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Associations of plasma trimethylamine *N*-oxide, choline, carnitine, and betaine with inflammatory and cardiometabolic risk biomarkers and the fecal microbiome in the Multiethnic Cohort Adiposity Phenotype Study

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ABSTRACT

Background: Trimethylamine *N*-oxide (TMAO), a compound derived from diet and metabolism by the gut microbiome, has been associated with several chronic diseases, although the mechanisms of action are not well understood and few human studies have investigated microbes involved in its production.

Objectives: Our study aims were *1*) to investigate associations of TMAO and its precursors (choline, carnitine, and betaine) with inflammatory and cardiometabolic risk biomarkers; and *2*) to identify fecal microbiome profiles associated with TMAO.

Methods: We conducted a cross-sectional analysis using data collected from 1653 participants (826 men and 827 women, aged 60–77 y) in the Multiethnic Cohort Study. Plasma concentrations of TMAO and its precursors were measured by LC-tandem MS. We also analyzed fasting blood for markers of inflammation, glucose and insulin, cholesterol, and triglycerides (TGs), and further measured blood pressure. Fecal microbiome composition was evaluated by sequencing the 16S ribosomal RNA gene V1–V3 region. Associations of TMAO and its precursors with disease risk biomarkers were assessed by multivariable linear regression, whereas associations between TMAO and the fecal microbiome were assessed by permutational multivariate ANOVA and hurdle regression models using the negative binomial distribution.

Results: Median (IQR) concentration of plasma TMAO was 3.05 μmol/L (2.10–4.60 μmol/L). Higher concentrations of TMAO and carnitine, and lower concentrations of betaine, were associated with greater insulin resistance (all $P < 0.02$). Choline was associated with higher systolic blood pressure, TGs, lipopolysaccharide-binding protein, and lower HDL cholesterol (P ranging from <0.001 to 0.03), reflecting an adverse cardiometabolic risk profile. TMAO was associated with abundance of 13 genera (false discovery rate < 0.05), including *Prevotella, Mitsuokella, Fusobacterium, Desulfovibrio,* and bacteria belonging to the families Ruminococcaceae and Lachnospiraceae, as well as the methanogen *Methanobrevibacter smithii*.

Conclusions: Plasma TMAO concentrations were associated with a number of trimethylamine-producing bacterial taxa, and, along with its precursors, may contribute to inflammatory and cardiometabolic risk pathways. *Am J Clin Nutr* 2020;111:1226–1234.

Keywords: TMAO, choline, carnitine, betaine, inflammation, cardiometabolic, biomarkers, microbiome

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Supplemental Tables 1–6 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

Data described in the article, code book, and analytic code will be made available upon request pending application and approval.

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Abbreviations used: APS, Adiposity Phenotype Study; *cnt*, carnitine utilization gene cluster; CRP, C-reactive protein; *cut*, choline utilization gene cluster; CVD, cardiovascular disease; DBP, diastolic blood pressure; ESI, electrospray ionization; FDR, false discovery rate; FMO, flavin-containing monooxygenase; Fred Hutch, Fred Hutchinson Cancer Research Center; FWHM, full width at half maximum; HILIC, hydrophilic interaction liquid chromatography; hsCRP, high-sensitivity C-reactive protein; ICC, intraclass correlation coefficient; LBP, lipopolysaccharide-binding protein; MEC, Multiethnic Cohort; MET, metabolic equivalent of task; *mttB*, trimethylamine methyltransferase; PERMANOVA, permutational multivariate ANOVA; QC, quality control; rRNA, ribosomal RNA; SBP, systolic blood pressure; TG, triglyceride; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; UHCC, University of Hawaii Cancer Center; USC, University of Southern California.

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Introduction

There is an increasing recognition of the role of gut microbiome–derived metabolites in disease etiology. The compound trimethylamine *N*-oxide (TMAO) has been found to be associated with several chronic diseases, including cardiovascular disease (CVD) (1), colorectal cancer (2), diabetes (3–5), and chronic kidney disease (6–8). Although earlier work has shown involvement of TMAO in atherogenic processes (9), the range of different diseases suggests other mechanisms may be involved. Growing experimental evidence in animal models demonstrates a contribution of TMAO to inflammation (10) and metabolic dysfunction (11), highlighting the need for additional epidemiologic research in this area.

