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Trimethylamine N-oxide, choline and its metabolites are associated with the risk of non-alcoholic fatty liver disease

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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 non-alcoholic 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 with the lowest tertile, the plasma TMAO concentration (OR = 2·02 (95 % CI 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 AUC 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 and 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: **Non-alcoholic fatty liver disease: Trimethylamine N-oxide: L-Carnitine: Betaine: Choline**

Non-alcoholic 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 in comparison with 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 hepatocellular carcinoma[4]. Moreover, disconcertingly, individuals are often afflicted by NAFLD at a young age, signifying an extended time frame for the development of severe complications, including cancer and CVD[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 metabolised by the hepatic enzyme

Abbreviations: DMG, dimethylglycine; FMO, flavin mono-oxygenase; NAFLD, non-alcoholic fatty liver disease; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

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flavin mono-oxygenase (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, CVD, diabetes mellitus, cancer and renal function[9].

A recent meta-analysis conducted by Theofilis *et al.*[13] comprehensively evaluated the levels of TMAO in NAFLD, revealing that NAFLD patients exhibit notably elevated circulating TMAO concentrations compared with those without NAFLD. Nonetheless, it is essential to acknowledge that the results were characterised 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 Fe 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, TMA, 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 Fe 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 1 January 2018 to 28 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 of 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 min. 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 randomised 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, TAG, LDL-cholesterol, HDL-cholesterol and fasting blood glucose levels were assessed using a sophisticated Hitachi 7600-210 automated analyser.

Liquid chromatography-tandem mass spectrometry was employed to quantify plasma TMAO, choline and their related

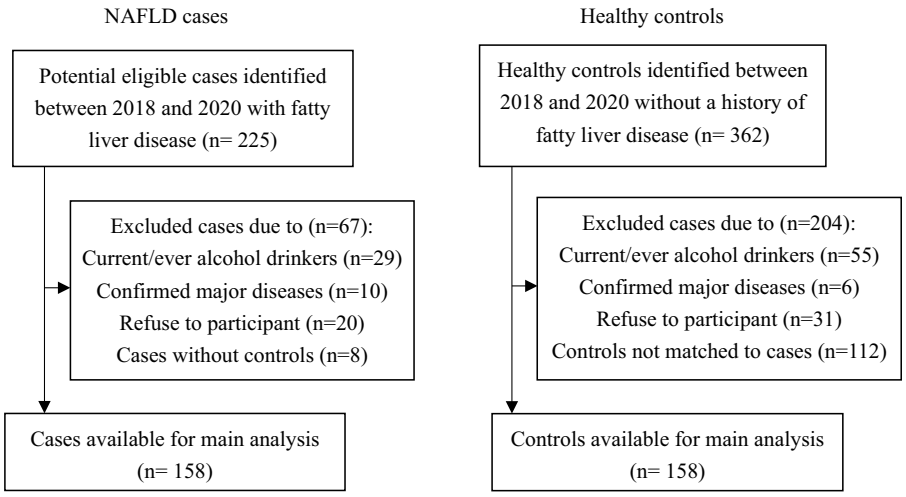

Fig. 1. Flow chart of the included cases and controls. NAFLD, non-alcoholic fatty liver disease.

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 utilised for this purpose. The internal standard utilised 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, twelve 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 CV for all biomarkers assessed ranged from 1·2 to 3·4 %, while the between-batch CV ranged between 3·4 and 7·1 %.

Abdominal MRI and diagnosis of non-alcoholic fatty liver disease

The diagnosis of NAFLD was ascertained through the application of magnetic resonance proton density fat fraction (MR-PDFF) analysis conducted by proficient radiologists. MRI was meticulously performed using a 1·5 T GE scanner equipped with an eight-channel, torso phased-array coil (Optima MR360; GE HealthCare). To construct the proton density fat fraction map, five circular regions of interest of approximately 100 mm^2 were manually delineated on the proton density fat fraction maps using the AW4.6 workstation (GE HealthCare). Among these regions of interest, 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 utilising face-to-face interviews. The questionnaire encompassed various domains, including socio-demographic 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 utilised to calculate BMI (kg/m^2). Blood pressure was assessed using two consecutive measurements performed with the participants in a seated position following at least 10 min of rest. The mean value of these readings was utilised for subsequent analyses. For the assessment of body composition, the bioelectrical impedance analysis method was employed utilising a sophisticated body composition device, namely the InBody S10 (BioSpace).

