Trimethylamine N-oxide (TMAO), choline and its metabolites are associated with the risk of nonalcoholic fatty liver disease

Running title: TMAO, choline and its metabolites and NAFLD

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Abstract

It is inconclusive whether trimethylamine N-oxide (TMAO) and choline and related metabolites, namely, trimethylamine (TMA), L-carnitine, betaine, and dimethylglycine (DMG), are associated with nonalcoholic fatty liver disease (NAFLD). Our objective was to investigate these potential associations. Additionally, we sought to determine the mediating role of TMAO. In this 1:1 age- and sex-matched case–control study, a total of 150 pairs comprising NAFLD cases and healthy controls were identified. According to the fully adjusted model, after the highest tertile was compared to the lowest tertile, the plasma TMAO concentration (odds ratio [OR]=2.02 [95% confidence intervals: 1.04-3.92]; P-trend=0.003), L-carnitine concentration (OR=1.79 [1.01-3.17]; P-trend=0.020), and DMG concentration (OR=1.81 [1.00-3.28]; P-trend=0.014) were significantly positively associated with NAFLD incidence. However, a significantly negative association was found for plasma betaine (OR= 0. 50 [0.28-0.88]; *P*-trend=0.001). The restricted cubic splines model consistently indicated positive dose–response relationships between exposure to TMAO, L-carnitine, and DMG and NAFLD risk, with a negative association being observed for betaine. The corresponding areas under the curve increased significantly from 0.685 (0.626-0.745) in the traditional risk factor model to 0.769 (0.716-0.822) when TMAO and its precursors were included (L-carnitine, betaine, choline) (*P*=0.032). Mediation analyses revealed that 14.7% and 18.6% of the excess NAFLD risk associated with L-carnitine and DMG, respectively, was mediated by TMAO (the *P* values for the mediating effects were 0.021 and 0.036, respectively). These results suggest that a higher concentration of TMAO is associated with increased NAFLD risk among Chinese adults and provide evidence of the possible mediating role of TMAO.

Keywords: Nonalcoholic fatty liver disease; Trimethylamine N-oxide; L-carnitine; Betaine; Choline

Nonalcoholic fatty liver disease (NAFLD) has emerged as the most prevalent hepatic disorder worldwide and is estimated to afflict approximately 38% of the world's population, with an annual incidence of 46.13 new cases per 1000 person-years [1,2]. In China, the cumulative nationwide incidence of NAFLD is 29.2%, which experienced a notable increase from 25.4% in 2008-2010 to 32.3% in 2015-2018 [3]. Despite the lower incidence of NAFLD-related cirrhosis or hepatocellular carcinoma (HCC) in comparison to other aetiologies, such as hepatitis, the exceptionally high prevalence and vast population at risk have propelled NAFLD to become the swiftest growing causative factor for HCC [4]. Moreover, disconcertingly, individuals are often afflicted by NAFLD at a young age, signifying an extended timeframe for the development of severe complications, including cancer and cardiovascular diseases [1]. Public health interventions for the prevention of NAFLD are needed with special emphasis. Although genetic, epigenetic, and environmental risk factors for NAFLD have been identified, the underlying causes are still controversial and largely unknown [5]. Hence, the urgent need arises to ascertain novel aetiological factors, particularly those that are modifiable, as they could contribute to the formulation of an evidence-based strategy for the primary prevention of NAFLD.

An animal study demonstrated the potential causative role of the gut microbiota in the development of NAFLD [6]. Furthermore, accumulating evidence underscores the involvement of the gut microbiome in the aetiology of NAFLD through the mediation of NAFLD metabolites, such as trimethylamine N-oxide (TMAO) [7], which is naturally found in seafood, dairy products, egg yolks, muscle, and organ meats in a preformed state and is also a metabolite originating from precursors, including phosphatidylcholine, choline, betaine, and L-carnitine [8, 9]. Within the vast expanse of the large intestine microbiome, there exists the capacity to convert carnitine and choline into trimethylamine (TMA), which is subsequently metabolized by the hepatic enzyme flavin monooxygenase (FMO) family, such as FMO-1 and FMO-3, ultimately culminating in the formation of TMAO [8, 10]. Notably, the beneficial effect of the human gut microbiota on glucose metabolism could be strongly mediated by microbial metabolites, particularly TMAO, and be contingent on diet [11,12]. Intriguingly, a multitude of studies have suggested that circulating concentrations of TMAO

and choline-related metabolites are significantly associated with various health outcomes, including all-cause mortality, cardiovascular disease (CVD), diabetes mellitus (DM), cancer, and renal function [9].

A recent meta-analysis conducted by Theofilis P *et al.*[13] comprehensively evaluated the levels of TMAO in NAFLD, revealing that NAFLD patients exhibit notably elevated circulating TMAO concentrations compared to those without NAFLD. Nonetheless, it is essential to acknowledge that the results were characterized by inconsistency and substantial heterogeneity, as indicated by an I^2 of 94%. In addition, the association between TMAO and choline-related metabolites and NAFLD risk, as well as hepatic iron and fat contents, has seldom been evaluated.

To address this discrepancy, we conducted a matched case–control investigation aimed at exploring the potential contributions of plasma TMAO, choline and its related metabolites (namely, trimethylamine, L-carnitine, and betaine) to the development of NAFLD among Chinese adults. In addition, we sought to elucidate the associations of these metabolites with hepatic iron and fat contents. Furthermore, our study delved into the mediating role of TMAO in the correlation between choline and its related metabolites and the risk of NAFLD.