TMAO is obtained from the diet, both directly from foods such as fish and shellfish (12), as well as through the microbial metabolism of choline, carnitine, and betaine to trimethylamine (TMA) (13–15), which is oxidized to TMAO by hepatic flavin-containing monooxygenases (FMOs), particularly FMO3 (16). Several bacterial species involved in TMA production have been identified by culture-based methods, and include those belonging to the phyla Firmicutes, Proteobacteria, and Actinobacteria (15, 17–19). Newer bioinformatic approaches have been utilized to identify bacterial genes involved in these conversions, including *cutC/D* from the choline utilization (cut) gene cluster and the two-component Rieske-type oxygenase/reductase *cntA/B*, which metabolize choline and carnitine, respectively (20, 21). The ability to reduce TMA by other microbes, namely methanogenic archaea (22), adds yet another layer of complexity in determining circulating TMAO concentrations. Although many of these previous studies indicated which bacteria have the potential to produce TMA in the gut environment, it is less clear whether these will also be associated with TMAO in population-based studies.

In this study, we used data from a subset of the Multiethnic Cohort (MEC) Study to address 2 aims: *1*) to investigate associations of TMAO and its precursors (choline, carnitine, and betaine) with inflammatory and cardiometabolic biomarkers to better understand potential disease mechanisms of these compounds; and *2*) to identify fecal microbiome profiles associated with TMAO.

Methods

Study participants

The MEC is an ongoing prospective cohort study that recruited 215,251 men and women from Hawaii and Los Angeles, CA between 1993 and 1996 (23). MEC participants were aged 45–75 y at the time of recruitment and were predominantly from 5 self-reported racial/ethnic groups: African American, Japanese American, Latino, Native Hawaiian, and white. The current study includes participants of the MEC-Adiposity Phenotype Study (MEC-APS), a substudy that recruited 1861 participants from the MEC to investigate associations between multiomics data and body fat distribution. MEC-APS participants were selected so as to have a similar distribution of men and women across the 5 racial/ethnic groups, with stratified sampling based on BMI (in kg/m^2) categories (18.5–21.9, 22.0–24.9, 25.0–26.9, 27.0–29.9, 30.0-34.9, 35.0–40.0). Additional inclusion and exclusion criteria for the MEC-APS have been reported in detail previously (24). Notably, individuals were excluded for current or recent (<2 y) smoking, insulin or thyroid medication, dialysis, serious health conditions, and antibiotics use in the last 3 mo, as well as colonoscopy, chemotherapy, radiation therapy, or significant weight change (>9 kg) in the last 6 mo.

In the current analysis, we excluded participants that did not have TMAO and biomarker measures ($n = 9$), or had missing data on key covariates (aspirin use, diet, percentage body fat, and physical activity) ($n = 136$). Participants were also excluded if they did not have fecal microbiome measures ($n = 63$), leaving 1653 available for the current analysis (**Supplemental Figure 1**). Carnitine data were available for 1371 of these participants.

Questionnaires

Before the clinic visit, participants filled out a mailed questionnaire containing items related to demographics, health and medication history, physical activity, and a quantitative FFQ (25). Metabolic equivalent of tasks (METs) were calculated based on the reported average time spent in light, moderate, and strenuous activity during the past year. Questions related to usual eating habits of >180 food items during the last year were included in the FFQ, which has been validated and calibrated against 24-h dietary recalls within the MEC, and incorporates many ethnic-specific foods (25). Participants were asked how often they ate each food item (8 possible responses ranging from "never or hardly ever" to "2 or more times a day") and the usual serving size, which was accompanied by pictures of 3 different portion sizes to assist in estimation. Food groups were calculated as grams per day based on relevant food items, as well as portions of mixed dishes. The questionnaire was filled out by the participant at home and reviewed by study staff during the clinic visit. For the current analysis, we included food groups and nutrients that are major sources of TMAO (fish and shellfish) and its precursors (red and processed meats, eggs, fiber) (26).

