controls	DEXA	No correlation between NAFLD and lower BMD. Subgroup analysis demonstrated that women with higher ALT levels had a lower BMD and higher hs-CRP levels	1. no biopsy for the distinguish of NAFLD; 2. conflicting results
Cui et al., 2013 (71)	Cross-sectional	China	224 adults; 99 men (46 with NAFLD, 53 without NAFLD), and 125 women (73 with NAFLD 52 without NAFLD)	DEXA	Men with NAFLD had significantly lower TH and FN BMD and women with NAFLD had Lower right TH BMD	1. cross-sectional design; 2. confounding factors; 3. no liver biopsies
Xia et al., 2016 (72)	Cross-sectional	China	1659 adults (755 men; 904 women)	DEXA	LFC and ALT were inversely associated with lower BMD regarding multiple skeletal sites in middle-aged men, but no association was found in postmenopausal women	1. cross-sectional design; 2. no liver biopsies; 3. sex steroid hormones were not evaluated
Lee et al., 2016 (73)	Cross-sectional	South Korea	6634 adults (3306 men: 1288 with NAFLD; 2018 without NAFLD; 3328 women: 1217 with NAFLD; 2112 without NAFLD)	DEXA	FN BMD was negatively correlated with NAFLD in men and LS BMD was positively correlated with NAFLD in women	1. cross-sectional design; 2. no liver biopsies; 3. confounding factors
Yang et al., 2016 (39)	Cross-sectional	South Korea	859 adult men (249 with and 610 without NAFLD)	DEXA	NAFLD was negatively associated with right TH BMD and serum osteocalcin in Korean men.	1. cross-sectional design; 2. only men; 3. no biopsies; 4. confounding factors
Kim et al., 2017 (74)	Cross-sectional	South Korea	231 adults (160 women and 71 men); 129 with NAFLD	DEXA and transient elastography	Correlation between significant liver fibrosis and lower BMD among patients with NAFLD, using TE	1. cross-sectional deign; 2. no liver biopsies; 3. difficulties in the interpretation of elastography; 4. no bone turnover markers; 5. use of hormonal replacement therapy, HOMA-IR index and CRP levels were not accessed
Ahn et al., 2018 (75)	Cross-sectional	South Korea	4264 adults (1908 men 2356 women)	DEXA and FLI	Correlation between high FLI with lower BMD in men (TH, FM and whole body BMD)	1.cross-sectional design; 2. FLI, no biopsies where used; 3. FLI index differentiation between races; 4. no relationship between FLI and osteoporotic fractures was found because of the small number of fractures among patients in the study; 5. the effect of diabetes and anti-diabetic drugs on NAFLD was not evaluated.
Chen et al., 2018 (76)	Cross-sectional	China	938 postmenopausal women (365 with NAFLD, of those	DEXA	Moderate/severe NAFLD was independently correlated with osteoporosis and not mild, MetS was found to be an independent factor for osteoporosis combined addictive	1. cross-sectional design; 2. retrospective study; 3. ultrasound or the diagnosis of NAFLD; 4. no metabolic markers; 5. only one center

(Continued)

TABLE 1 Continued

	Study design	Origin	Study population	Methods	Outcome	Limitations
			132 with moderate/ severe NAFLD)		effect of moderate and severe NAFLD and MetS on osteoporosis	
Wang et al., 2018 (77)	Cross-sectional	China	2659 adults (950 men and 1709 women) of these 2045 with NAFLD	Ultrasound, questionnaire	NAFLD was correlated with the risk of osteoporotic fractures in men over 55 years old, but not in women. NAFLD was correlated with osteoporotic fractures in men without dyslipidemia	1. cross-sectional design; 2. recall bias; 3. self-reported fractures-missing vertebral; 4. confounding factors
Chen et al., 2018 (78)	Retrospective cohort study	China	4318 adults with NAFLD and 17272 without		Association between NAFLD and increased risk of new onset osteoporosis	1.confounding factors; 2. delay of diagnosis
Umehara, 2018 (79)	Cross-sectional	USA	6089 adults (1690 with NAFLD and 4399 without NAFLD)	DEXA	NAFLD was not significantly associated with BMD. NAFLD with higher ALT was negatively correlated with FN BMD	1. cross-sectional design; 2. confounding factors; 3. no fractures report; 4. ALT does not directly access the severity of NAFLD
Sung et al., 2020 (80)	Retrospective cohort study	South Korea	4536 adults (1006 men: 434 with NAFLD and 572 without NAFLD; 3530 women: 446 with NAFLD and 3084 without NAFLD)	DEXA	NAFLD was correlated with lower risk of BMD decrease in women	1. no biopsies, 2. young sample, 3. no bone metabolic markers
Ciardullo et al., 2021 (81)	Cross-sectional	USA	1784 adults (925 men and 859 women, 488 men and 391 women with liver steatosis and 126 men and 74 women with liver fibrosis)	DEXA, TE	No association between hepatic steatosis and hepatic fibrosis with osteoporosis	1. cross-sectional design; 2. no liver biopsies; 3. fracture risk was not accessed
Lee et al., 2021 (82)	Cross-sectional	Korea	2525 adults (FLI defined: 233 with NAFLD, 279 with NAFLD and fibrosis, CNS defined: 544 with NAFLD 614 with NAFLD and fibrosis)	Frax score	Association between NAFLD and a higher 10-year probability of major osteoporotic fracture in men >50, while this association was more pronounced in those with sarcopenia	1. cross-sectional design; 2. underestimated FRAX score due to missing data, 3. no biopsies
Xie et al., 2022 (83)	Cross-sectional	China	1980 adults (281 with NAFLD, 489 with severe steatosis)	DEXA Fibroscan	Negative correlation between NAFLD and BMD in persons aged 20 to 59 on subgroup analysis. A U-shaped relationship was found in black participants. In people aged 40-49 years, a positive relationship was found between BMD and advanced fibrosis and cirrhosis	1. cross-sectional design; 2. diagnosis with elastography; 3. missing data regarding medication, history of fracture; 4. no T scores and Z scores were reported
Yu et al., 2022 (84)	Cross-sectional	China	1243 diabetic patients (760 with NAFLD and 483 without NAFLD)	DEXA, ultrasound, FIB 4, NFS	Association between NAFLD (high risk for liver fibrosis) and osteoporosis in postmenopausal women with diabetes mellitus, but not in men	1. cross-sectional design; 2. no liver biopsies; 3. only middle and high risk according to NFS
Hassan et al., 2023 (85)	Cross-sectional	Egypt	100 adults (50 with NAFLD)	DEXA lumbar BMD	Association between NAFLD and lower BMD	1. small sample; 2. ultrasound for the diagnosis of NAFLD/no liver biopsy, 3. minimal steatosis could not be diagnosed; 4. the role of diabetes was not accessed; 5. only LS BMD was measured

(Continued)

TABLE 1 Continued

	Study design	Origin	Study population	Methods	Outcome	Limitations
Liu et al., 2023 (86)	Cross-sectional	USA	817 (381 with NAFLD 436 without NAFLD)	DEXA femoral BMD	NAFLD was correlated with higher BMD and lower risk of osteoporosis	1. cross-sectional study; 2. Possible ethnic disparities; 3. Questionnaires/recall bias; 4. No liver biopsies; 5. LS BMD was not accessed