Methods and materials

This study strictly adhered to the principles outlined in the Declaration of Helsinki and received approval from the institutional review board of the First Affiliated Hospital of Chengdu Medical College. Before participation, all the subjects provided written informed consent. The investigation was conducted in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Study population

We conducted a 1:1 matched case–control study examining the association between plasma TMAO, choline and its related metabolites and NAFLD. Cases were defined as patients who were admitted to the Department of Hepatology of Xinjiang Corps Hospital and were diagnosed with new-onset NAFLD from January 1, 2018, to February 28, 2020. Included were all patients who were older than 18 years but without confirmed heart disease, stroke,

cancer, excessive alcohol consumption, autoimmune liver disease, or other disorders potentially linked to fatty liver disease. The control group subjects were recruited mainly through recruitment advertisements distributed through WeChat or recommended by doctors from the physical examination centre. We randomly selected one control per case without a history of NAFLD. We applied the same exclusion criteria to controls as to cases except for a diagnosis of NAFLD. We matched the controls to the patients on age (± 2 years) and sex. **Figure 1** presents the flow chart of participant recruitment and the reasons for exclusion.

Assessment of Blood Biomarkers

A 5 mL blood sample was extracted from the cephalic vein of each participant in the early hours of the morning following an overnight fast. The clotted samples were centrifuged at 1000×g for 15 minutes. The resulting clear aliquots were meticulously separated and preserved at a frigid temperature of − 80 °C until further analysis. To ensure a rigorous and unbiased approach, the serum samples from each matched case–control set, comprising one case and one control, were positioned adjacently in a randomized sequence and subjected to simultaneous testing. All personnel involved in the testing procedure were kept unaware of the case/control status of the samples, maintaining strict blinding throughout the analysis.

Serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and fasting blood glucose levels were assessed using a sophisticated Hitachi 7600-210 automated analyser.

Liquid chromatography-tandem mass spectrometry (LC-TMS) was employed to quantify plasma TMAO, choline and their related metabolites, encompassing TMA, L-carnitine, betaine, and dimethylglycine (DMG), which are betaine-related metabolites. [(14)] The stable isotope dilution liquid chromatography-tandem mass spectrometry (6460 Series Triple Quadrupole LC/MS; Agilent) method, as previously described [(15)], was utilized for this purpose. The internal standard utilized was d9-TMAO. A silica column (4.6×250 mm, 5 μm Luna silica; catalogue no. 00G-4274-E0; Phenomenex) was used in the analysis. To ensure rigorous quality control, 12 duplicated samples sourced from a pool of plasma samples collected from cohort participants during the same study period were distributed across six

batches of test samples (two per batch). The within-batch coefficients of variation (CVs) for all biomarkers assessed ranged from 1.2% to 3.4%, while the between-batch CVs ranged between 3.4% and 7.1%.

Abdominal magnetic resonance imaging (MRI) and diagnosis of NAFLD

The diagnosis of NAFLD was ascertained through the application of magnetic resonance proton density fat fraction (MR-PDFF) analysis conducted by proficient radiologists. MR imaging was meticulously performed using a 1.5 T GE scanner equipped with an 8-channel, torso phased-array coil (Optima MR360; GE HealthCare, Milwaukee, WI, USA). To construct the PDFF map, five circular regions of interest (ROIs) of approximately 100 mm2 were manually delineated on the PDFF maps using the AW4.6 workstation (GE HealthCare, Milwaukee, WI, USA). Among these ROIs, three were uniformly positioned on the right lobe, while the remaining two were placed on the left lobe, strategically avoiding major vessels, ligaments, and bile ducts [(16)]. The diagnosis of fatty liver disease was established based on MRI findings, where the mean proportion of liver fat exceeded 5.5% [(17)]. Patients with fatty liver disease were subsequently diagnosed with NAFLD after meticulous assessment and exclusion of excessive alcohol consumption; NAFLD was defined as a daily intake of alcohol exceeding 20 g for men and 10 g for women.

Covariate collection

Trained nurses meticulously gathered all covariate data during the enrolment process. Comprehensive information was obtained through a structured questionnaire utilizing face-to-face interviews. The questionnaire encompassed various domains, including sociodemographic characteristics such as age, sex, marital status, and education level. Furthermore, lifestyle behaviours, such as smoking, alcohol consumption, and physical activity, were meticulously documented. Additionally, the participants' medical history, including hypertension, diabetes, heart disease, or any other major conditions diagnosed by a medical professional, was carefully recorded. Body weight and height were meticulously measured and subsequently utilized to calculate body mass index (BMI, kg/m^2). Blood pressure (BP) was assessed using two consecutive measurements performed with the

participants in a seated position following at least 10 minutes of rest. The mean value of these readings was utilized for subsequent analyses. For the assessment of body composition, the bioelectrical impedance analysis (BIA) method was employed utilizing a sophisticated body composition device, namely, the InBody S10 (BioSpace, Seoul, Korea).

Statistical analysis

According to our primary association analysis, 33.3% of people had higher serum TMAO, choline and their related metabolites in the top tertile, and the estimated OR between the serum TMAO concentration and NAFLD risk was 2.14 [18]. The type I error rate was <0.05 ($\alpha = 0.05$), the power of the test was 90% ($\beta = 0.10$), and the response rate was 90%. Based on these assumptions, we required a sample size of 129 paired cases and controls.

Continuous variables are presented as the mean and standard deviation, provided they were normally distributed. Otherwise, these variables are represented as the median and the interquartile range. On the other hand, categorical variables are depicted in terms of frequencies and percentages. To assess the disparities between groups, statistical comparisons were performed employing one-way ANOVA, the Kruskal–Wallis H test, and Pearson's chi-square test, as appropriate for the specific data type.