Study clinic visit

At the study clinic visit to the University of Hawaii Cancer Center (UHCC) or the University of Southern California (USC), participants had anthropometrics measured, fasting blood samples drawn, and underwent whole-body DXA and abdominal MRI scanning. Venous blood was collected after an overnight fast (>8 h) in two 10-mL heparinized vacutainer tubes and two 10-mL dry tubes. Blood was processed into components within 4 h of collection and frozen at −80°C until analysis at the UHCC Analytical Biochemistry Shared Resource lab. The DXA scan (Hologic Discovery A) was performed to measure total and regional body fat mass (24). DXA image files from both study sites were centrally analyzed at the University of California, San Francisco to estimate percentage body fat.

Systolic (SBP) and diastolic blood pressure (DBP) were measured in a sitting position after 20 min of rest. Trained technicians measured blood pressure in the left arm of the participant using a digital monitor (Omron HEM-907XL). If the first 2 measurements differed by ≥10 mm Hg, a third measurement was taken and the closest 2 were averaged.

Blood cardiometabolic biomarker analysis

Serum was analyzed on a Cobas MiraPlus chemistry analyzer (Roche) for glucose using kits from Randox Laboratories

(#GL1611) and for high-sensitivity C-reactive protein (hsCRP), triglycerides (TGs), and total and HDL cholesterol using kits from Pointe Scientific (C7568, T7532, and H7545). LDL cholesterol was derived from total cholesterol and HDL cholesterol among individuals with TGs < 400 mg/dL using the Friedewald equation (27). ELISAs were used to measure serum insulin (EMD Millipore EZHI-14K) and plasma lipopolysaccharide-binding protein (LBP) (Cell Sciences CKH113). The HOMA-IR estimate was derived from glucose and insulin measurements (28, 29). Blind duplicate quality control (QC) samples (10% of study samples) were included at random in each batch and yielded CVs and intraclass correlation coefficients (ICCs) as follows: glucose: 10% and 90%; hsCRP: 61% and 86%; TGs: 18% and 95%; total cholesterol: 14% and 72%; HDL cholesterol: 18% and 80%; insulin: 14% and 96%; and LBP: 19% and 72%, respectively.

Plasma TMAO, choline, carnitine, and betaine analysis

Plasma TMAO, choline, carnitine, and betaine were analyzed by LC–tandem MS (Surveyor HPLC coupled to a TSQ Quantum™ mass spectrometer, Thermo Scientific Inc.) after electrospray ionization (ESI) in positive mode using selected reaction monitoring modified from published methods (30, 31). Plasma (0.025 mL) was mixed with 0.01 mL of an aqueous internal standard solution consisting of choline-(trimethyl-d_9) hydrochloride, betaine-(trimethyl-d_9) hydrochloride, L-carnitine-d_3 hydrochloride (all from Sigma), and trimethylamine-d_9 *N*-oxide (Cambridge Isotopes). Proteins were then precipitated by the addition of 0.215 mL acetonitrile. After mixing on a vortex for 5 min and centrifuging at ambient temperature for 5 min at 14,000 × *g*, 0.01 mL of the clear supernatant was injected onto a ZIC-c hydrophilic interaction liquid chromatography (HILIC) column (100 × 2.1 mm, 3 μm, 100 A) with a ZIC-c HILIC guard column (20 × 2.1 mm; The Nest Group). The mobile phases consisted of A = MeCN/EtOH/H_2O/100 mM NH_4OAc, pH 4.8 (40/6.8/40/13.2, by vol) and B = MeCN/EtOH/H_2O/100 mM NH_4OAc, pH 4.8 (80/6.8/12.7/0.5, by vol). Linear gradient elution was performed at a flow rate of 0.2 mL/min as follows (%A): 0–2.0 min at 20%, 2.0–5.0 min to 100%, 5.0–6.0 min hold at 100%, 6.0–6.1 min linear gradient to 20%, and equilibrate at 20% for 5.9 min.