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 ($\alpha = 0{\cdot}05$), the power of the test was 90 % ($\beta = 0{\cdot}10$) 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 that they are 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 χ^2 test, as appropriate for the specific data type.

The conditional logistic regression method was elegantly employed to determine ORs and their corresponding 95 % CI 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 utilised to address potential confounding factors. The inclusion criteria for age (years), BMI (kg/m^2), systolic blood pressure (mmHg), diastolic blood pressure (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 TAG 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 were employed to investigate the plausible non-linear 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 curve analysis was conducted, facilitating the calculation of AUC 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 utilised 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 ($\beta = a \times b$) reaches statistical significance[19].

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

	Controls		Cases		*P*
Number of subjects	158		158		
	n	%	*n*	%	
Age (years)					
Mean	61·2		61·6		0·569
SD	6·02		6·43		
Female	92	58·2	92	58·2	1·000
BMI (kg/m^2)					
Mean	22·1		24·9		**<0·001**
SD	2·53		2·53		
Systolic blood pressure (mmHg)					
Mean	120·5		128·7		**<0·001**
SD	17·4		16·0		
Diastolic blood pressure (mmHg)					
Mean	73·3		79·2		**<0·001**
SD	9·52		8·93		
Marital status					0·678
Married	144	91·1	147	93·0	
Others	14	8·9	11	7·0	
Education level					**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					0·932
Yes	13	8·2	11	7·0	
No	145	91·8	147	93·0	
Physical activity (min/week)					
Mean	109·5		102·1		**0·017**
SD	28·34		26·69		
History of hypertension	39	24·7	54	34·2	0·064
History of type 2 diabetes	14	8·9	21	13·3	0·210
	Mean	SD	Mean	SD	
Body fat (%)	24·1	7·60	28·8	7·71	**<0·001**
Total cholesterol (mmol/l)	5·45	0·985	5·58	0·998	0·239
HDL-cholesterol (mmol/l)	1·61	0·412	1·40	0·366	**<0·001**
LDL-cholesterol (mmol/l)	3·60	0·843	3·79	0·959	0·060
TAG (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**
	Medians	IQRs	Medians	IQRs	
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**

TMAO, trimethylamine-N-oxide; DMG, dimethylglycine.
Values in bold indicate significant *P*-values.
* Continuous values are means ± SD or medians (IQR).

Statistical analyses were performed using R version 3.6.0 (R Foundation for Statistical Computing). 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 (SD) 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 BMI, systolic blood pressure, diastolic blood pressure, body fat, TAG levels, and fasting glucose but had lower education, physical activity and HDL-cholesterol 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, total cholesterol and LDL-cholesterol.

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 TMA 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

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

		Crude model			Adjusted model		
	No. (cases/controls)	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**		

TMAO, trimethylamine N-oxide; NAFLD, non-alcoholic fatty liver disease; DMG, dimethylglycine.
Crude and adjusted OR (95 % CI) from the conditional logistic regression model. Covariates include age, BMI, systolic blood pressure, diastolic blood pressure, marital status, education level, smoking status, physical activity, total cholesterol and TAG.
Significance of bold values at $P < 0.05$.

decreased risk of NAFLD (all *P* trends < 0·005). After adjustment for covariates, including age, BMI, systolic blood pressure, diastolic blood pressure, marital status, education level, smoking status, physical activity, total cholesterol and TAG, the associations were not significant. Compared with those of the lowest tertile, the OR (95 % CI) 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 TMA and choline (Table 2).

Figure 2 presents the results of the multivariable-adjusted restricted cubic spline analysis. A positive dose–response association was observed for NAFLD risk with serum levels of TMAO (*P* for linear < 0·001, *P* for non-linear < 0·001), L-carnitine (*P* for linear < 0·001, *P* for non-linear = 0·005) and DMG (*P* for linear < 0·001, *P* for non-linear = 0·339), whereas a linear negative association with serum betaine was observed (*P* for linear < 0·001, *P* for non-linear = 0·321). No associations were detected for serum levels of choline (*P* for linear = 0·239, *P* for non-linear = 0·875) or TMA (*P* for linear = 0·294, *P* for non-linear = 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) (Fig. 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