The conditional logistic regression method was elegantly employed to determine odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) for NAFLD across tertiles of plasma TMAO, choline and related metabolites. The cut-off points for these tertiles were meticulously determined based on the distributions among the control subjects. Both crude and adjusted models were thoughtfully utilized to address potential confounding factors. The inclusion criteria for age (years), BMI (kg/m^2), systolic blood pressure (SBP, mmHg), diastolic blood pressure (DBP, mmHg), marital status (married, others), education level (<9, 9-12, or ≥12 years), current smoking status (yes, no), physical activity (minutes per week), total cholesterol level (mmol/L), and triglyceride level (mmol/L) in the multivariable logistic models allowed for comprehensive adjustment. The linear trend test was conducted based on the ordinal values of tertiles (i.e., 1, 2, and 3) for each of the studied biomarkers in relation to the risk of developing NAFLD, adding further depth to the statistical analysis.

Restricted cubic splines (RCSs) were employed to investigate the plausible nonlinear associations between plasma TMAO, choline and their related metabolites and the risk of NAFLD, rendering a continuous scale analysis possible. Moreover, a receiver operating characteristic (ROC) curve analysis was conducted, facilitating the calculation of areas under the curve (AUCs) to assess the discriminative capacity of plasma TMAO, choline and their related metabolites in predicting the occurrence of NAFLD.

Mediation analyses were additionally performed to explore the potential role of TMAO as a mediator of the association between choline and its related metabolites and NAFLD risk. To execute this analysis, we utilized the CAUSALMED procedure, which allowed us to calculate the total, direct, and indirect mediation effects of TMAO. This was achieved through the employment of the variance–covariance matrix and the maximum likelihood method. In the causal process, the product of the "a" path quantifies the effect of independent variables (L-carnitine, betaine or DMG) on the mediator (TMAO), and the product of the "b" path quantifies the effect of the mediator (TMAO) on the dependent variable (NAFLD), controlling for independent variables [19]. Mediation is presented if the product of the coefficients (β=a*b) reaches statistical significance [19].

Statistical analyses were performed using R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria). A two-sided *P* value of < 0.05 was considered to indicate statistical significance.

Results

The baseline characteristics and serum levels of TMAO, choline and their related metabolites in the patients and controls are presented in **Table 1.** The mean (standard deviation) ages of the included participants were 61.6±6.4 years and 61.2 ± 6.0 years, and 58.2% of them were women. Compared with controls, NAFLD cases had significantly greater BMIs; SBP; DBP; body fat; triglyceride levels; and fasting glucose but had lower education, physical activity, and HDL-c levels. There were no statistically significant differences between the cases and controls in age, sex, marital status, smoking status, history of hypertension or type 2 diabetes, TC, and LDL-c.

The plasma concentrations of TMAO, L-carnitine, and DMG were significantly greater, but the concentration of betaine was lower in NAFLD cases than in controls (all *P* values < 0.05; Table 1). Moreover, there was no statistically significant difference in the serum concentrations of trimethylamine or choline between the patients and controls.

The associations between TMAO, choline and their related metabolites and the risk of NAFLD are presented in **Table 2.** After adjustment, higher levels of TMAO, L-carnitine, and DMG were significantly associated with an increased risk of NAFLD, whereas higher levels of plasma betaine were related to a decreased risk of NAFLD (all *P* trends <0.005). After adjustment for covariates, including age, BMI, SBP, DBP, marital status, education level, smoking status, physical activity, total cholesterol, and triglycerides, the associations were not significant. Compared with those of the lowest tertile, the ORs (95% CIs) of NAFLD for the highest tertile of TMAO, betaine, L-carnitine, and DMG were 2.02 (1.04, 3.92), 0.50 (0.28, 0.88), 1.79 (1.01-3.17), and 1.81 (1.00, 3.28), respectively. No statistically significant association was detected for the risk of NAFLD with any other biomarkers tested, including trimethylamine and choline (**Table 2**).

Figure 2 presents the results of the multivariable-adjusted RCS analysis. A positive dose–response association was observed for NAFLD risk with serum levels of TMAO (*P* for linear < 0.001, *P* for nonlinear <0.001), L-carnitine (*P* for linear < 0.001, *P* for nonlinear =0.005), and DMG (*P* for linear < 0.001, *P* for nonlinear = 0.339), whereas a linear negative association with serum betaine was observed (*P* for linear < 0.001, *P* for nonlinear = 0.321). No associations were detected for serum levels of choline (*P* for linear = 0.239, *P* for nonlinear = 0.875) or trimethylamine (*P* for linear =0.294, *P* for nonlinear = 0.543).

Figure 3 shows the discriminatory value of plasma TMAO, choline and their related metabolites for NAFLD. Notably, the AUC increased significantly from 0.685 (95% CI=0.626-0.745) in the traditional risk factor model to 0.769 (95% CI=0.716-0.822) when TMAO, choline, L-carnitine, and betaine were included (*P*=0.032).

We further analysed the mediating effects of TMAO on the association between NAFLD risk and three significant choline-related metabolites (L-carnitine, betaine, and DMG) (**Figure 4**). TMAO served as a significant mediator of L-carnitine (β: 0.021, 95% CI: 0.011, 0.029) and

DMG (β: 0.012, 95% CI: 0.007, 0.017) but not betaine (β: -0.002, 95% CI: -0.007, 0.003). Overall, 14.7% and 18.6% of the increased NAFLD risk associated with L-carnitine and DMG, respectively, was mediated by TMAO (*P* for mediation effect = 0.036 and 0.021, respectively).