The general MS conditions were as follows: source, ESI; ion polarity, positive; spray voltage, 4500 V; sheath and auxiliary and ion sweep gas, nitrogen; sheath gas pressure, 45 arbitrary units; auxiliary gas pressure, 5 arbitrary units; ion sweep gas pressure, 0 arbitrary units; ion transfer capillary temperature, 350°C; scan type, high-resolution selected reaction monitoring; collision gas, argon; collision gas pressure, 1.0 mTorr, source collision induced dissociation (CID) 5V; scan width, 0.01 u; scan time, 1 s; Q1 peak width was set at 0.7 u full width at half maximum (FWHM) and Q3 peak width at 0.70 u FWHM. Mass spectrometric monitoring was started 0.0 min after sample injection by multiple reaction monitoring using transitions (only ions measured were listed, collision energies applied in parentheses) for choline from *m/z* 104.109 to *m/z* 45.203 (21 eV), 58.186 (33 eV), and 60.188 (16 eV); for betaine from *m/z* 118.089 *to m/z* 42.216 (53 eV), 58.181 (25 eV), and 59.186 (18 eV); for choline trimethyl-d_9 from *m/z* 113.000 to *m/z* 49.200 (35 eV), 66.203 (32 eV), and 69.235 (17 eV); for betaine trimethyl-d_9 from *m/z* 127.000 to *m/z* 64.217 (35 eV), 66.219 (30 eV), and 68.209 (20 eV); for TMAO from *m/z* 76 to *m/z* 42.276 (42 eV) and 58.205 (19 eV); for TMAO-d_9 from *m/z* 85.00 to *m/z* 46.284 (38 eV), 66.228 (19 eV), and 68.276 (13 eV); for carnitine from *m/z* 162.098 to *m/z* 60.176 (16 eV), 85.086 (20 eV), and 103.083 (16 eV); and for carnitine-d_3 from *m/z* 165.060 to *m/z* 61.209 (40 eV), 103.128 (16 eV), and 105.154 (18 eV). Final concentrations were obtained by external calibration. Pooled QC samples (n = 111 replicates over 2 y) had the following means and CVs: TMAO (1.6 μM), 15.6%; choline (15.0 μM), 15.0%; betaine (43.0 μM), 13.6%; and carnitine (39.1 μM), 9.6%. Blind duplicate QC samples embedded at random positions throughout batches yielded acceptable ICCs: TMAO, 93%; choline, 76%; betaine, 86%; and carnitine, 73%.

Stool sample collection and fecal microbiome analysis

Participants received a stool collection kit during their clinic visit. Stool samples were collected at the participants' home into a vial containing RNAlater and frozen overnight (32). Participants were then asked to bring the sample to the UHCC or USC study center. The UHCC and USC labs stored the samples at −80°C until bulk shipments were made every 3 mo to the Fred Hutchinson Cancer Research Center (Fred Hutch), where they were stored at −80°C until processing.

Laboratory and bioinformatic processing procedures have been described previously (33). Briefly, DNA from stool samples was extracted at Fred Hutch, amplified for the V1–V3 region of the 16S ribosomal RNA (rRNA) gene, and shipped to Research and Testing Laboratory LLC (Lubbock, TX) for sequencing. Gut microbial composition of stool samples was assessed with 2 × 300-bp paired-end sequencing on the Illumina MiSeq platform. QC of sequences and inference of phylogenetic relations were done using Quantitative Insights Into Microbial Ecology version 1.8 (34) pipelines. All failed sequence reads and low-quality sequence ends were filtered. Chimeric and nonbacterial sequences were also removed. Filtered sequences were grouped into operational taxonomic units at 97% similarity and aligned for phylogenetic analysis. α- and β-diversity measures were rarefied to 9000 sequences per sample.

