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Trimethylamine N-Oxide levels are associated with NASH in obese subjects with type 2 diabetes

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Author contributions

Study design: PL-M, AJL, AH-V and SC-Q; acquisition of data: HV-R, XSL, DMS, STH, EO-M, BL-C, SM-R, MO-A, PG-R, IG-G, FG-P, FC-P, CA-S, EL-C, and SLH; pathology analysis: RH-P, drafting of the manuscript: PL-M, AJL, TV-M, AH-V and SC-Q; analysis and interpretation of data: PL-M and LM-K, study supervision: AJL, AH-V and SC-Q. All authors contributed to the manuscript for important intellectual content and approved the submission.

Disclosure: Dr. Hazen reports being named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. Dr. Hazen also reports being a paid consultant for P&G, having received research funds from P&G, and Roche Diagnostics, and being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland HeartLab and P&G. The other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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Abstract

Aims.—Trimethylamine N-oxide (TMAO), choline and betaine serum levels have been associated with metabolic diseases including type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD). These associations could be mediated by insulin resistance. However, the relationships among these metabolites, insulin resistance and NAFLD have not been thoroughly investigated. Moreover, it has recently been suggested that TMAO could play a role in NAFLD by altering bile acid metabolism. We examined the association between circulating TMAO, choline and betaine levels and NAFLD in obese subjects.

Methods.—Serum TMAO, choline, betaine and bile acid levels were measured in 357 Mexican obese patients with different grades of NAFLD as determined by liver histology. Associations of NAFLD with TMAO, choline and betaine levels were tested. Moreover, association of TMAO levels with non-alcoholic steatohepatitis (NASH) was tested separately in patients with and without T2D.

Results.—TMAO and choline levels were significantly associated with NAFLD histologic features and NASH risk. While increased serum TMAO levels were significantly associated with NASH in patients with T2D, in non-T2D subjects this association lost significance after adjusting for sex, BMI and insulin resistance. Moreover, circulating secondary bile acids were associated both with increased TMAO levels and NASH.

Conclusions.—In obese patients, circulating TMAO levels were associated with NASH mainly in the presence of T2D. Functional studies are required to evaluate the role of insulin resistance and T2D in this association, both highly prevalent in NASH patients.

Keywords

TMAO; non-alcoholic fatty liver disease; type 2 diabetes

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the leading cause of end-stage liver disease and liver transplantation [1-3]. NAFLD is a progressive condition that can evolve from a benign state or simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma [4]. NAFLD is strongly associated with obesity, insulin resistance (IR) and type 2 diabetes (T2D) [5-7]. All these metabolic diseases are also associated with changes in gut microbiome composition and microbiota-derived metabolites [8, 9].

Trimethylamine N-oxide (TMAO) is the product of choline metabolization by the host gut microbiome, after trimethylamine oxidation by flavin monooxygenase (FMO3) in the liver; TMAO is then excreted by the kidney [10-12]. Recent studies in humans have reported significant correlations between serum levels of TMAO and its precursors choline and

betaine with NAFLD and other cardiometabolic traits including T2D and metabolic syndrome [10, 13-17]. Moreover, several independent studies have reported associations between circulating TMAO levels and heightened adverse cardiovascular disease (CVD) outcomes in diabetic patients [16, 18, 19]. Dietary TMAO supplementation has shown different effects in the development of fatty liver in animal models; while TMAO was found to increase hepatic triglyceride accumulation and lipogenesis in mice fed with a high-fat diet [20], TMAO attenuated steatohepatitis induced by a high-fat and cholesterol diet in rats [21].

It has recently been suggested that TMAO could play a role in NAFLD by altering the bile acid profile [22]. In mice, TMAO was found to upregulate bile acid synthesis, which in turn activates farnesoid X receptor (FXR), inducing de novo lipogenesis in the liver [20]. Moreover, circulating bile acid profile abnormalities have been reported in subjects with metabolic diseases such as non-alcoholic steatohepatitis (NASH), insulin resistance and T2D [23]. However, Legry *et al* reported that bile acid levels are associated with insulin resistance, but not with NASH in subjects with obesity [24]. The authors suggest that the previously observed association of bile acids with NASH is mediated by insulin resistance.