ALT, alanine transaminase; BMD, bone mineral density; CRP, C-reactive protein; DEXA, dual x-ray absorptiometry; FLI, fatty liver index; FN, femoral neck; hs:high-sensitivity; HOMA-IR, homeostatic model assessment for insulin resistance; LS, lumbar spine; LFC, liver fat context; MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease; NFS, nafld fibrosis score; TE, transient elastography; TH, total hip.

liver function should be carefully surveilled following initiation of raloxifene treatment. Contrary, the findings from Luo et al. in a choline-deficient high-fat diet NASH mouse model showed an improvement in NASH after the administration of raloxifene (100). In line with this animal study, Barrera et al. examined a recent SERM, i.e. bazedoxifene acetate (BZA), in ovariectomized female mice fed a Western diet for 10-12 weeks. In this study, BZA administration, either alone or in combination with a conjugated estrogen (CE), resulted in attenuated liver steatosis along with increases in subcutaneous and visceral white adipose tissue induced by a high-fat diet (101). Moreover, Kim et al. studied the effects of BZA and TSEC on metabolic dysfunction in ovariectomized mice fed with a high-fat diet, demonstrating that BZA and TSEC promoted hepatic lipid oxidation and improved glucose homeostasis by raising the activity of Sirtuin1 (SIRT1), PPARα and hepatic AMPK of a different mechanism of action compared to E2 and CE (102). Interestingly, a natural SERM (genistein) given as monotherapy at high doses or in combination with CE in ovariectomized mice reduced fed with a high-fat diet significantly reduced the microvesicular fat infiltration in hepatocytes and hepatic TG accumulation induced by the high-fat diet (103). In an attempt to elucidate the underlying molecular mechanism, genistein was shown to decrease the expression of peroxisome proliferator-activated receptor-gamma (*PPARγ*) which is known to play a crucial role in the progression of hepatic steatosis (103).

4.3 Calcitonin and NAFLD

Calcitonin is a 32 amino acids hormone which acts by inhibiting the osteoclasts and stimulating the renal calcium excretion; hence, is regarded as an anti-osteoporotic treatment (not as effective as other anti-osteoporotic drugs) (104). Although, the nasal spray of calcitonin may be used in patients that cannot tolerate other therapies, there are concerns that calcitonin may provoke

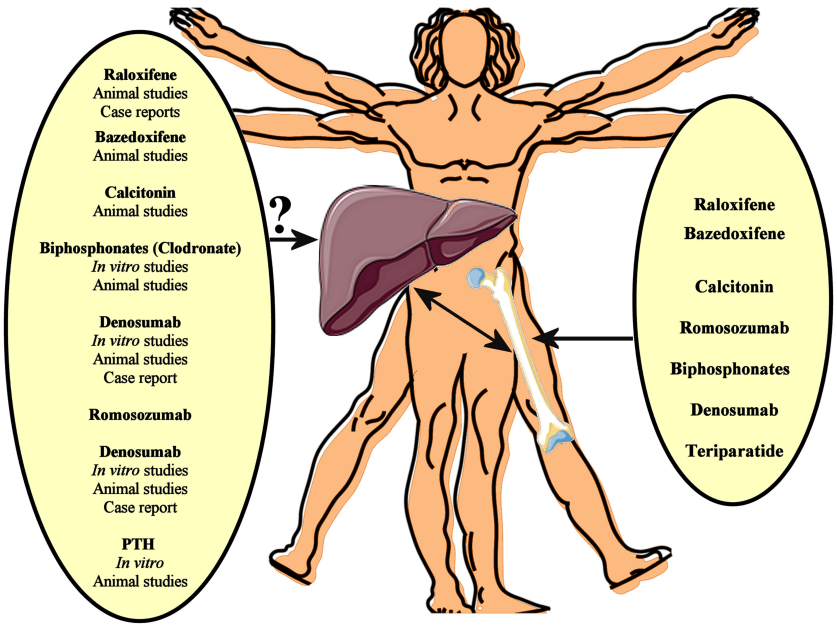

FIGURE 1
Several pharmacotherapies, including denosumab, bisphosphonates, teriparatide, raloxifene, calcitonin, and romosozumab, have well-established efficacy in the treatment of osteoporosis, reducing the risk of osteoporotic fractures. Given that osteoporosis and NAFLD frequently co-exist, such medications against osteoporosis may affect NAFLD progression by impacting on pathogenetic mechanisms/pathways shared by both these chronic diseases. *In vitro* data, animal studies and case reports support a beneficial effect of anti-osteoporotic drugs on the NAFLD/NASH progression. However, interventional studies could finally evaluate the potential impact of these anti-osteoporotic drugs on NAFLD.

malignancies, thus, it was withdrawn from the market in Europe and Canada (92). Gydesen et al. investigated the effect of a dual amylin and calcitonin receptor agonist (DACRA) on rats fed with high-fat diet, showing that this treatment resulted in improved glucose homeostasis, higher weight loss, enhanced insulin action and decreased lipid accumulation in the liver and skeletal muscles, whilst improved food preferences was also noted in these rats (105, 106). Finally, Polymeris et al. demonstrated that serum calcitonin levels increased after a 75-g oral glucose tolerance test in healthy adults, suggesting that calcitonin may be stimulated by hyperinsulinemia (107).

4.4 Denosumab and NAFLD

Denosumab is a human monoclonal immunoglobulin G2 antibody used as a treatment of osteoporosis. which binds to RANKL, thus inhibiting RANK activation and the formation and survival of osteoclasts. As shown by the 10-year FREEDOM Extension study, denosumab can be safely used as an anti-osteoporotic treatment for 10 years with low rates of adverse events, whilst significantly increasing the lumbar spine BMD and decreasing the incidence of fractures (108, 109). However, multiple vertebral fractures have been reported after the discontinuation of this drug, and, thus, the transition to another anti-osteoporotic therapy is important after the discontinuation of denosumab (110).

As RANK/RANKL and OPG are also expressed in other tissues (e.g. in the liver and fibroblasts), it has been suggested that the RANK/RANKL/OPG system may play a physiologic role in organs/tissues other than bone tissue (44). Indeed, the inhibition of the RANKL/RANK signaling pathway has been reported as a potential target for the treatment of T2DM and insulin resistance in humans (111). Interestingly, Zhong et al. showed that RANKL levels were gradually higher when going from control mice to high-fat diet induced NAFLD and NASH, whilst RANKL appeared to also play a role in Runx2-prompted macrophage migration (112). *In vitro*, this study also showed that Runx2 regulated the production of RANKL in hepatic stellate cells (112). Furthermore, using a mouse model that expressed human RANKL, Rinotas et al. showed that RANKL overexpression increases insulin resistance and promotes the development of NAFLD, with these effects being exerted -at least partially- by acting at a post-receptor level, as well as by upregulating the secretion of inflammatory cytokines through NFκB activation (113). Of note, the administration of denosumab appeared to reverse the negative effect of RANKL on insulin resistance (113). In line with this finding, Kiechl et al. showed in a mouse model that RANKL blockage improved hepatic insulin resistance by preventing the activation of NFκB which is known to play a role in hepatic steatosis and NAFLD (114).