Discussion

A matched case–control study of Chinese adults suggested that plasma TMAO, L-carnitine and DMG levels were positively associated with the risk of NAFLD, whereas betaine was negatively associated with NAFLD risk. Furthermore, the incorporation of traditional risk factors, in conjunction with TMAO, L-carnitine, betaine, and DMG, leads to a substantial enhancement in the discriminatory capacity for NAFLD diagnosis. In addition, TMAO may play a pivotal role as a crucial mediator in the intricate relationship between NAFLD risk and L-carnitine or DMG levels.

Foods associated with significant benefits in relation to glucose metabolism were major contributors to microbial metabolites, particularly TMAO [11,12]. Animal models have consistently indicated that the ingestion of trimethylamine containing nutrients (i.e., choline, carnitine, γ-butyrobetaine, etc.) can activate the gut microbial TMA–FMO3–TMAO pathway, subsequently impacting cardiometabolic disease incidence [20]. A meta-analysis involving 7 studies with 7583 individuals reported that NAFLD patients tended to have higher levels of TMAO (standardized mean difference [SMD]: 0.66, 95% CI -0.12 to 1.21; P = 0.02, I^2: 94%) than did patients without NAFLD [13]. Another meta-analysis further indicated that L-carnitine supplementation could reduce the levels of aspartate transaminase (mean difference [MD]: - 15.89, 95% CI: - 29.87 to -1.91) and alanine amiotransferase (MD: - 26.38, 95% CI: - 45.46 to -7.30), as well as triglycerides (MD: - 6.92, 95% CI: - 13.82 to -0.03), in NAFLD patients [21]. A cross-sectional study further showed that ln-transformed serum levels of TMAO and choline and the betaine/choline ratio measured in 60 NAFLD patients were positively associated with elevated steatosis and total NAFLD activity (all *P* values trend <0.05) [22].

The increasing trends in the incidence of NAFLD underscore the importance of timely

identification of NAFLD to mitigate potential hepatic and extrahepatic complications. Furthermore, the pathophysiologic underpinnings of NAFLD are multifactorial and not fully understood. Foods rich in trimethylamine precursors, such as red meat, eggs, and fish, undergo metabolism within the digestive system, resulting in the production of choline, L-carnitine, and betaine. The surplus TMA precursors that cannot be absorbed are converted by gut bacteria into TMA, which is subsequently oxidized by FMO-1 and FMO-3, produced by the liver, to form TMAO. This TMAO is transported to various organ tissues and is eventually excreted by the kidneys [(23)]. TMAO potentially affects carbohydrate, TG, and cholesterol metabolism by influencing the total bile acid pool size through the reduction of bile acid production via the suppression of the crucial enzymes CYP1A1 and CYP27A1, as well as by restricting bile acid enterohepatic circulation through the repression of the organic anion transporter and the expression of the multidrug resistance protein family [(24)].

Insulin resistance appears to be the most potent contributor to the development of NAFLD [(25)]. TMAO may negatively affect insulin signalling by reducing the mRNA levels of key insulin pathway components in high-fat diet-fed mice. This finding suggested that TMAO may hinder liver glycogen synthesis and transport capacity, exacerbate insulin resistance, and promote tissue inflammation by upregulating gluconeogenesis-related genes [(26)]. On the other hand, activation of the bile acid nuclear receptor FXR changes the structure of the gut microbiota, thus affecting the metabolism of bile acid and inducing the activation of intestinal GFR5 to increase the secretion of glucagon-like peptide-1 (GP-1) in intestinal endocrine L-cells to control glucose homeostasis and improve liver insulin sensitivity and liver metabolism. FXR-deficient mice exhibit impaired insulin signalling and glucose homeostasis disorders. Therefore, FXR not only plays an important regulatory role in lipid metabolism but is also a key transcription factor in glucose homeostasis [(27; 28)]. TMAO can inhibit the activation of FXR by changing the size of the bile acid pool, which may weaken or inhibit the beneficial effect of FXR, affect the glucose metabolism of the host, and promote the occurrence and development of insulin resistance and NAFLD. Gut bacteria can convert the intake of choline into TMA, further generating TMAO in the liver, which reduces the bioavailability of choline and increases the lipid content of the newborn liver, leading to

NAFLD and even NASH. The role of choline deficiency in the development and progression of NAFLD is related to mitochondrial-related oxidative stress, lipid metabolism abnormalities, and epigenetic factors [29]. TMAO can be used as a chemical chaperone to reduce the unfolded protein response, thereby reducing endoplasmic reticulum stress [30]. As a result, TMAO likely alters hepatic TG levels, cholesterol transport, glucose and energy balance, and bile acid production and transport, indicating that TMAO is a potential risk factor for NAFLD.

L-carnitine has essential intracellular and metabolic functions and can stimulate mitochondrial functions. It is essential for long-chain fatty acid beta-oxidation, the regulation of the mitochondrial acyl-CoA/CoA ratio and the stabilization of cell membranes [31]. L-carnitine has long been considered a safe human nutritional supplement, but recently, it was found that L-carnitine, a methyl food, can generate TMAO in the body. A study showed that a high intake of methyl foods such as L-carnitine can cause oxidative stress in the livers of mice. After liver injury, mice exhibited significant increases in alanine transaminase and glutamic transaminase activity and in the lipid peroxide malondialdehyde [32].