Increased TMAO serum concentrations have been found to be associated with both T2D and NASH. However, whether the association of TMAO levels with NAFLD is confounded by insulin resistance and/or T2D is unclear. In this study, we analyzed the relationships between TMAO, betaine and choline levels with insulin resistance and NAFLD in a cohort of 357 Mexican obese patients

Materials and methods

Study population

A total of 357 male and female non-related Mexican patients with obesity (BMI ≥ 35 kg/m²) who underwent bariatric surgery, aged 20 to 60 years, were included. The characteristics of the study population have been previously described [25-27]. For biochemical evaluation, blood samples were drawn from all patients after 8-10 hours of fasting. Biochemical measurements including serum concentrations of glucose, insulin, triglycerides, total and HDL cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), adiponectin and leptin were measured as previously described [25, 26]. Insulin resistance was estimated using the updated homeostasis model assessment of insulin resistance (HOMA2-IR) in non-type 2 diabetes subjects [28]. T2D was defined as either previous diagnosis, use of glucose-lowering medication and/or fasting serum glucose levels ≥ 126 mg/dL [29]. Glomerular filtration Rate (GFR) was estimated with the CKD-EPI formula [30].

All participants provided informed consent. This study complied with the principles of the Declaration of Helsinki and was approved by the Instituto Nacional de Medicina Genómica and Hospital General Rubén Leñero Ethics Committees.

Metabolomic quantification (TMAO, choline and betaine levels)

Serum samples were collected in sterile containers (BD Microtainer) before the surgery and centrifuged at 2300×g for 10 minutes. The supernatant serum was stored at -80°C . Stable

isotope dilution liquid chromatography with tandem mass spectrometry (LC/MS-MS) was used to quantify circulating trimethylamine N-oxide, choline and betaine levels in positive ion multiple reaction monitoring (MRM) mode using characteristic parent to daughter ion transitions: m/z 76.10 \rightarrow 59.10, m/z 104.00 \rightarrow 60.05, m/z 118.10 \rightarrow 58.10, respectively. Stable isotope-labeled internal standards, d9-trimethyl-trimethylamine N-oxide, d9-choline, d9-betaine (all from Cambridge Isotope Laboratories, Tewksbury, MA, USA) dissolved in methanol were spiked to serum samples to precipitate protein and were similarly monitored in MRM mode at m/z 85.00 \rightarrow 66.15, m/z 113.10 \rightarrow 69.20, m/z 127.00 \rightarrow 66.10, respectively, as described previously [31, 32]. Varying concentrations of standards and a fixed amount of internal standards were spiked into control serum to prepare calibration curves for quantification of serum analytes. Supernatants were analyzed after injection onto a Luna silica column (2.0 x 150 mm, 5 μ m; Phenomenex, Torrance, CA, USA) at a flow rate of 0.35 ml/min composed of solvent A, 0.1% propionic acid in water, and solvent B, 0.1% acetic acid in methanol, using a Shimadzu Nexera Ultra High Performance Liquid Chromatograph system interfaced with Shimadzu 8050 Triple Quadrupole Mass Spectrometer (Shimadzu, Kyoto, Japan).

Measurement of bile acids

Serum bile acids were quantified using Tandem (liquid/gas phase) chromatography (LC-MS/MS) on a Shimadzu Nexera X2 UHPLC system coupled with the ABSciex QTRAP 4000 mass spectrometer equipped with an ESI ion source at the UC Davis West Coast Metabolomic Center. Briefly, the sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained standards at fixed concentrations. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM Ammonium Bicarbonate. Following LC/MS runs, the metabolites were identified based on the combination of chromatographic and mass spectra properties by automated comparison to the metabolomic library entries of purified standards. Batch normalization was performed using the median ratio for each metabolite in duplicate across runs.

Liver histology

Liver biopsies were obtained during surgery. Samples were stabilized in RNAlater (Ambion/Applied Biosystems, CA, USA) and stored at -80°C until histologic evaluation. Liver biopsy specimens were fixed in 10% formaldehyde embedded in paraffin, stained with hematoxylin-eosin and Masson's trichrome, and evaluated by an experienced pathologist. The histological evaluation was performed according to the Kleiner scoring system [33]. Steatosis was scored as 0-3, lobular inflammation 0-3, and hepatocellular ballooning 0-2. The sum of these pathological features was used to estimate the NAFLD activity score (NAS). Participants without steatosis were classified as non-fatty liver (non-NAFL). Fatty liver (NAFL; NAS = 2) was defined by steatosis with or without inflammation; borderline NASH (NAS = 3 to 4) was defined as the presence of some but not all features of definite steatohepatitis; and NASH (NAS = 5) was defined by the presence of steatosis, inflammation, and hepatocellular ballooning. Fibrosis was staged as grade 0-4 [33, 34].