Moreover, Takeno et al. presented a case report, showing NASH improvement following denosumab treatment in a woman with growth hormone deficiency and NASH (115). Observational studies have also noted an association between serum RANKL levels and NAFLD, with. Lu et al. reporting a correlation between elevated RANKL levels and higher NAFLD risk in women with PCOS (116). In addition, RANKL levels have been associated with hyperglycemia and higher T2DM risk. Recently, taking into account that increased hepatic expression of RANKL may play a role in the progression of NAFLD, Polyzos et al. proposed the use of denosumab for the treatment of NAFLD (117). However, interventional studies are required to support this suggestion.

4.5 Romosozumab and NAFLD

Romosozumab is a monoclonal antibody which inhibits sclerostin, an inhibitor of the Wnt signaling pathway signaling, and increases bone formation whilst reducing bone resorption. Since there are studies that have documented a relation between romosozumab treatment and cardiovascular and cerebrovascular events, it is currently recommended not to use romosozumab in patients with myocardial infarction or stroke in the last year (118).

As aforementioned, the Wnt/beta-catenin pathway appears to have an important role in the development and the progression of NAFLD (66), hence, romosozumab has also been proposed as a potential treatment for NAFLD (119). In line with this, Kim et al. using two different mouse models, namely sclerostin-deficient mice and mice treated with a sclerostin-neutralizing antibody, showed significantly increased bone mass, as well as decreased hepatic lipid accumulation and liver inflammation (120). Furthermore, Zhou et al. also revealed that sclerostin levels were reduced in NAFLD mice compared to controls (121). Finally, Oh et al. reported higher sclerostin mRNA levels in both patients with obesity and mice fed with a high-fat diet., whilst further showed that sclerostin administration amplified lipid accumulation in hepatocytes (122). On the other hand, Polyzos et al. reported decreased sclerostin levels in patients with NAFLD and NASH (123), while Rhee et al. founded that patients with advanced liver cirrhosis had higher sclerostin levels compared to healthy controls and patients with early cirrhosis (124).

Overall, the role of sclerostin and Wnt/beta-catenin in the development and progression of NAFLD appears to be complex and further research on the potential clinical impact of romosozumab on NAFLD is required to elucidate the role of this anti-osteoporotic treatment in the context of NAFLD/NASH.

4.6 Teriparatide and NAFLD

Teriparatide [rhPTH(1-34), the bioactive portion (1-34) of endogenous human PTH] is an anti-osteoporotic/osteoanabolic treatment (89). Feng et al. in their recent study in animal models of NAFLD using intermittent PTH administration, showed an amelioration of hepatic steatosis. They demonstrated, using an *in vitro* model of hepatic steatosis, that PTH through its receptor, induces in hepatocytes the expression of genes involved in β-oxidation and reduces the expression of genes involved in lipid uptake and *de novo* lipogenesis (125).

A recent metanalysis of 10 studies by Jaroenlapnopparat et al. demonstrated that high PTH levels was correlated with NAFLD, and their relation was statistically important. They also showed an

association between PTH level and NASH, which was not statistically important (126).

5 Perspectives and conclusion

NAFLD and osteoporosis are highly prevalent diseases which frequently co-exist with increasing incidence globally. Although common molecular pathogenetic mechanisms/pathways (e.g. the RANKL-OPG-RANK pathway and Wnt pathway) are supported by emerging data, not all epidemiological studies point towards a positive link between these two chronic diseases. To date, a limited number studies demonstrated an associative relationship between NAFLD and osteoporosis; however, conclusive evidence for causative link(s) and their direction are still missing. Further research, both basic/translational and clinical aiming to elucidate the interplay between the liver and bones is essential, including large prospective cohort and interventional studies which could target specific patient populations with NAFLD and osteoporosis.

In this context, recent studies have been further linking sarcopenia with both NAFLD and osteoporosis, thus highlighting sarcopenia as a potential mediating factor between these two diseases. This is also supported by the fact that molecules causatively implicated in sarcopenia, such as sclerostin, RANKL, and 25(OH)-vitamin-D, already constitute therapeutic targets in osteoporosis, whilst are also considered to play a role in the pathophysiology of NAFLD (122–124). Similarly, other therapeutic targets for osteoporosis, such as kathepsin K, also seem to be implicated in NAFLD progression (127, 128). Thus, it can be proposed that there is scope to focus future research in this field among patients with coexisting NAFLD, osteoporosis and sarcopenia since this group may benefit from anti-osteoporotic drugs involved in the overlapping pathophysiological mechanisms underlying these conditions. Since no globally approved pharmacological treatment for NAFLD is available yet, whilst there is an arsenal of approved anti-osteoporotic medication, observational data as well as interventional studies could evaluate the potential impact of these anti-osteoporotic drugs on NAFLD (Figure 1), with focus on certain phenotypic characteristics of the patient population (e.g. sarcopenic or not). Such targeted studies may shed light in the complex and yet not fully clarified links that form the liver-bone axis.

Author contributions

MC: Investigation, Writing – original draft. IK: Writing – review and editing. TA: Methodology, Writing – original draft. C-MF: Writing – original draft. EM: Writing – original draft. KC: Writing – original draft. YA: Writing – original draft. AP: Writing – original draft. EK: Conceptualization, Investigation, Methodology, Supervision, Writing – original draft, Writing – review and editing. HR: Investigation, Methodology, Supervision, Writing – original draft, Writing – review and editing.

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Long-Term pemafibrate treatment exhibits limited impact on body fat mass in patients with hypertriglyceridemia accompanying NAFLD

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Aim: Short-term use of pemafibrate (PEM), a selective modulator of peroxisome proliferator-activated receptor alpha, has been reported to improve abnormal liver function in patients with nonalcoholic fatty liver disease with hypertriglyceridemia (HTG-NAFLD). This study aimed to clarify the effects and predictive factors of long-term 72-week PEM administration on body composition, and laboratory tests in HTG-NAFLD patients.

Methods: Fifty-three HTG-NAFLD patients receiving a 72-week PEM regimen were retrospectively enrolled. Routine blood and body composition results were analyzed immediately before and at the end of the study period.

Results: PEM treatment significantly improved liver enzyme levels such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, and gamma-glutamyl transferase, along with lipid profiles including triglyceride, total cholesterol, and low-density lipoprotein cholesterol. PEM did not have any detectable impact on body composition parameters. The factors of female, higher AST ($\geq$ 46 U/L) and fat mass ($\geq$ 31.9%), as well as lower soft lean mass (< 61.6%), skeletal muscle mass (< 36%), and skeletal muscle mass index (< 6.9 kg/m^2) were significantly associated with the treatment response status of a > 30% decrease in ALT. All patients completed the treatment without any adverse effects.