Betaine may normalize the downstream pathways involved in insulin signal transduction, gluconeogenesis, and glycogen synthesis [33]. A study in mice revealed that betaine can restore the function of adipose tissue and sensitivity to insulin, and these effects may be attributed to the alleviation of endoplasmic reticulum stress [34]. Moreover, other studies have shown that the effect of betaine supplementation in the diet on liver steatosis in mice is related to an increase in adenosine 5‘-monophosphate -activated protein kinase (AMPK) activation in the liver [35]. It was speculated that AMPK controls the balance of liver glucose and body lipids through multiple effects on genes and short-term regulation of specific enzymes. It has been suggested that betaine supplementation can alleviate liver steatosis, which may also be caused by fatty acid oxidation and increased lipid output [36]. In addition, based on the study of betaine in terms of genome methylation, this mechanism may involve the upregulation of genes involved in de novo synthesis and fatty acid oxidation. In mice with NAFLD, several related gene expression disorders were recovered by betaine supplementation [36]. In conclusion, the role of betaine in rodents with NAFLD seems to involve multiple metabolic

pathways, and its important role is to regulate the expression of genes involved in fatty acid and lipid metabolism, thus improving the development of liver steatosis. Conversely, this may protect mitochondria from lipid toxicity caused by fatty acid oxidation failure and alleviate ER stress [36]. Other effects may be caused by the indirect effect of betaine. For example, fibroblast growth Factor 21 (FGF21) is a new metabolic regulator that is produced mainly in the liver and participates in the regulation of lipid metabolism, including lipolysis, fatty acid oxidation, and ketogenesis [37]. Betaine can increase the expression of FGF21 in the liver [38], thereby enhancing the oxidation of fatty acids.

It has been reported that TMAO can cause liver inflammation and damage, and a correlation between circulating TMAO levels and the presence and severity of nonalcoholic fatty liver disease has also been reported [39]. After 3 days of fatty degeneration in the liver tissue caused by intraperitoneal injection, the concentration of metabolites in the liver tissue of adult male Wistar rats changed, with increasing triglyceride levels but decreasing betaine and trimethylamine levels. These findings suggested that the increase in the TMAO concentration in plasma is related to the development of NAFLD. An increase in the serum concentration of TMAO is accompanied by an increase in the risk of NAFLD. Researchers have conducted a cross-sectional study among more than 3000 ordinary residents in Guangzhou, China, and the results showed that the severity of NAFLD was correlated with an increase in TMAO and Betaine concentrations and a decrease in the betaine/choline ratio [30]. This discovery indicates that TMAO not only promotes the development of NAFLD but is also is closely related to the severity of NAFLD [22]. Therefore, an increase in the serum TMAO concentration can increase the risk of NAFLD, and the TMAO concentration is an independent predictor of NAFLD.

The present study has significant strengths, including its meticulously designed 1:1 age- and sex-matched case–control approach and the use of magnetic resonance imaging (MRI) to definitively confirm the presence of fatty liver disease. However, certain limitations warrant consideration. Like with other case–control designs, our study may be susceptible to selection and recall biases, potential reversal causality, and residual confounding factors. While we took measures to minimize the risk of reversal causality by promptly collecting blood

samples upon diagnosis, the cross-sectional nature of the study necessitates vigilance in its interpretation. Additionally, approximately 17% of the controls were recruited from hospitals, although it is crucial to emphasize that they were selected from inpatients whose medical conditions were not influenced by dietary modifications. Despite adjusting for known confounders associated with NAFLD risk, the possibility of unmeasured or residual confounding factors remains, and as such, we cannot entirely discount the potential influence of additional confounders. Finally, the inclusion of hospital-based cases and controls introduces the potential for selection bias, particularly in relation to admission bias. We attempted to mitigate this bias by enlisting controls from communities within the same city or from the same hospital.

In summary, this 1:1 age- and sex-matched case–control study of Chinese adults suggested that higher plasma TMAO, L-carnitine and DMG levels are associated with increased NAFLD risk and that higher betaine levels are related to reduced NAFLD risk. In particular, the associations of NAFLD risk with L-carnitine or DMG might be mediated by TMAO. However, further cohort studies with larger sample sizes are needed to confirm the associations found in the present study.

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Abbreviations: TMAO, trimethylamine N-oxide; TMA, trimethylamine; DMG, dimethylglycine; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; HCC, hepatocellular carcinoma; FMO, flavin monooxygenase; CVD, cardiovascular disease; DM,

diabetes mellitus; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; TC, total cholesterol; TG, triglyceride; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; LC-TMS, liquid chromatography-tandem mass spectrometry; CVs, coefficients of variation; MR-PDFF, magnetic resonance proton density fat fraction; ROIs, regions of interest; BMI, body mass index; BP, blood pressure; BIA, bioelectrical impedance analysis; CIs, confidence intervals; SBP, systolic blood pressure; DBP, diastolic blood pressure; RCSs, restricted cubic splines; ROC, receiver operating characteristic; AUCs, areas under the curve; MD, mean difference; GP-1, glucagon-like peptide-1; FGF21, fibroblast growth Factor 21; MRI, magnetic resonance imaging; AMPK, adenosine 5‘-monophosphate -activated protein kinase.