Hepatic FMO3 gene expression

Total RNA was extracted from 131 liver biopsy using Trizol reagent (Invitrogen, Life Technologies, CA, USA), and 500 ng RNA was reverse transcribed with TaqMan Reverse Transcription Reagent (Applied Biosystems). Real-time PCR was performed in a Light Cycler 480 II (Roche), using LNA TaqMan probes from the Universal Probe Library (Roche). The following primers and probe were used to assess hepatic *FMO3* mRNA levels: gagaaaagcgcaaatggtt (forward), atgggatgttgggctttg (reverse) and probe #63. Gene expression values were normalized to the value of the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*) using the following primers tgaccttgattatttgcatacc (forward) and cgagcaagacgttcagtctt (reverse), probe #73. Relative quantification of gene expression was calculated with the Light- Cycler ® 480 Software 1.5.1.

Statistical analyses

Data that were not normally distributed (Kolmogorov-Smirnov test; e.g. TMAO, choline and betaine levels) were logarithmically transformed. Skewed variables were back-transformed for presentation in tables and figures. ANOVA was used to evaluate differences in continuous variables among NAFLD study groups, while categorical variables were compared by the chi-square test. Associations of TMAO, choline and betaine metabolites with liver histological characteristics, biochemical parameters and hepatic *FMO3* gene expression were tested using Pearson correlation analyses. Comparisons of metabolite levels between non-NASH (non-NAFL and NAFL) and NASH individuals were carried out using linear regression models. Logistic regression models were used to test for associations between metabolite level tertiles and NASH in the entire sample and stratifying by T2D. Linear and logistic regression models were adjusted for sex and BMI, while associations stratified by T2D were also adjusted for HOMA2-IR. To test for associations of TMAO with bile acid levels, partial correlations adjusted for sex, BMI and T2D were performed. *P*-values less than 0.05 were considered as statistically significant. All statistical analyses were performed using the statistical package SPSS 24.0 software (SPSS, Inc., Chicago, IL).

Results

Clinical characteristics of the study population

A total of 357 patients with liver biopsy were included in the study (38 non-NAFL and 319 with NAFLD: 88 with NAFL, 122 with borderline NASH and 109 with NASH). The baseline characteristics of the study population stratified by liver histology are summarized in Table 1. BMI, insulin, HOMA2-IR, triglycerides and liver enzymes significantly increased, while HDL-C levels decreased according to NAFLD severity ($P < 0.05$). Although the proportion of individuals with T2D and of men increased with NAFLD severity, the differences were borderline significant. In spite of only 3% of individuals had fibrosis grade 2 or 3, the number of subjects with fibrosis (F1, F2 and F3) increased significantly according to NAFLD severity (Table 1).

Elevated serum TMAO and choline levels are associated with an unfavorable metabolic profile and NAFLD severity

We then investigated whether serum TMAO, choline and betaine levels were associated with metabolic traits (Figure 1A). TMAO levels were associated with an unfavorable metabolic profile showing positive and significant correlations with insulin and insulin resistance (HOMA2-IR) in individuals without T2D (Figure 1A). Moreover, TMAO was significantly correlated with lower HDL-C as well as with higher TG and AST levels. In addition, choline levels correlated significantly with higher TG levels. Conversely, betaine levels showed a significant negative correlation negatively with glucose levels in patients without T2D.

TMAO levels showed positive and significant correlations with NAFLD histologic characteristics including steatosis grade ($r=0.170$, $P=0.001$), hepatocellular ballooning ($r=0.135$, $P=0.011$) and NAS score ($r=0.173$, $P=0.001$), and a borderline significant correlation with lobular inflammation ($r=0.098$, $P=0.064$), but not with fibrosis (Figure 1A). Choline levels also correlated positively with lobular inflammation ($r=0.113$, $P=0.032$), hepatocellular ballooning ($r=0.181$, $P=0.001$) and NAS score ($r=0.154$, $P=0.004$). In contrast, no significant correlations between betaine levels and histologic characteristics of NAFLD were observed (Figure 1A). Because of the known gender differences in NAFLD severity, we stratified the analyses by gender (84 men and 273 women). All correlations between TMAO and choline with NAFLD histologic features showed the same trend in both genders, however not all correlations reached statistical significance (Supplementary Table 1).