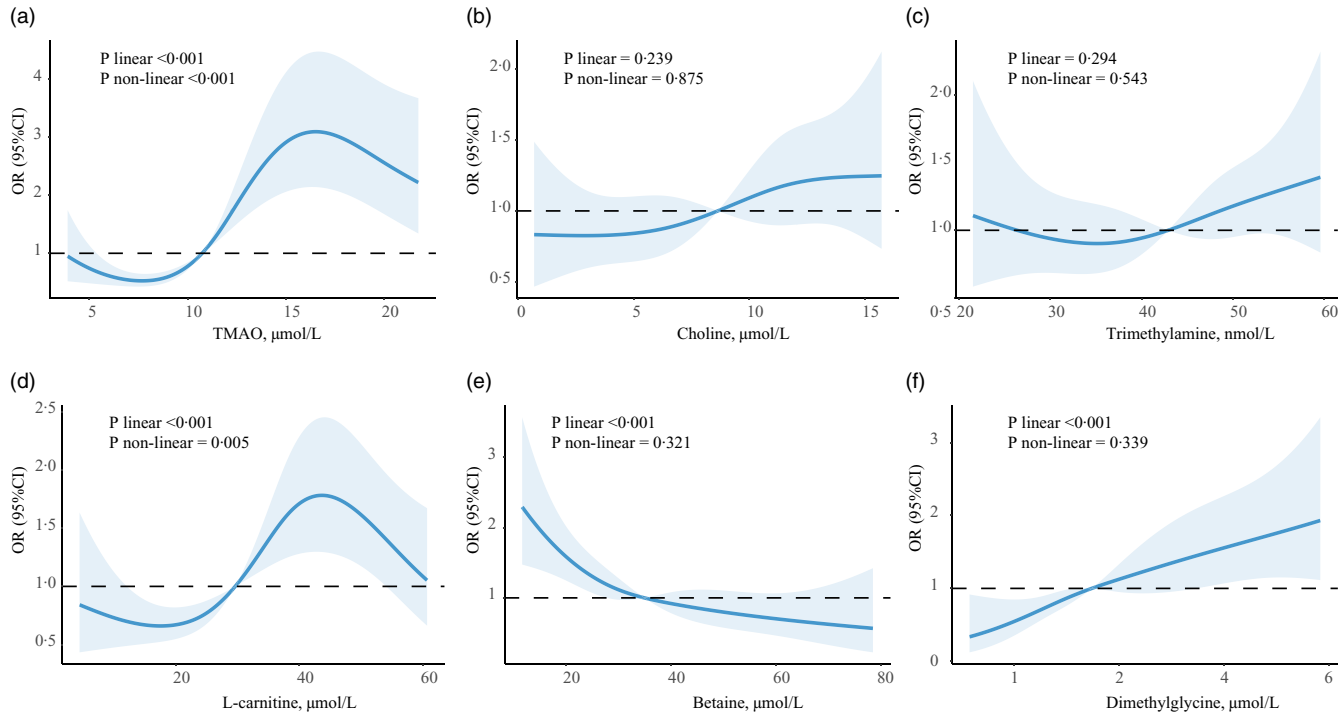

Fig. 2. Restricted cubic splines nested in logistic regression analyses for associations of NAFLD risk with serum levels of (a) TMAO, (b) choline, (c) trimethylamine, (d) L-carnitine, (e) betaine and (f) DMG. NAFLD, non-alcoholic fatty liver disease; TMAO, trimethylamine N-oxide; DMG, dimethylglycine.

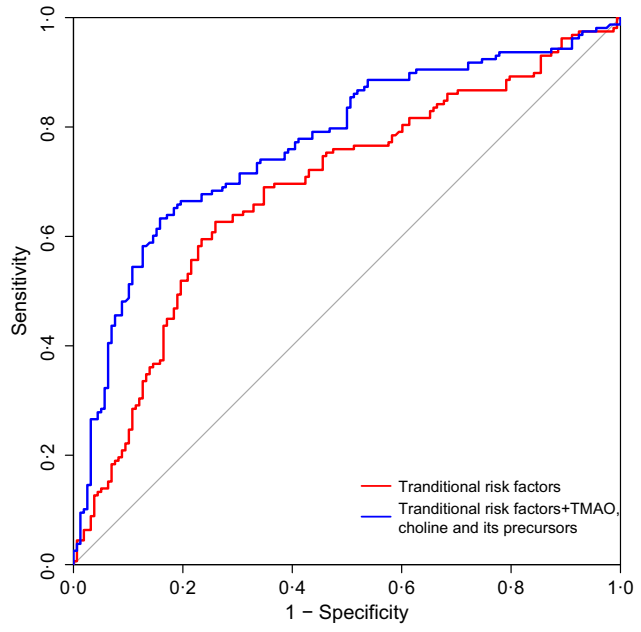

Fig. 3. Receiver operating characteristic curves of traditional risk factors (blue) plus TMAO, choline and its related metabolites (red) for NAFLD. NAFLD, non-alcoholic fatty liver disease; TMAO, trimethylamine N-oxide.