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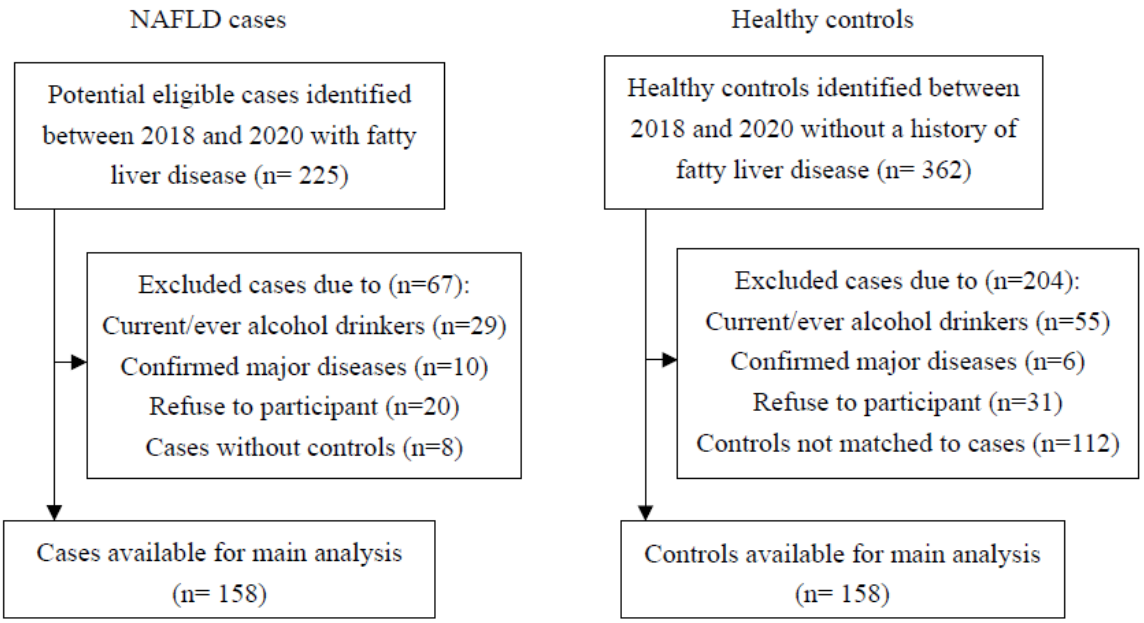

Fig.1. Flow chart of the included cases and controls.

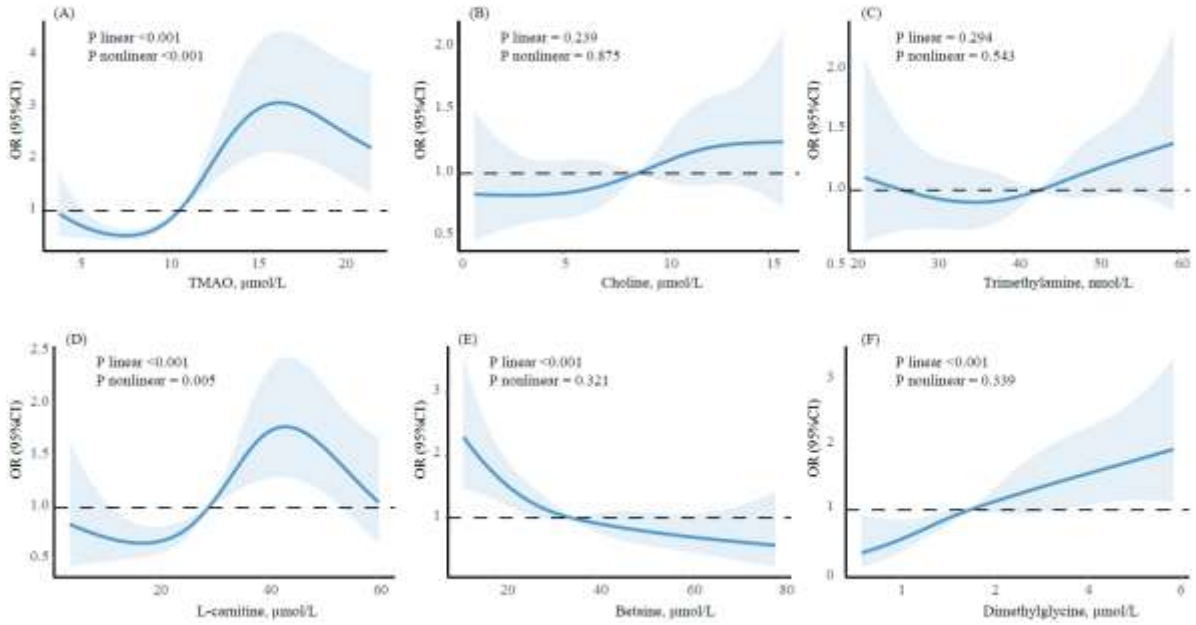

Fig.2. Restricted cubic splines nested in logistic regression analyses for associations of NAFLD risk with serum levels of (A) TMAO, (B) choline, (C) trimethylamine, (D) L-carnitine, (E) betaine, and (F) DMG.