On stratifying by NASH, TMAO and choline levels were higher in NASH as compared to non-NASH patients ($P=0.004$ and $P=0.003$, respectively). Betaine levels showed no significant differences between groups (Figure 1B). On stratifying by tertiles, TMAO and choline tertile 3 subjects had a significantly higher risk of NASH as compared to TMAO and choline tertile 1 subjects (OR=2.77, 95%CI=1.38-5.55; $P=0.004$ and OR=4.09, 95%CI 2.02-8.30; $P=9.1 \times 10^{-5}$, respectively; Figure 1C).

Elevated serum TMAO levels are associated with NAFLD severity in patients with T2D

Because TMAO levels were found to be associated with insulin resistance, we compared TMAO levels in NASH and non-NASH patients stratifying by T2D. TMAO levels were significantly higher in NASH patients both with and without T2D ($P=0.009$ and 0.025 , respectively). However, this association lost significance after adjusting for sex, BMI and HOMA2-IR in non-T2D subjects ($P=0.083$; Figure 2A). On stratifying according to TMAO level tertiles, only patients with highest TMAO levels (tertile 3) had a significantly higher risk of NASH than TMAO tertile 1 subjects in the T2D group (OR=4.42; 95% CI=1.16-16.84; $P=0.029$ Figure 2B).

Elevated serum TMAO levels can be caused by increased TMAO synthesis (mediated by hepatic *FMO3* activity) or decreased TMAO renal clearance. In this study, no significant correlation was observed between hepatic *FMO3* gene expression and TMAO levels ($r=0.047$, $P=0.598$). Conversely, TMAO levels showed a negative and significant correlation with estimated Glomerular Filtration Rate (eGFR) ($r=-0.289$, $P=3.1 \times 10^{-8}$) in the whole

population. Interestingly, this correlation was stronger in T2D subjects ($r=-0.436$, $P=2.9 \times 10^{-9}$).

Serum TMAO levels are associated with circulating bile acids

Because the relationship between TMAO, T2D and NAFLD has been suggested to be mediated through bile acid metabolism, we sought to identify correlations between TMAO and circulating bile acids. As shown in Figure 3, TMAO levels correlated significantly with total secondary bile acids ($r=0.288$, $P=5.1 \times 10^{-5}$), both conjugated and unconjugated. Interestingly, total secondary bile acids concentrations were significantly higher in NASH than non-NASH subjects (Table 2).

Discussion

In the present study, we sought to assess TMAO, choline and betaine levels in NAFLD and NASH. In agreement with previous studies [15, 20, 35], we observed that higher TMAO and choline levels were associated with NAFLD traits and NASH, but not with fibrosis, possibly due to the reduced number of patients with advanced fibrosis. Notably, circulating TMAO levels were significantly higher in NASH patients both with and without T2D. However, in the latter group, the association lost significance after adjusting for insulin resistance, suggesting it may be mediated by this trait. Interestingly, an increased NASH risk was observed in T2D patients with elevated TMAO levels. This is consistent with increased CVD risk in diabetic subjects with high TMAO levels previously observed by several independent groups [16, 18, 19].

Because this is a cross-sectional study, causality of increased TMAO levels in NASH cannot be established. Studies in murine model have shown that modifying TMAO serum levels by liver-specific *Fmo3* gene silencing or dietary TMAO supplementation affects carbohydrate and lipid metabolism [16, 20, 36-38]. Furthermore, these effects seem to be mediated by insulin and inflammatory signaling, bile acid metabolism and lipogenesis, resulting in the accumulation of fat in liver, hepatotoxicity and liver function impairment [20, 37, 38]. Altogether, this evidence suggests increased TMAO levels could have a causal effect on these metabolic traits.

FMO3 is the main enzyme converting bacterial-derived trimethylamine to trimethylamine N-oxide (TMAO) in the liver [11]. However, in the present study there was no correlation between hepatic *FMO3* expression and serum TMAO levels and *FMO3* liver expression did not differ in non-NASH and NASH patients. In consistency, *FMO3* gene variation has not been associated with TMAO levels in population-based studies [39]. This suggests that TMAO levels are not mainly mediated by *FMO3*-dependent TMAO synthesis in humans. In contrast, serum TMAO levels correlated negatively with eGFR, suggesting TMAO renal clearance is an important mediator of elevated TMAO levels [40].