Conclusions: Long-term PEM treatment had a positive impact on liver enzymes and lipid profiles, but it did not result in significant changes in body composition among HTG-NAFLD patients. In predicting the response to PEM treatment, the evaluation of AST and body composition may be useful.

KEYWORDS

non-alcoholic fatty liver disease, hypertriglyceridemia, pemafibrate, selective PPARα modulator, body composition analysis, treatment response, metabolic dysfunction-associated steatotic liver disease

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver condition characterized by the accumulation of triglyceride (TG) in over 5% of the liver (1). NAFLD has emerged as the most prevalent liver disease worldwide, leading to significant morbidity and mortality. In fact, its global prevalence is surpassing previous estimates and continues to rise at an alarming pace (2). The progression from simple steatosis to non-alcoholic steatohepatitis (NASH) can lead to the development of liver cirrhosis and potentially hepatocellular carcinoma (HCC) (3).

While no specific pharmacological treatments are currently approved for NAFLD, ongoing clinical trials are investigating various drug candidates targeting energy intake, energy disposal, lipotoxic liver injury, inflammation, and fibrosis (4). One such candidate is pemafibrate (PEM), a selective peroxisome proliferator-activated receptor-alpha (PPARα) modulator recently approved in Japan in 2018 for dyslipidemia. To evaluate the pharmacological and toxicological effects of PEM, comprehensive transcriptome analyses have been conducted on primary human hepatocytes and mouse liver tissue, which showed the induction of PPARα target genes involved in key hepatic processes, including TG hydrolysis, fatty acid uptake, fatty acid β-oxidation, and ketogenesis (5). In animal NASH models, PEM administration produced notable improvements in obesity, dyslipidemia, liver dysfunction, and NASH-associated pathological features (6). In another recent study, PEM treatment led to improvements in liver function tests, fibrotic biomarkers, and FibroScan-AST (FAST) score in NAFLD with hypertriglyceridemia (HTG-NAFLD) patients, suggesting a potential to prevent disease progression (7).

Several studies in mice have investigated the effects of PEM on adipose tissue but provided conflicting results. Araki et al. reported that PEM activated thermogenesis in mouse inguinal white adipose tissue (iWAT) and brown adipose tissue (BAT) by increasing plasma fibroblast growth factor 21 (FGF21) levels. The drug induced the expression of adipose triglyceride lipase (Atgl) and hormone-sensitive lipase (Hsl) in epididymal white adipose tissue (eWAT), leading to lipolysis activation and a presumed ability to decease fat mass (8). On the other hand, Zhang reported that the administration of a clinical volume of PEM to mice increased FGF21 levels but did not alter fatty acid uptake, fatty acid synthesis, TG synthesis, lipolysis (Atgl, Hsl), fatty acid beta-oxidation, or browning genes in eWAT or BAT (9). These incongruent results raised clinical questions about the potential of PEM to reduce body fat mass. Although short-term PEM treatment of HTG-NAFLD patients did not impact body fat mass or other related body composition parameters in our earlier study (10), long-term observations were considered necessary to confirm the body compositional changes from PEM.

In this investigation, we sought to clarify the effects of long-term PEM treatment on body fat mass and laboratory tests including liver function and lipid profile in HTG-NAFLD patients, as well as exploring predictive factors for PEM treatment responsiveness.

Materials and methods

Patients and clinical examinations

This was a retrospective analysis of prospectively registered patients. The present case-control study was approved by Nagano Municipal Hospital (ID number: 0038) and was performed following the Helsinki declaration of 1975 (1983 revision). We prospectively registered the 53 Japanese NAFLD patients who treated with PEM at Nagano Municipal Hospital (Nagano, Japan) between September 2019 and April 2023. Body composition and blood test data before, 24 weeks, and 72 weeks after PEM administration were used to retrospectively examine the therapeutic effect of long-term PEM administration in the HTG-NAFLD patients. Additionally, we defined responders as those showing an improvement of ALT >30% at 72 weeks according to previous studies (10–12), and conducted an analysis to identify predictive factors for responders using blood test and body composition data obtained before PEM administration.

The inclusion criteria for the HTG-NAFLD patients were as follows: (1) presence of hepatorenal contrast and increased hepatic echogenicity on abdominal ultrasonography; and (2) fasting serum

levels of TG > 150 mg/dL (13). The main exclusion criteria included patients with (1) average alcohol consumption of <30 g/day in men and <20 g/day in women; (2) absence of other causes of liver dysfunction, such as viral hepatitis, drug-induced liver injury, autoimmune liver disease, Wilson's disease, hereditary hemochromatosis, and citrin deficiency (14). Before PEM commencement, all patients had well-preserved liver function (i.e., not Child-Pugh class B or C) and no signs of HCC, gallstones, or renal impairment (i.e., serum creatine [Cre] concentration ≥ 2.5 mg/dL).

Patients were defined as hypertensive if their systolic/diastolic pressure was > 140/90 mmHg or if they were taking anti-hypertensive drugs (15). Type 2 diabetes is diagnosed with a fasting plasma glucose level ≥126 mg/dL on two occasions, HbA1c ≥6.5%, OGTT plasma glucose ≥200 mg/dL after 2 hours, or random plasma glucose ≥200 mg/dL with classic symptoms of hyperglycemia or if they were taking insulin or oral hypoglycemic agents (16). The diagnosis of liver cirrhosis (LC) was based on imaging findings and the formula for predicting LC proposed by Ikeda et al. (17). All laboratory data and body composition measurements were obtained in a fasting state. Fibrosis-4 (FIB-4) was calculated according to the following formula: FIB-4 = (age [years] × AST [U/L])/(platelet count [x10^9/L] × alanine aminotransferase [ALT] [U/L]$^{1/2}$) (18). The interval between patient visits for blood sampling was 4-12 weeks, at which time the patient was also interviewed about side effects.

Body composition analysis

Body composition analysis was conducted on 53 patients at the initiation of PEM administration using an InBodyS10 multi-frequency impedance body composition analyzer (InBody Japan, Tokyo). The analysis included measurements of fat mass, soft lean mass, and skeletal muscle (SKM) mass. Subsequently, 40 patients were assessed at 3 time points: immediately before PEM administration (baseline) and at 24 and 72 weeks later. Fat mass (%), soft lean mass (%), and SKM mass (%) were calculated by dividing the amounts calculated by body composition analysis by body weight. Skeletal muscle mass index (SMI) was determined as appendicular SKM mass (kg)/height (m)^2.

Statistical analysis

Clinical data are expressed as the number (percentage) or median (interquartile range). Statistical analyses were performed using StatFlex Ver. 7.0 (Artech Co., Ltd., Osaka, Japan). Wilcoxon matched-pairs signed-rank testing was employed for evaluating parameters before and after PEM treatment. The Friedman test was adopted for evaluating parameters before, at 24 weeks, and at 72 weeks of PEM treatment. The Mann–Whitney test and chi-square test were employed to compare responders and non-responders to PEM treatment. The diagnostic accuracy for identifying predictive factors of treatment responsiveness was assessed using the area under the receiver operating characteristic (ROC) curve (AUROC). AST, ALT, SKM mass (%), and SMI were employed as parameters in the ROC analysis of this study. The Youden index identified cut-off values, with the nearest clinically applicable value to the cut-off considered the optimal threshold for clinical convenience. All statistical tests were evaluated at the 0.05 level of significance.