We also used stool samples to quantify total bacteria and *Methanobrevibacter smithii*, the predominant methanogen in the human gut, as described previously in detail (35). Briefly, quantification was done using TaqMan real-time PCR (Applied Biosystems, QuantStudio 5) with primers 8FM and 530R, and a Bac338 NED-labeled probe for total bacteria; and using PCR primers directed at archaeal 16S rRNA genes with a fluorescein-labeled (6-FAM) probe for *M. smithii*. Standard curves for *M. smithii* were generated from DNA extracted from a pure culture using the same primer probe set. Data were analyzed using QuantStudio software version 1.2.x (Applied Biosystems) to quantify the copy numbers of the 16S rRNA gene for total bacteria and *M. smithii*.

Statistical analysis

Descriptive statistics of the study population were calculated for each quartile of plasma TMAO concentration, and comparisons across quartiles were done using chi-squared tests for categorical variables and either ANOVA or Kruskal–Wallis tests

for continuous variables. The primary outcomes for our first aim were the inflammatory and cardiometabolic biomarkers, which were regressed on plasma TMAO, choline, carnitine, and betaine using multivariable linear regression. Normality was assessed using histograms and probability plots. Concentrations of TMAO, choline, carnitine, and betaine, as well as CRP, HOMA-IR, and TGs, had skewed distributions and were thus log transformed for all models to improve normality. All linear regression models were adjusted for the following potential confounding factors selected a priori: age (continuous), sex, race-ethnicity (5 groups), physical activity (METs; continuous), percentage body fat (continuous), and aspirin use (no, previous, current). For CRP, we also adjusted for the phase in which samples were analyzed by the laboratory. There was no evidence of collinearity between the included covariates in the models (all variance inflation factors <3.0).

For our second aim, we investigated whether TMAO and its precursors explained variation in fecal microbial community profiles. To identify associations between plasma TMAO and the fecal microbiome, we examined overall microbiome community structure and taxa at the genus level. Both analyses included adjustment for age, sex, race-ethnicity, physical activity, percentage body fat, and the 16S rRNA sequencing batch. The variation in the microbiome explained by TMAO and its precursors was assessed by permutational multivariate ANOVA (PERMANOVA) (999 permutations) for both unweighted and weighted UniFrac distance measures with the "adonis2" function in the R package "vegan" (36). Marginal effects were calculated using the "margin" option. We applied hurdle regression with a negative binomial distribution using the "hurdle" function in the R package pscl (37) as the primary analysis for genera. The hurdle model is a 2-component model incorporating a truncated count component which separately models the positive count data (i.e., abundance) and a zero hurdle component which models the zero counts (i.e., presence/absence). For genera that did not have zero counts, we applied the standard negative binomial model. Genera were modeled as raw counts and only those present in ≥20% of participants were included in the analysis. In addition to the aforementioned covariates, we further adjusted for total sample sequence count to account for sequencing depth. To assess the relation of TMAO and precursor compounds with *M. smithii*, we used hurdle regression models with a negative binomial distribution adjusted for age, sex, race-ethnicity, physical activity, percentage body fat, and total bacterial count.

Statistical tests were 2-sided with significance at $P < 0.05$. Adjusted P values were also calculated to correct for the false discovery rate (FDR) by using the Benjamini–Hochberg procedure (38). Analyses were conducted in R version 3.4.4 (R Foundation for Statistical Computing).

Results

Participant characteristics

Plasma TMAO differed by race-ethnicity and participants with higher TMAO had lower physical activity and were more likely to be current users of aspirin (**Table 1**). Higher concentrations of choline and carnitine were seen in the upper quartiles of TMAO, with a similar pattern across racial/ethnic groups (**Supplemental Table 1**). Dietary intake of several food groups also differed by TMAO, with greater consumption of fish, shellfish, and red meat in the higher quartiles of TMAO.