The molecular mechanisms underlying the association of TMAO with metabolic abnormalities and particularly with NASH have not been fully elucidated. Moreover, reports of the effects of TMAO supplementation on NAFLD in animal models have been inconsistent. While TMAO supplementation attenuated steatohepatitis in rats fed a high-fat

and high-cholesterol diet [21], in mice fed a high-fat diet, TMAO aggravated liver steatosis by hepatic inhibition of *FXR* signaling, downregulating *CYP7A1*-mediated bile acid synthesis and upregulating lipogenesis [20]. A previous study in a reduced number of NAFLD patients reported a positive correlation of TMAO with serum bile acid levels [20]. In agreement with this study, we observed positive and significant correlations of TMAO levels with circulating bile acids. However, while Tan et al. [20] observed an association of TMAO with primary bile acid levels, in our cohort TMAO was significantly associated with secondary bile acids (DCA, UDCA, GDCA and GLCA). In addition, secondary bile acid levels were significantly higher in patients with NASH. Because secondary BA and TMA (the precursor of TMAO) are synthesized by gut bacteria [41, 42], it is necessary to further analyze the role of the gut microbiome in the relationship between TMAO, secondary bile acids, NAFLD and T2D. Unfortunately, fecal samples for gut microbiota analysis were not available in our patients, being a limitation of the study.

Several studies in animals and in different human populations have shown that a choline-deficient diet may cause NAFLD [43-48], and fibrosis particularly in postmenopausal women [49]. In contrast, other studies have reported that higher serum choline levels are associated with increased risk of steatosis, NASH and fibrosis [15, 50], without reporting gender differences. In agreement with the latter studies, we observed increased choline levels are associated with NASH in Mexican patients with obesity. While we did not observe a gender effect, it is important to point out that information on menopause status was not available in our sample. It is unknown whether dietary choline and serum choline levels are correlated, and thus studies on factors affecting choline metabolism such as gut microbiota are required to understand these inconsistencies.

Betaine supplementation has been found to decrease the risk of NAFLD and fibrosis both in rodent models [51-57] and in human randomized controlled trials [58]. However, the results of studies seeking associations of betaine serum levels with NAFLD have not been consistent. While studies in patients from Argentina (European ancestry) and China reported lower betaine serum levels were associated with NAFLD and NASH [59], a study in European individuals with histologic diagnosis of NAFLD and the present study did not observe this association [35]. These inconsistencies could be related with differences in the BMI of study participants. Both studies reporting the association (Argentiniens and Chinese) included patients with overweight and non-morbid obesity. In contrast, the other two studies (Europeans and the present study) included patients with morbid obesity. Because reports of circulating betaine levels and NAFLD in humans are limited, further prospective studies are needed to analyze the possible influence of morbid obesity on the effect of betaine in NAFLD.

Additional limitations of the study should be acknowledged. Firstly, this study included mainly morbidly obese subjects, so further studies are required to analyze these associations in non-morbidly obese subjects. Secondly, our sample size was small for the T2D stratified analysis; however the sample size is similar to those reported in previous studies analyzing the association of TMAO with NAFLD [15, 20, 35]. Finally, because dietary parameters were not assessed, it is not possible to analyze the role of nutrients in TMAO and choline levels and how they affect the association of these metabolites with NAFLD.

In conclusion, in Mexican patients with obesity, circulating TMAO levels were associated with NASH mainly in patients with T2D. Functional studies are required to evaluate the role of insulin resistance and T2D in this association, both highly prevalent in NASH patients, and if increased TMAO levels have causal role in these metabolic traits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
IR	insulin resistance
T2D	type 2 diabetes
TMAO	trimethylamine N-oxide
FMO3	flavin monooxygenase
FXR	farnesoid X receptor
HDL-C	HDL cholesterol
AST	aspartate aminotransferase
ALT	alanine aminotransferase
GGT	gamma glutamyl transpeptidase
CYP7A1	cytochrome P450 family 7 subfamily A member 1