Results

Clinical characteristics of HTG-NAFLD patients treated with PEM

53 patients completed the full 72-week PEM regimen, 40 of which underwent comprehensive 72-week body composition analysis. The pretreatment clinical characteristics of the cohort are summarized in Table 1. Median age was 57 years, with 35 patients (66.0%) being male. One person was using 0.1 mg/day, another person was using 0.4 mg/day, and the remaining 51 people were using 0.2 mg/day of PEM. Body composition analysis revealed the following median values: body weight 71.0 kg, body mass index (BMI) 26.8 kg/m^2, fat mass 38.8%, soft lean mass 57.7%, SKM mass 33.0%, and SMI 7.4 kg/m^2. The elevated complication rates of type 2 diabetes mellites (DM) (49.1%) and hypertension (HT) (41.5%) were typical for the Japanese NAFLD population (19). The median values for AST, ALT, alkaline phosphatase (ALP), gamma-glutamyltransferase (GGTP), and FIB-4 index were 42 U/L, 55 U/L, 226 U/L, 56 U/L, and 1.45, respectively.

Absence of significant treatment-induced changes in body composition parameters over baseline at 24 and 72 weeks

PEM can theoretically influence PPARα and its targets throughout the entire body, including adipose tissue and muscle, to alter body composition. However, body composition parameters, including BMI, fat mass (%), soft lean mass (%), SKM mass (%), and SMI showed no significant changes from baseline to 24 weeks and 72 weeks (Figure 1A). In a sub-analysis focused on the PEM responders with an improvement of ALT >30%, no statistically significant changes in body composition parameters from baseline at 24 and 72 weeks were observed (Figure 1B).

Seventy-two-week treatment with PEM significantly improved liver function and lipid profiles

We analyzed the changes in clinical parameters from before to 72 weeks of PEM treatment in patients with HTG-NAFLD (Table 2). The median values of AST, ALT, ALP, and GGTP all showed significant improvements at 72 weeks over baseline (AST: 42 to 30 U/L, ALT: 55 to 27 U/L, ALP: 226 to 60 U/L, GGTP: 56 to 34 U/L; all $p < 0.001$). A significant increase in serum albumin (Alb) was observed as well (4.5 to 4.6 g/dL, $p = 0.018$). Lipid profiles,

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TABLE 1 Clinical characteristics of HTG-NAFLD patients treated with PEM.

	Baseline
Age (years)	57 (46-67)
Male	35 (66.0%)
Body composition	
Body weight (kg)	71.0 (58.3-79.8)
BMI (kg/m^2)	26.8 (25.1-30.0)
Fat mass (%)	38.8 (30.1-42.5)
Soft lean mass (%)	57.7 (54.3-66.4)
SKM mass (%)	33.0 (30.8-39.0)
SMI (kg/m^2)	7.4 (6.6-8.3)
Complications	
LC	4 (7.5%)
Type 2 DM	26 (49.1%)
HT	22 (41.5%)
Obesity (BMI ≥ 25)	41 (77.4%)
Laboratory data	
Alb (g/dL)	4.5 (4.3-4.8)
AST (U/L)	42 (28-61)
ALT (U/L)	55 (36-78)
ALP (U/L)	226 (183-294)
GGTP (U/L)	56 (39-95)
Platelets (×10^3/μL)	251 (171-301)
FIB-4 index	1.45 (0.9-2.9)
TG (mg/dL)	181 (126-264)
TC (mg/dL)	198 (169-233)
LDL-C (mg/dL)	114 (98-144)
HDL-C (mg/dL)	41 (31-55)
FBS (mg/dL)	117 (104-149)
HbA1c (%)	6.1 (5.7-6.6)
Cre (mg/dL)	0.80 (0.65-0.90)
CK (U/L)	107 (64-151)

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CK, creatine kinase; Cre, creatinine; DM, diabetes mellitus; FBS, fasting blood sugar; FIB-4, Fibrosis-4; GGTP, gamma-glutamyltransferase; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; HT, hypertension; HTG-NAFLD, hypertriglyceridemia accompanying non-alcoholic fatty liver disease; LC, liver cirrhosis; LDL-C, low-density lipoprotein cholesterol; PEM, pemafibrate; SKM, skeletal muscle; SMI, skeletal muscle mass index; TC, total cholesterol; TG, triglyceride.

including TG and total cholesterol (TC), showed improvements at 72 weeks compared with baseline, as previously reported (TG: 181 to 118 mg/dL; p < 0.001, TC: 198 to 187 mg/dL; p = 0.006). No significant changes in Cre values were seen. All patients were able to complete PEM treatment without noticeable adverse effects.

Comparison between responders and non-responders to PEM treatment in HTG-NAFLD patients

We next compared the baseline clinical features of PEM responders with an improvement of ALT >30% (n = 34) and non-responders (n = 19) among the HTG-NAFLD patients. Responders had a significantly higher frequency of female (73.5 vs. 42.1%, p = 0.023). The presence of LC, HT, and type 2 DM did not significantly affect PEM treatment response (Table 3). In comparisons of the clinical parameters of responders and non-responders before PEM treatment, body composition analysis revealed that responders had significantly higher fat mass (%), lower soft lean mass (%), lower SKM mass (%), and lower SMI prior to PEM treatment (fat mass: 39.7 vs. 31.5%; p = 0.039, soft lean mass: 57.3 vs. 64.7%; p = 0.040, SKM mass: 32.5 vs. 38.3%; p = 0.034, SMI: 7.0 vs. 7.8 kg/m^2; p = 0.029) (Figure 2A). Responders also exhibited significantly higher levels of AST and ALT before PEM treatment (AST: 49 vs. 30 U/L; p <0.001, ALT: 65 vs. 43 U/L; p = 0.048) (Figure 2B).

The respective AUROC values for AST, ALT, fat mass (%), soft lean mass (%), SKM mass (%), and SMI were 0.77, 0.65, 0.67, 0.67, 0.68, and 0.68, respectively. The most appropriate ROC cut-off values for discriminating between responders and non-responders were AST: 46 U/L (sensitivity: 55.9%, specificity: 94.8%), ALT: 25 U/L (sensitivity: 94.0%, specificity: 57.9%), fat mass (%): 31.9% (sensitivity: 79.4%, specificity: 57.9%), soft lean mass (%): 61.6% (sensitivity: 73.5%, specificity: 63.2%), SKM mass (%): 36% (sensitivity: 76.5%, specificity: 63.3%) and SMI: 6.9 kg/m^2 (sensitivity: 41.2%, specificity: 84.3%) (Figure 3).