Associations between TMAO compounds and biomarkers

We identified several associations of TMAO, choline, carnitine, and betaine with inflammatory and cardiometabolic biomarkers (**Table 2**). Plasma carnitine was positively associated with CRP (β: 0.34, $P = 0.04$), whereas choline was positively associated with LBP (β: 1.78, $P = 0.03$). HOMA-IR was significantly associated with increased concentrations of TMAO, carnitine, and betaine, and showed a suggestive trend for choline. Choline was also inversely associated with HDL cholesterol (β: −14.30, $P < 0.001$) and had a suggestive positive trend with LDL cholesterol (β: 9.52, $P = 0.06$). Choline (β: 0.13, $P = 0.005$), carnitine (β: 0.18, $P = 0.001$), and betaine (β: −0.17, $P < 0.001$) were associated with TGs. Betaine was inversely associated with DBP (β: −2.15, $P = 0.01$), whereas choline was positively associated with SBP (β: 3.96, $P = 0.02$). A majority of significant associations (7 of 10) remained significant after correcting for multiple comparisons. Parameter estimates were slightly attenuated after further adjustment for total energy intake and dietary intake of seafood and red and processed meat (**Supplemental Table 2**).

TMAO and the fecal microbiome

PERMANOVA analysis showed that TMAO, choline, carnitine, and betaine each explained ∼0.1% of the total variation in the fecal microbiome (**Table 3**). TMAO ($R^2 = 0.0009$, $P = 0.007$) and betaine ($R^2 = 0.0009$, $P = 0.012$) were significantly associated with the microbiome for unweighted UniFrac, and betaine ($R^2 = 0.0013$, $P = 0.0049$) was associated with the microbiome for weighted UniFrac.

Out of 161 genera present in the sample of MEC-APS participants, 141 were present in ≥20% of participants. The abundance of 13 genera was significantly associated with plasma TMAO at FDR $Q < 0.05$ (**Table 4**). These included 3 Bacteroidetes (*Prevotella 7*, *Prevotella 2*, an uncultured Prevotellaceae); 6 Firmicutes (*Mitsuokella*, *Ruminococcaceae UCG-011*, *Ruminococcaceae NK4A214* group, *[Ruminococcus]* torques group, [*Bacteroides*] *pectinophilus* group, *Eisenbergiella*); 3 Proteobacteria (*Bilophila*, *Desulfovibrio*, uncultured Rhodospirillales); and 1 Fusobacteria (*Fusobacterium*). All but *Ruminococcaceae UCG-011* were positively associated with TMAO. Among these genera, 3 were also associated with choline (uncultured Prevotellaceae, *Ruminococcaceae UCG-011*, *Prevotella 2*) and 6 with betaine (uncultured Prevotellaceae, uncultured Rhodospirillales, [*Bacteroides*] *pectinophilus* group, *Fusobacterium*, *Bilophila*, and *Prevotella 7*) (**Supplemental Table 3**).

Abundances of 4 TMAO-associated genera (uncultured Rhodospirillales, [*Bacteroides*] *pectinophilus* group, *Prevotella* 2, and *Fusobacterium*) were also significantly associated with HOMA-IR (**Supplemental Table 4**). No genus in the zero hurdle model was significantly associated with TMAO after FDR correction (**Supplemental Table 5**).

TABLE 1 Characteristics of 1653 study participants in the Adiposity Phenotype Study by quartiles of TMAO[1]