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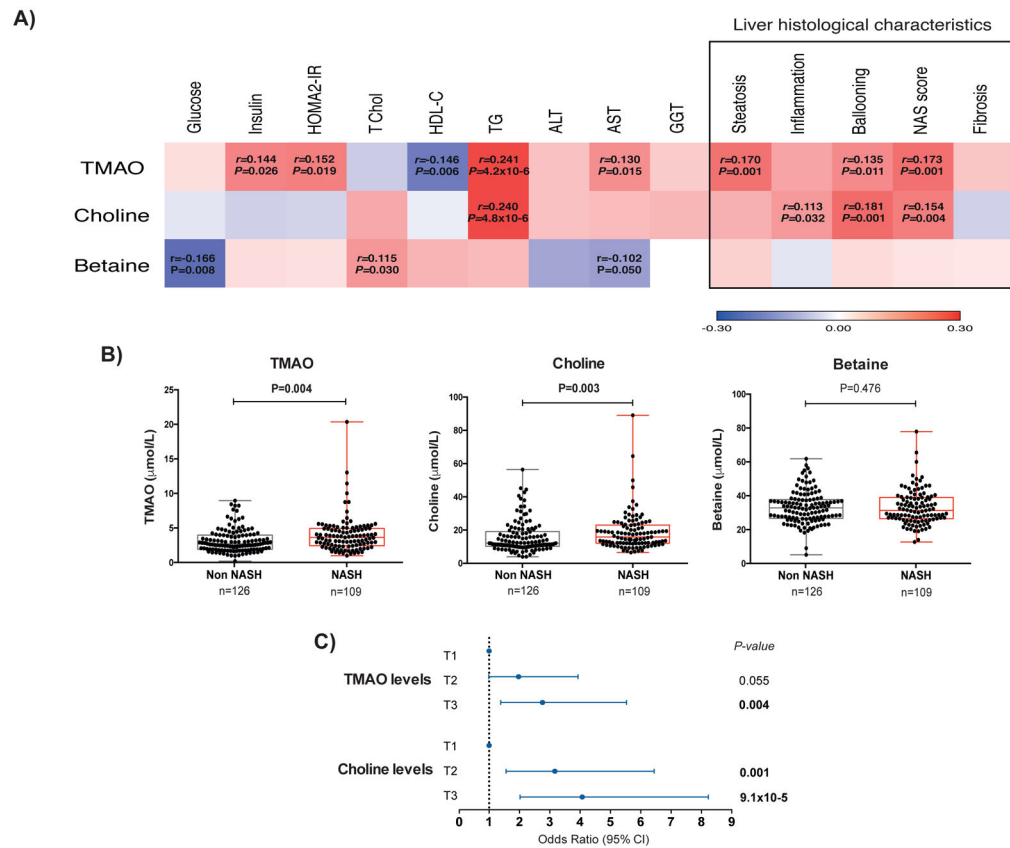


Figure 1. Associations between serum TMAO, choline and betaine levels with metabolic traits and histological features of NAFLD.

(A) Heat map of correlations between serum metabolites, metabolic traits and histologic features of NAFLD. (B) Comparison of serum TMAO, choline and betaine levels between non-NASH (non-NAFL and NAFL) and NASH subjects. Graphs showed the median values and interquartile ranges. (C) Association of TMAO and choline level tertiles with NASH. *P* values were calculated using logistic regressions adjusted by sex and BMI.