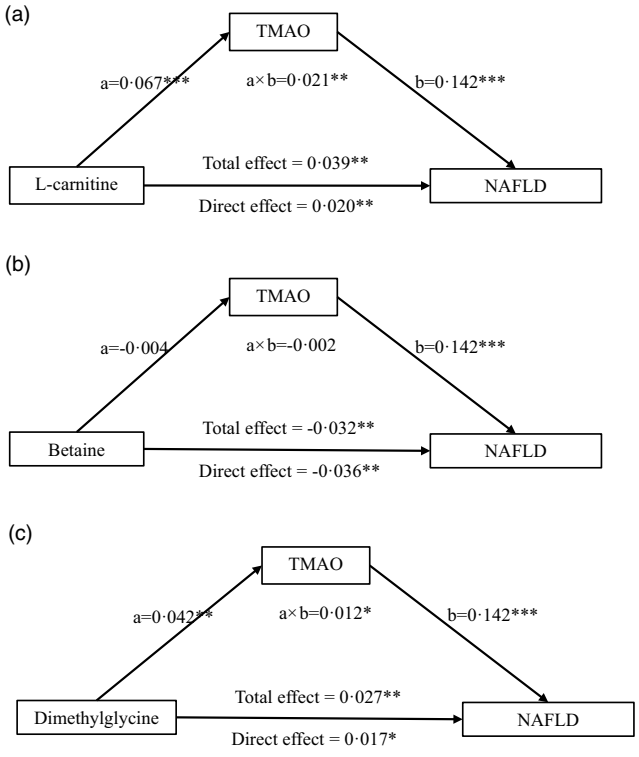

Fig. 4. Indirect effects of trimethylamine-N-oxide on the association of NAFLD risk with serum levels of (a) L-carnitine, (b) betaine and (c) DMG. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. NAFLD, non-alcoholic fatty liver disease; DMG, dimethylglycine.

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 TMA-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 seven studies with 7583 individuals reported that NAFLD patients tended to have higher levels of TMAO (standardised mean difference: 0·66, 95 % CI −0·12, 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: −15·89, 95 % CI −29·87, −1·91) and alanine aminotransferase (mean difference: −26·38, 95 % CI −45·46, −7·30), as well as TAG (mean difference: −6·92, 95 % CI −13·82, −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 sixty 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 pathophysiological underpinnings of NAFLD are multifactorial and not fully understood. Foods rich in TMA 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 oxidised 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, TAG 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 up-regulating gluconeogenesis-related genes[26]. On the other hand, activation of the bile acid nuclear receptor farnesoid X receptor (FXR) changes the structure of the gut microbiota, thus affecting the metabolism of bile acid and inducing the activation of intestinal grain filling rate 5 to increase the secretion of glucagon-like peptide-1 in intestinal endocrine L-cells to control glucose homoeostasis and improve liver insulin sensitivity and liver metabolism. FXR-deficient mice exhibit impaired insulin signalling and glucose homoeostasis disorders. Therefore, FXR not only plays an important regulatory role in lipid metabolism but is also a key transcription factor in glucose homoeostasis[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 nonalcoholic steatohepatitis. 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 stabilisation 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 lipid peroxide malondialdehyde[32].

Betaine may normalise 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 AMP-activated protein kinase activation in the liver[35]. It was speculated that AMP-activated protein kinase 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 up-regulation 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 endoplasmic reticulum stress[36]. Other effects may be caused by the indirect effect of betaine. For example, fibroblast growth factor 21 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 fibroblast growth factor 21 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 NAFLD has also been reported[39]. After 3 d of fatty degeneration in the liver tissue

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caused by intraperitoneal injection, the concentration of metabolites in the liver tissue of adult male Wistar rats changed, with increasing TAG levels but decreasing betaine and TMA 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 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 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 minimise 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 emphasise 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.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007114524000631.

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