Discussion

This prospectively registered, retrospectively observed cohort assessed the impact of an extended 72-week PEM regimen on body composition in patients with HTG-NAFLD. Similarly to short-term PEM administration (10), no notable alterations in body composition were observed, and consistent outcomes emerged across treatment-responsive subgroups. Regarding the effects of PEM treatment, the intended metabolic shifts were achieved in terms of reductions in AST, ALT, GGTP, ALP, TG, and TC levels along with an increase in Alb levels. Furthermore, this investigation identified that 72-week PEM treatment responders with a > 30% decrease in ALT were predominantly women with higher AST, ALT, and fat mass (%) along with lower soft lean mass (%), SKM mass (%), and SMI, which corroborated previous findings of short-term PEM treatment (10).

The metabolism of liver fatty acids and TG is closely regulated through a delicate balance of *de novo* lipogenesis, glyceroneogenesis, very low-density lipoprotein assembly and secretion, lipolysis, and fatty acid oxidation at both the transcriptional and post-transcriptional levels (20, 21). Multiple studies have suggested that compromised PPARα function and impaired fatty acid oxidation play significant roles in the

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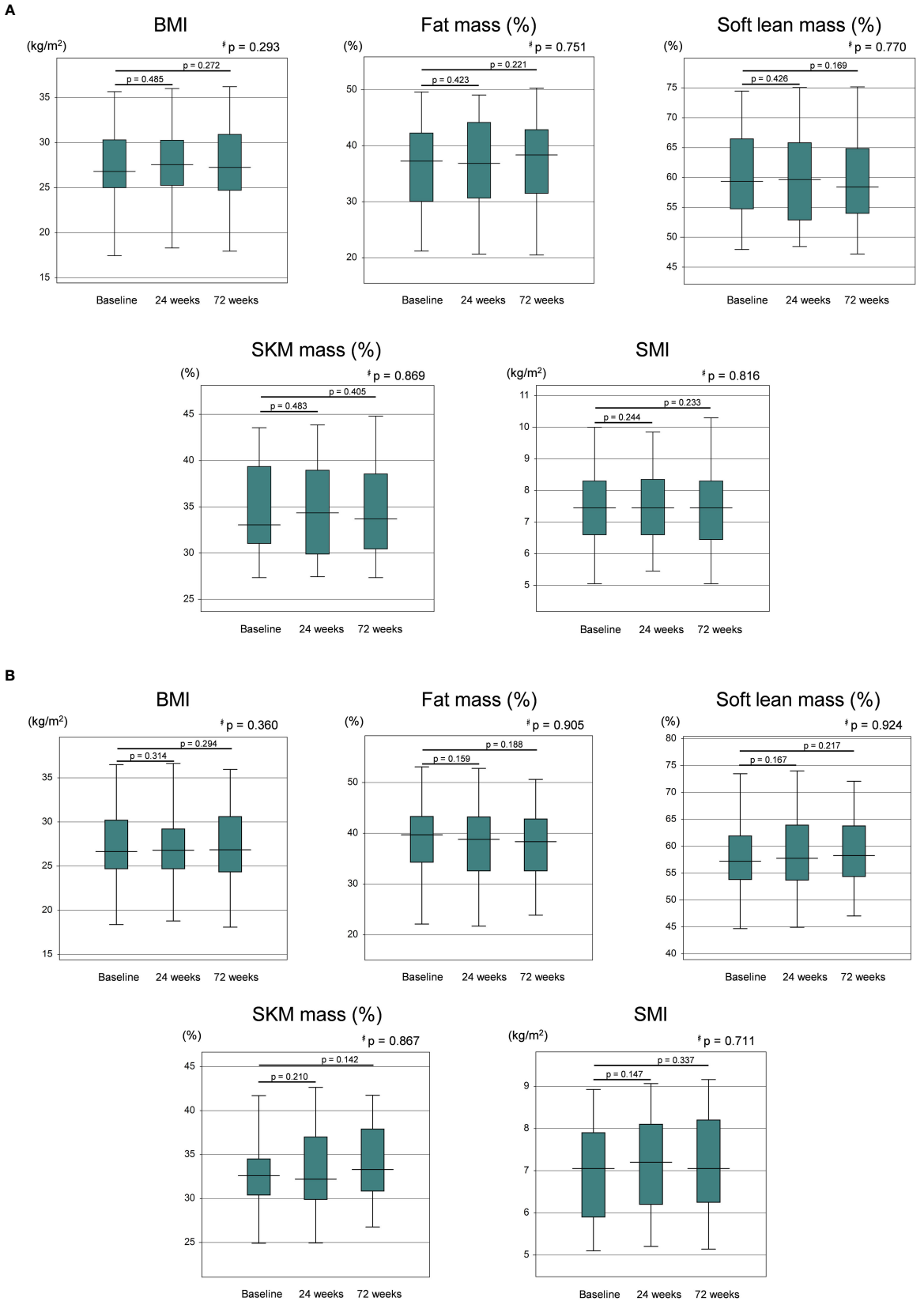

FIGURE 1
(A) Levels of BMI, fat mass (%), soft lean mass (%), SKM mass (%), and SMI at baseline, 24 weeks, and 72 weeks of PEM treatment. **(B)** Levels of BMI, fat mass (%), soft lean mass (%), SKM mass (%), and SMI at baseline, 24 weeks, and 72 weeks of PEM treatment in responders (Wilcoxon matched-pairs signed-rank test). p values are indicated by ♯for the Friedman test. BMI, body mass index; PEM, pemafibrate; SKM, skeletal muscle; SMI, skeletal muscle mass index.

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TABLE 2 Characteristics of 53 patients with HTG-NAFLD at baseline and after 72 weeks of PEM treatment.

PEM treatment			
	Baseline	72 weeks	p-value
Alb (g/dL)	4.5 (4.3-4.8)	4.6 (4.4-4.9)	**0.018**
AST (U/L)	42 (28-61)	30 (22-36)	< **0.001**
ALT (U/L)	55 (36-78)	27 (20-42)	< **0.001**
ALP (U/L)	226 (183-294)	60 (44-86)	< **0.001**
GGTP (U/L)	56 (39-95)	34 (25-53)	< **0.001**
Platelets ($\times10^3/\mu L$)	251 (171-301)	248 (194-303)	**0.009**
FIB-4 index	1.45 (0.9-2.9)	1.30 (0.8-2.2)	0.069
TG (mg/dL)	181 (126-264)	118 (88-169)	< **0.001**
TC (mg/dL)	198 (169-233)	187 (174-206)	**0.006**
LDL-C (mg/dL)	114 (98-144)	117 (93-126)	**0.004**
HDL-C (mg/dL)	41 (31-55)	48 (36-56)	0.097
Cre (mg/dL)	0.80 (0.65-0.90)	0.76 (0.62-0.83)	0.426

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cre, creatinine; FIB-4, Fibrosis-4; GGTP, gamma-glutamyltransferase; HDL-C, high-density lipoprotein cholesterol; HTG-NAFLD, hypertriglyceridemia accompanying non-alcoholic fatty liver disease; LDL-C, low-density lipoprotein cholesterol; PEM, pemafibrate; TC, total cholesterol; TG, triglyceride. p<0.05 is indicated in bold.

development of NASH (22, 23). In this context, it is plausible that PEM, which activates PPARα, improves the pathogenesis of NASH. In patients with NAFLD, PEM treatment has been reported to improve liver fibrosis markers along with findings on magnetic resonance elastography, transient elastography, and hepatic shear wave velocity, indicating a positive effect on liver fibrosis (7, 12, 24, 25). Notably, those studies indicated that PEM did not lead to a reduction in BMI and that the therapeutic benefits of PEM were independent of weight loss. Our previous data from a 24-week PEM treatment study revealed that the treatment response to PEM was more favorable in patients with higher body fat (10). We therefore hypothesized that longer term PEM administration would alter body composition by stimulating PPARα and its target genes in adipose and muscle tissue in this 72-week PEM study. In contrast to the discernible alterations observed in liver enzymes and lipid profiles, however, no significant changes were detected in fat mass or other relevant body composition parameters. Our results are consistent with the previous mouse study showing that a clinical dose of PEM treatment does not induce PPARα target genes in extrahepatic tissues, including BAT and eWAT (9).