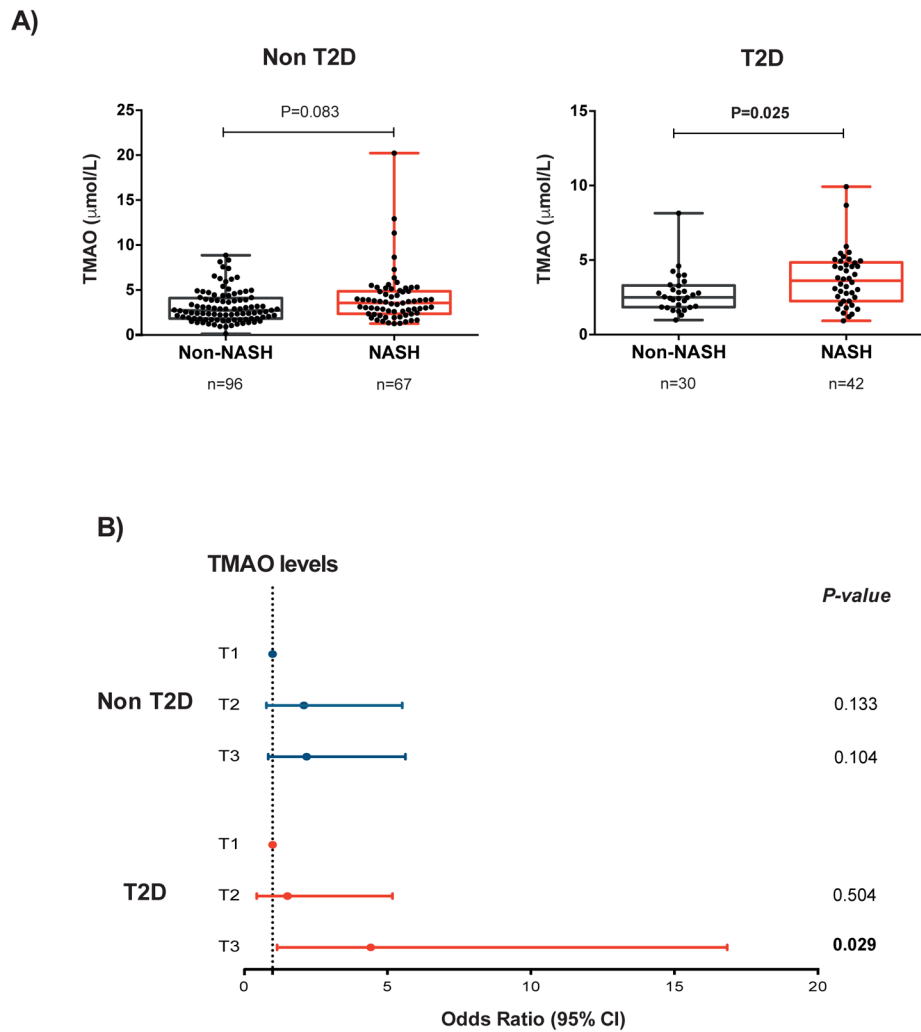


Figure 2. Associations of TMAO levels with NASH stratified by the presence of T2D
 (A) Comparisons of serum TMAO levels between non-NASH (non-NAFL and NAFL) and NASH subjects, in the absence of T2D (Non-T2D, n=163), and in the presence of T2D (n=72). Graphs show median values and interquartile ranges. (B) Association of TMAO level tertiles with NASH. *P-values* were calculated using logistic regressions adjusted by sex and BMI.

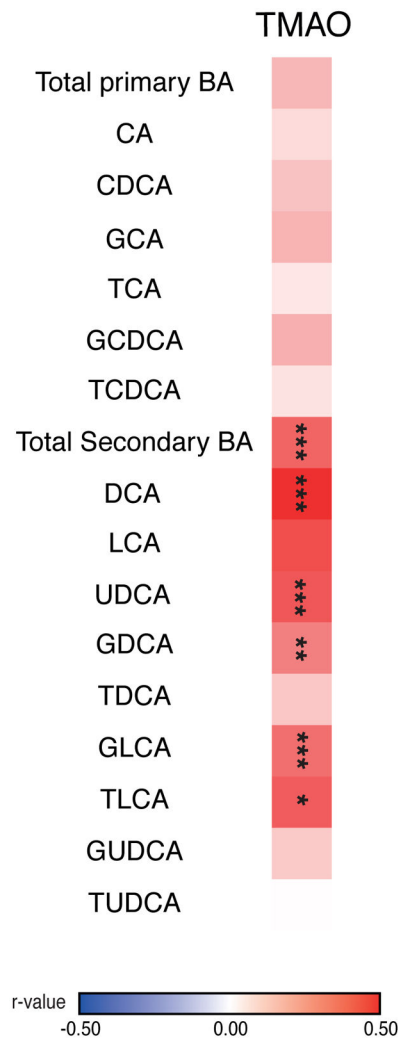


Figure 3. Correlations of serum TMAO levels with primary and secondary serum bile acids. *P*-values were adjusted by sex, BMI and T2D status. (**P*<0.05, ***P*< 0.005). Abbreviations: CA, cholate; CDCA, chenodeoxycholate; GCA, glycocholate; TCA, taurocholate; GCDCA, glycochenodeoxycholate; TCDC, taurochenodeoxycholate; DCA, deoxycholate; LCA, lithocholate; UDCA, ursodeoxycholate; GDCA, glycodeoxycholate; TDCA, taurodeoxycholate; GLCA, glycolithocholate; TLCA, tauroolithocholate; GUDCA, glycoursodeoxycholate; TUDCA, tauroursodeoxycholate.