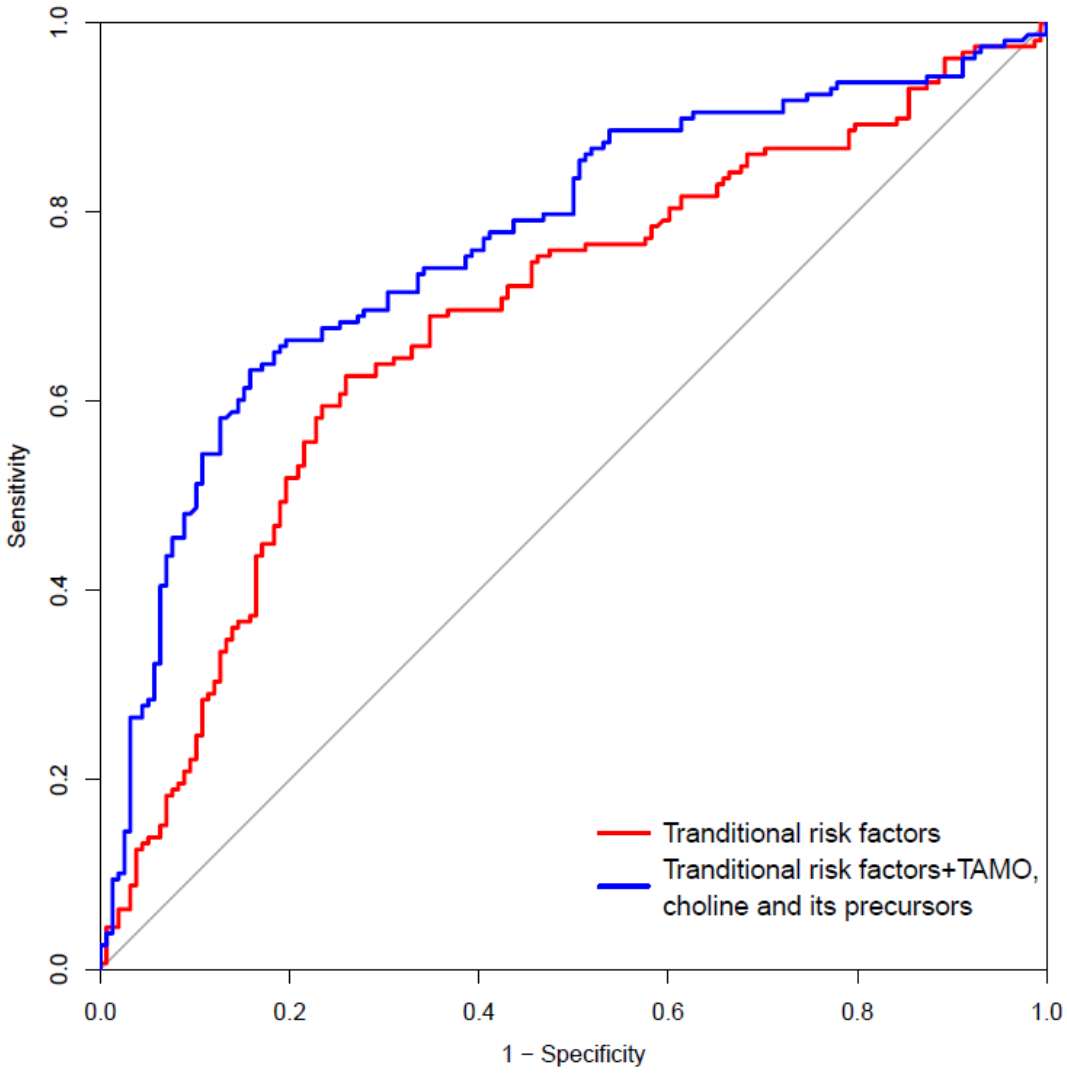

Fig.3. Receiver operating characteristic curves of traditional risk factors (blue) plus TMAO, choline and its related metabolites (red) for NAFLD.

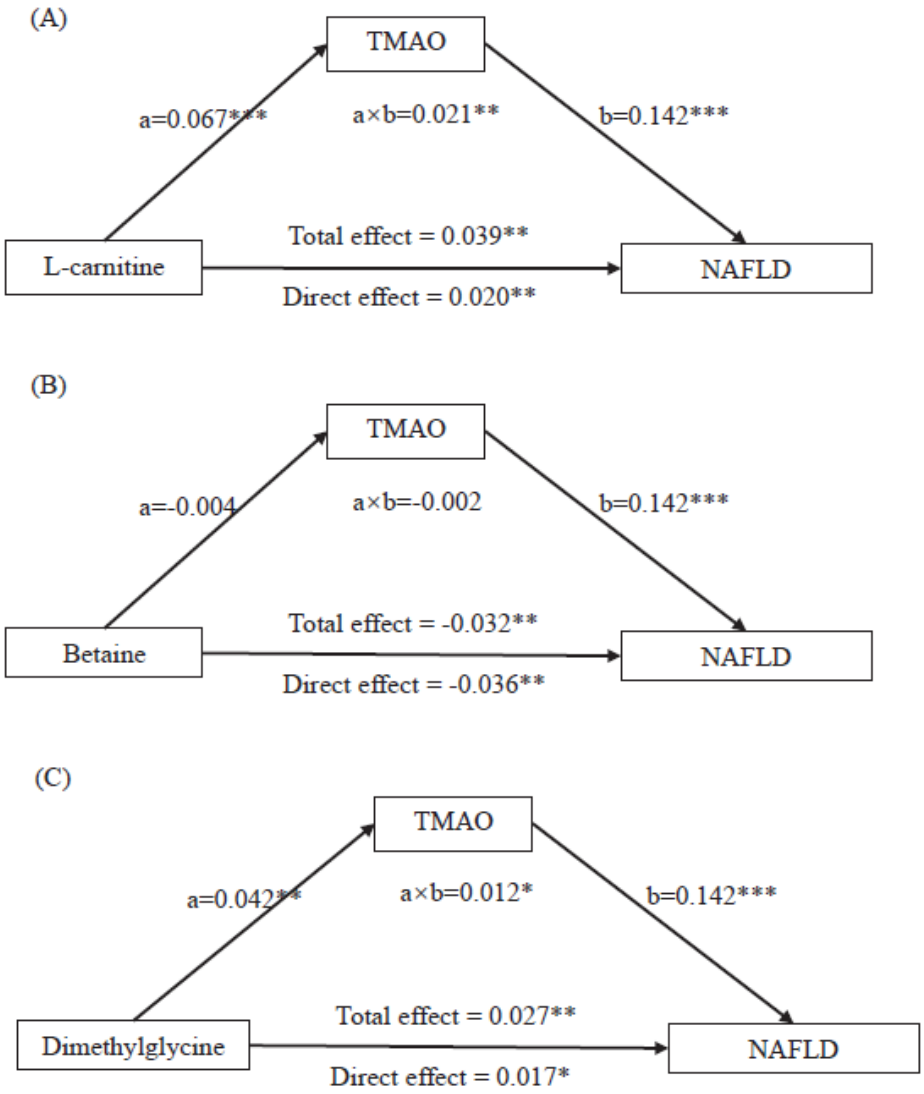

Fig.4. Indirect effects of trimethylamine-N-oxide on the association of NAFLD risk with serum levels of (A) L-carnitine, (B) betaine, and (C) DMG. *P<0.05; **P<0.01; ***P<0.001.