In earlier reports, the therapeutic effect of PEM was verified by changes in FAST score (26), normalization of ALT (27), a 30% reduction in ALT, and a 30% reduction in shear wave velocity (12). However, investigations of histopathological changes in human liver, adipose, and muscle tissue as well as variations in gene expression with PEM treatment in NAFLD have not been adequately investigated. Exploring the detailed effects of PEM on the human liver both intra- and extrahepatically is a future challenge.

This study had several limitations, primarily stemming from its retrospective, single-center, non-interventional design. To validate our findings, it will be necessary to conduct further large-scale, prospective investigations. And, the gold standard for body composition

TABLE 3 Patient background comparison of responders and non-responders to 72 weeks of PEM treatment.

	Non-Responder (n=19)	Responder (n=34)	
	n (%)	n (%)	p-value
Female	8 (42.1)	25 (73.5)	**0.023**
LC	3 (15.8)	1 (2.9)	0.089
HT	8 (42.1)	14 (41.2)	0.947
Type 2 DM	10 (52.6)	15 (47.1)	0.697

Patients were defined as responders if ALT decreased by > 30% at 72 weeks of PEM administration. DM, diabetes mellitus; HT, hypertension; LC, liver cirrhosis; PEM, pemafibrate. p<0.05 is indicated in bold.

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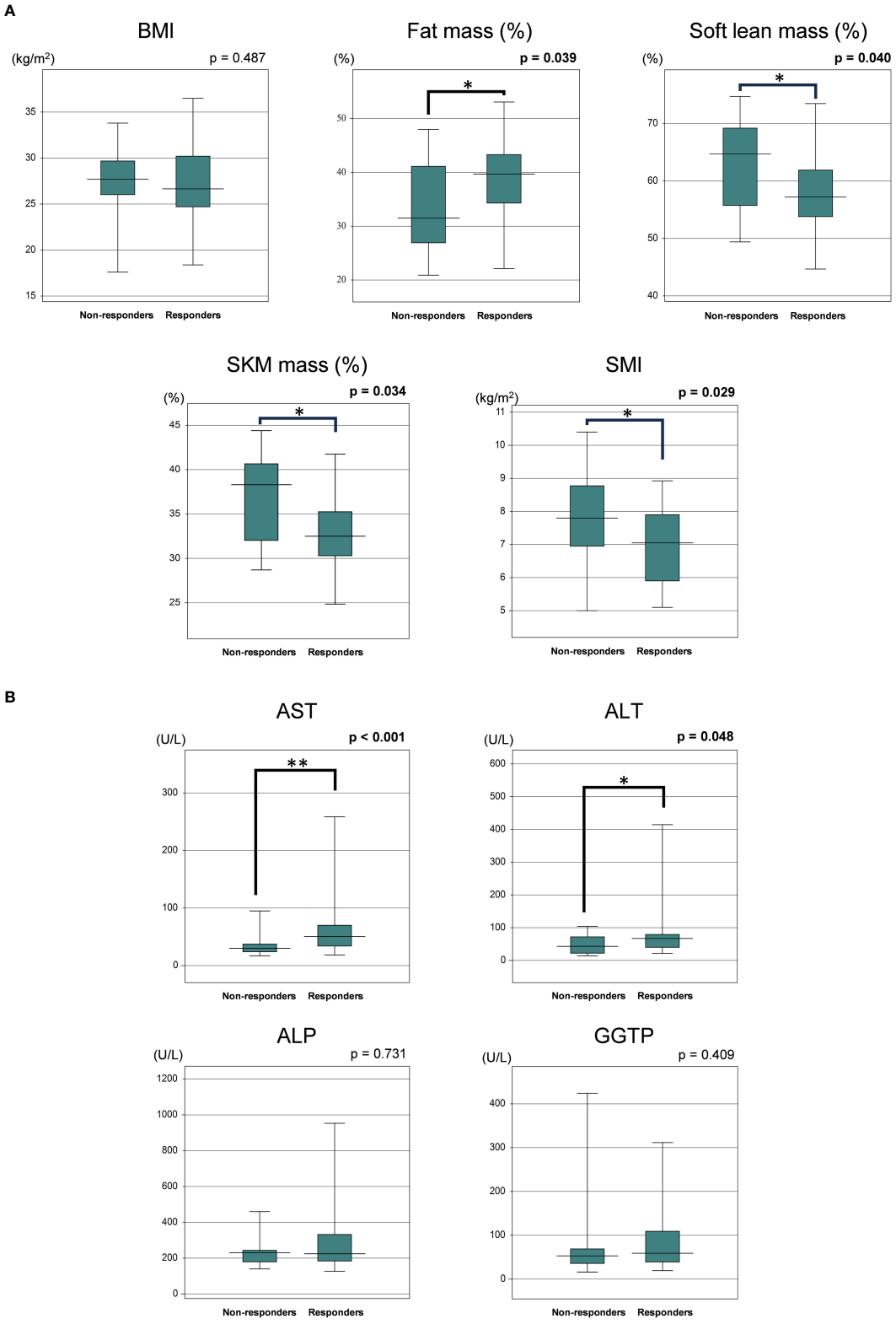

FIGURE 2
(A) Comparison of baseline body composition parameters of responders and non-responders to 72 weeks of PEM treatment. **(B)** Comparison of baseline laboratory data of responders and non-responders to 72 weeks of PEM treatment (Mann–Whitney U test). *p < 0.05, **p < 0.001. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGTP, gamma-glutamyltransferase; PEM, pemafibrate; SKM, skeletal muscle; SMI, skeletal muscle mass index.