Table 1.

Clinical and biochemical characteristics of study participants.

	Non-NAFL n=38	NAFL n=88	Borderline NASH n=122	NASH n=109	<i>P-value</i>
Age (yrs)	38.8±11.2	37.8±10.4	37.5±9.6	37.7±8.9	0.924
Sex (M/F)	6/32	15/73	28/94	35/74	0.050
BMI (kg/m ²)	41.4±5.5	43.1±6.4	45.0±6.5	44.5±5.9	0.006
Glucose (mg/dL)	91.5 (80.0-99.0)	89.0 (84.0-96.0)	92.0 (84.0-102.0)	91.0 (84.0-97.0)	0.458
Insulin (μIU/mL)	9.1 (4.8-11.9)	9.5 (7.3-13.8)	12.6 (8.0-18.5)	15.0 (12.6-20.2)	1.0x10⁻⁶
HOMA2-IR	1.2 (0.6-1.5)	1.2 (0.9-1.8)	1.6 (1.1-2.4)	2.0 (1.6-2.6)	1.5x10⁻⁶
T2D n (%)	6 (15.8)	24 (27.3)	35 (28.7)	42 (38.5)	0.049
T Chol (mg/dL)	164.7±27.7	176.6±36.1	172.6±44.0	175.3±42.0	0.461
HDL-C (mg/dL)	39.2±8.7	38.5±10.5	36.3±9.3	34.0±8.9	0.003
TG (mg/dL)	99.5 (77.8-135.3)	126.5 (102.3-164.0)	130.0 (97.8-164.3)	154.0 (120.0-214.0)	9.2x10⁻¹⁰
ALT (UI/L)	22.0 (16.0-31.3)	24.0 (18.0-34.5)	28.0 (20.0-42.0)	32.0 (23.0-47.0)	4.4x10⁻⁵
AST (UI/L)	24.0 (20.8-32.0)	24.5 (20.0-31.0)	29.0 (23.0-41.0)	30.0 (23.0-45.5)	1.5x10⁻⁴
GGT (UI/L)	14.0 (10.0-21.3)	17.0 (14.0-22.3)	19.0 (14.0-28.0)	21.0 (16.0-32.0)	8.5x10⁻⁵
F0/F1/F2/F3	34/4/0/0	81/6/0/0	89/28/4/1	77/24/4/2	0.013

Data are shown as the mean ± SD, median (interquartile range) or n (%) according to the distribution of variables. Glucose, Insulin, HOMA2-IR *P*-values were estimated considering only individuals without T2D. Data were analyzed using ANOVA, Kruskal Wallis or chi-square test. The *P*-values in bold indicate significant differences. Abbreviations: BMI, body mass index; HOMA2-IR, Homeostatic Model Assessment for Insulin Resistance; T2D, Type 2 Diabetes; T Chol, Total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; F, fibrosis.

Table 2.

Comparisons of secondary bile acid levels in non-NASH and NASH subjects.

	Non-NASH	NASH	<i>P-value</i>
Total secondary BA (nM)	57.51 (24.90-120.56)	72.28 (35.30-236.17)	0.008
DCA	12.52 (1.40-32.34)	17.20 (7.41-43.60)	0.027
UDCA	7.92 (0.12-18.05)	7.83 (3.17-21.67)	0.816
GDCA	15.85 (5.40-37.48)	19.56 (8.65-91.73)	0.035
TDCA	0.89 (0.20-2.99)	1.63 (0.53-4.73)	0.026
GLCA	3.70 (0.79-13.34)	6.54 (1.91-13.55)	0.423
GUDCA	5.36 (1.83-11.73)	8.33 (3.49-20.60)	0.024
TUDCA	0.53 (0.17-1.40)	0.91 (0.27-2.66)	0.004

Data are shown as median (interquartile range). The *P*-values in bold indicate significant differences. Abbreviations: BA, Bile acids; DCA, deoxycholate; UDCA, ursodeoxycholate; GDCA, glycodeoxycholate; TDCA, taurodeoxycholate; GLCA, glycolithocholate; GUDCA, glyoursodeoxycholate; TUDCA, tauroursodeoxycholate.

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