Table 1. Comparison of baseline characteristics and serum levels of TMAO, choline, and related metabolites between cases and controls [a]

	Controls	Cases	*P*
Number of subjects	158	158	
Age, years	61.2 ± 6.02	61.6±6.43	0.569
Female, n (%)	92 (58.2)	92 (58.2)	1.000
BMI, kg/m^2	22.1 ± 2.53	24.9 ± 2.53	**<0.001**
SBP, mmHg	120.5±17.4	128.7±16.0	**<0.001**
DBP, mmHg	73.3±9.52	79.2±8.93	**<0.001**
Marital status, n (%)			0.678
Married	144 (91.1)	147 (93.0)	
Others	14 (8.9)	11 (7.0)	
Education level, n (%)			**0.023**
<9 years	35 (24.6)	39 (27.7)	
9-12 years	55 (38.7)	71 (50.4)	
>12 years	52 (36.6)	31 (22.0)	
Current smoking, n (%)			0.932
Yes	13 (8.2)	11 (7.0)	
No	145 (91.8)	147 (93.0)	
Physical activity, min/week	109.5±28.34	102.1±26.69	**0.017**
History of hypertension, n (%)	39 (24.7)	54 (34.2)	0.064
History of type 2 diabetes, n (%)	14 (8.9)	21 (13.3)	0.210
Body fat, %	24.1±7.60	28.8±7.71	**<0.001**
TC, mmol/L	5.45±0.985	5.58±0.998	0.239
HDL-c, mmol/L	1.61±0.412	1.40±0.366	**<0.001**
LDL-c, mmol/L	3.60±0.843	3.79±0.959	0.060
Triglycerides, mmol/L	1.13±0.590	1.68±1.05	**<0.001**
Fasting glucose, mmol/L	4.94±0.87	5.30±1.51	**0.018**
TMAO, choline, and its related metabolites			
TMAO, μmol/L	8.59 (5.29, 18.59)	12.25 (6.52, 20.58)	**<0.001**
Choline, μmol/L	11.55 (8.58, 12.07)	12.04 (8.62, 12.13)	0.188
Trimethylamine, nmol/L	52.01 (28.11, 70.28)	53.14 (28.41, 76.38)	0.195
L-carnitine, μmol/L	45.28 (38.42, 60.04)	51.25 (40.42, 62.14)	**<0.001**
Betaine, μmol/L	43.31 (31.47, 51.04)	36.42 (30.17, 41.72)	**0.001**
DMG, μmol/L	3.11 (2.24, 4.08)	3.44 (2.41, 4.31)	**0.012**

[a] Continuous values are means ± SDs or medians (IQRs).

Abbreviations: TMAO: trimethylamine-N-oxide; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; TMAO, trimethylamine N-oxide; DMG, dimethylglycine.

Table 2. Associations between serum TMAO, choline and its related metabolites, and NAFLD risk

	No. (Cases/Controls)	Crude Model		Adjusted Model	
		OR (95% CI)	*P*	OR (95% CI)	*P*
TMAO, μmol/L					
T1	40/52	1.00		1.00	
T2	34/53	0.83 (0.46, 1.51)	0.551	0.84 (0.44, 1.62)	0.608
T3	84/53	2.06 (1.20, 3.52)	0.008	2.02 (1.04, 3.92)	0.038
P trend		**0.001**		**0.003**	
Choline, μmol/L					
T1	50/52	1.00		1.00	
T2	52/53	1.02 (0.59, 1.76)	0.942	1.02 (0.56, 1.86)	0.953
T3	56/53	1.10 (0.64, 1.89)	0.732	1.09 (0.60, 1.98)	0.783
P trend		0.524		0.607	
Trimethylamine, nmol/L					
T1	50/52	1.00		1.00	
T2	46/53	0.90 (0.52, 1.57)	0.717	0.88 (0.49, 1.6)	0.686
T3	62/53	1.22 (0.71, 2.08)	0.472	1.21 (0.67, 2.19)	0.528
P trend		0.317		0.388	
L-carnitine, μmol/L					
T1	44/52	1.00		1.00	
T2	37/53	0.83 (0.46, 1.47)	0.516	0.83 (0.44-1.53)	0.543
T3	77/53	1.72 (1.01, 2.92)	0.047	1.79 (1.01-3.17)	0.047
P trend		**0.021**		**0.020**	
Betaine, μmol/L					
T1	70/52	1.00		1.00	
T2	53/53	0.74 (0.44, 1.25)	0.265	0.75 (0.42, 1.34)	0.333
T3	35/53	0.49 (0.28, 0.86)	0.012	0.50 (0.28, 0.88)	0.017
P trend		**0.001**		**0.001**	
DMG, μmol/L					
T1	37/52	1.00		1.00	
T2	53/53	1.41 (0.80, 2.48)	0.240	1.39 (0.76, 2.55)	0.286
T3	68/53	1.80 (1.04, 3.14)	0.037	1.81(1.00, 3.28)	0.049
P trend		**0.010**		**0.014**	

Crude and adjusted ORs (95% CI) from the conditional logistic regression model. Covariates include age, BMI, SBP, DBP, marital status, education level, smoking status, physical activity, total cholesterol, and triglycerides.

Abbreviations: TMAO, trimethylamine N-oxide; OR, odds ratio; CI, confidence interval; DMG, dimethylglycine.