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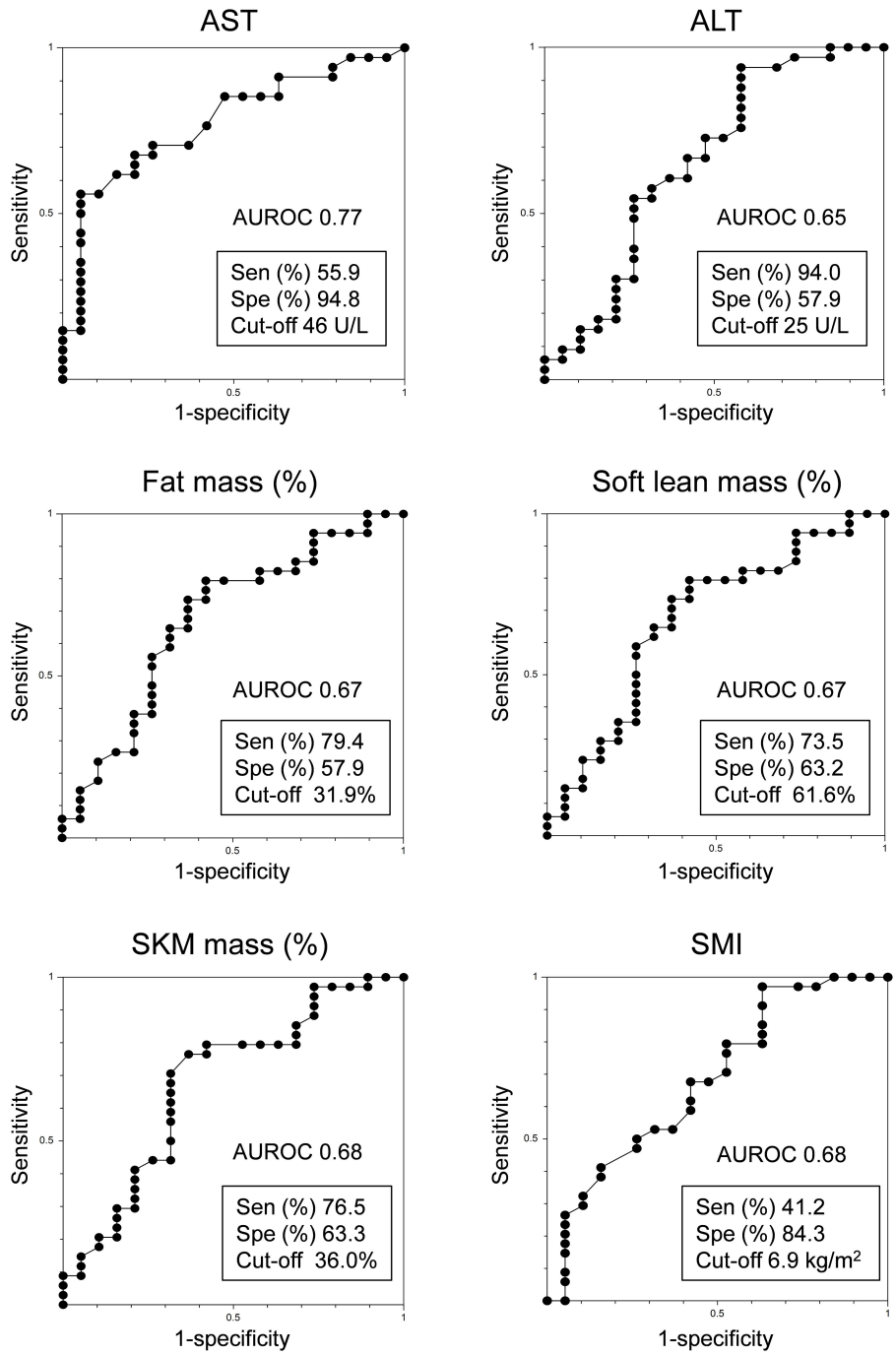

FIGURE 3
Receiver operating characteristic analysis of PEM responders in HTG-NAFLD patients. ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic curve; PEM, pemafibrate; Sen, sensitivity; SKM, skeletal muscle; SMI, skeletal muscle mass index; Spe, specificity.

measurement includes dual-energy X-ray absorptiometry (DXA) and magnetic resonance imaging (MRI). Therefore, it is essential to determine whether the results obtained from the multi-frequency impedance body composition analyzer used in this study are consistent with those obtained from MRI or DXA.

In conclusion, 72 weeks of PEM treatment resulted in improvements in liver enzymes and lipid profile, but not significant changes in body fat mass. Female, higher AST and body fat percentage, and lower soft lean mass percentage were associated with better response to long-term PEM treatment.

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 humans were approved by Committees for Medical Ethics of Nagano Municipal Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

TI: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. TK: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. HKu: Writing – review & editing, Resources, Project administration, Investigation, Data curation, Conceptualization. TO: Writing – review & editing, Investigation, Data curation. S-IW: Writing – review & editing, Software, Resources, Formal analysis, Data curation. HKo: Writing – review & editing, Investigation, Data curation. YY: Writing – review & editing, Methodology, Investigation. AS: Writing – review & editing, Investigation, Data curation. NT: Writing – review & editing, Supervision. TU: Writing – review & editing, Supervision, Resources.

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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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Glossary

Alb	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
Atgl	adipose triglyceride lipase
AUROC	area under the receiver operating characteristic curve
BAT	brown adipose tissue
BMI	body mass index
Cre	creatinine
DM	diabetes mellitus
DXA	dual-energy X-ray absorptiometry
eWAT	epididymal white adipose tissue
FAST	FibroScan-aspartate aminotransferase
FIB-4	Fibrosis-4
FGF21	fibroblast growth factor 21
GGTP	gamma-glutamyltransferase
HCC	hepatocellular carcinoma
Hsl	hormone-sensitive lipase
HT	hypertension
HTG	hypertriglyceridemia
LC	liver cirrhosis
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
PEM	pemafibrate
PPARα	peroxisome proliferator-activated receptor-alpha
ROC	receiver operating characteristic
SKM	skeletal muscle
SMI	skeletal muscle mass index
TC	total cholesterol
TG	triglyceride

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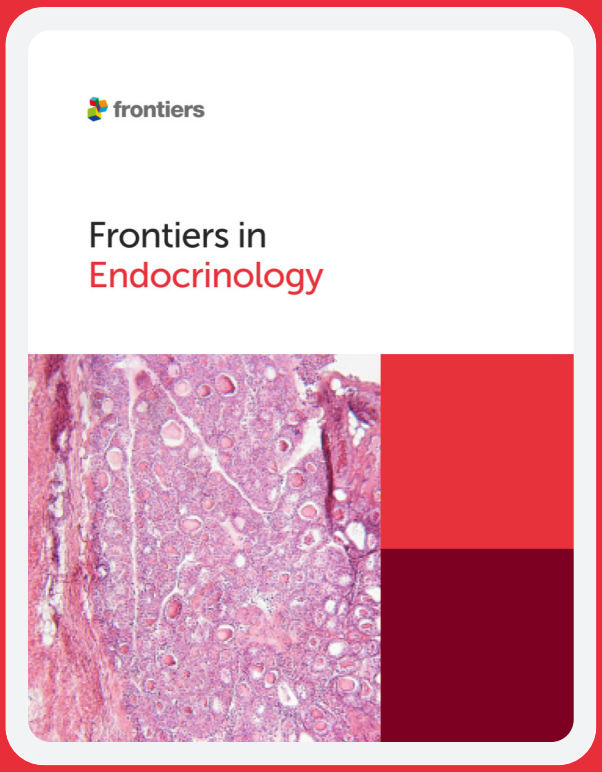

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