	TMAO				
	Quartile 1 (<2.10 μmol/L)	Quartile 2 (2.10–3.05 μmol/L)	Quartile 3 (3.06–4.60 μmol/L)	Quartile 4 (>4.60 μmol/L)	P^2
n	416	411	414	412	
Age, y	69.1 ± 2.8	69.0 ± 2.7	69.2 ± 2.7	69.3 ± 2.7	0.28
Female	220 (52.9)	214 (52.1)	202 (48.8)	191 (46.4)	0.21
Race-ethnicity					0.002
African American	66 (15.9)	71 (17.3)	61 (14.7)	62 (15.0)	
Native Hawaiian	68 (16.3)	66 (16.1)	65 (15.7)	69 (16.7)	
Japanese American	126 (30.3)	91 (22.1)	92 (22.2)	118 (28.6)	
Latino	91 (21.9)	81 (19.7)	74 (17.9)	68 (16.5)	
White	65 (15.6)	102 (24.8)	122 (29.5)	95 (23.1)	
Cigarette smoking history					0.90
Never	261 (62.7)	250 (60.8)	250 (60.4)	250 (60.7)	
Former	155 (37.3)	161 (39.2)	164 (39.6)	162 (39.3)	
Physical activity, METs	1.7 ± 0.3	1.7 ± 0.3	1.6 ± 0.3	1.6 ± 0.3	0.03
Body fat, %	33.4 ± 7.7	33.4 ± 7.9	33.1 ± 7.7	33.2 ± 7.7	0.61
Aspirin use					<0.001
No	254 (61.1)	225 (54.7)	221 (53.4)	197 (47.8)	
Previous	52 (12.5)	53 (12.9)	64 (15.5)	59 (14.3)	
Current	110 (26.4)	133 (32.4)	129 (31.2)	156 (37.9)	
TMAO precursors, μmol/L					
Choline	11.5 [9.9–13.3]	12.2 [10.5–14.1]	12.7 [10.8–14.8]	13.1 [11.0–15.9]	<0.001
Betaine	40.0 [31.7–47.7]	40.2 [33.5–49.1]	41.2 [33.5–51.0]	40.3 [31.4–48.8]	0.14
Carnitine	38.8 [33.0–44.5]	38.8 [32.9–43.9]	39.5 [34.4–45.5]	41.0 [35.6–46.9]	<0.001
Total energy intake, kcal/d	1648 [1306–2200]	1666 [1248–2159]	1749 [1341–2324]	1728 [1269–2288]	0.26
Dietary intake, g/d					
Fish	16.7 [7.4–28.7]	15.8 [7.0–26.9]	17.5 [8.0–32.5]	18.9 [10.0–33.9]	0.006
Shellfish	1.9 [0.7–4.1]	1.5 [0.5–3.7]	2.1 [0.7–4.3]	2.1 [0.7–4.7]	0.02
Unprocessed red meat	21.3 [11.7–38.5]	20.5 [12.0–35.6]	23.4 [12.2–38.6]	23.1 [12.9–39.7]	0.15
Processed red meat	11.0 [5.3–20.6]	11.2 [5.5–19.2]	11.7 [5.2–23.0]	14.0 [7.3–24.4]	0.006
Total red meat	34.0 [19.1–59.1]	34.2 [18.8–56.3]	35.4 [21.3–61.2]	39.3 [22.6–66.1]	0.02
Eggs	17.2 [9.6–33.6]	16.1 [8.4–28.5]	17.6 [8.8–34.2]	16.2 [9.2–28.4]	0.45
Fiber	19.9 [13.8–28.9]	20.3 [14.1–27.8]	21.3 [14.8–29.3]	19.8 [14.0–28.9]	0.36

[1] Values are *n* (%), means ± SDs, or medians [IQRs]. MET, metabolic equivalent task; TMAO, trimethylamine *N*-oxide.

[2] *P* values for differences between quintiles calculated using chi-squared tests for categorical variables and either ANOVA or Kruskal–Wallis tests for continuous variables.

M. smithii was detected in 40.7% of participants and abundance was inversely associated with TMAO (β: −0.426, $P < 0.001$) (**Supplemental Table 6**). There was no association for *M. smithii* with choline, carnitine, or betaine.

Discussion

In the present study, we identified several associations of TMAO, and its precursors choline, carnitine, and betaine, with inflammatory and cardiometabolic biomarkers. Notably, we

TABLE 2 Parameter estimates and *P* values for regression of plasma biomarkers on TMAO, choline, carnitine, and betaine[1]

	TMAO		Choline		Carnitine		Betaine	
	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*
CRP	0.02 (0.05)	0.73	0.28 (0.15)	0.06	0.34 (0.17)	0.04	−0.03 (0.11)	0.81
Lipopolysaccharide-binding protein	0.51 (0.29)	0.08	1.78 (0.81)	0.03	0.92 (0.95)	0.33	1.05 (0.60)	0.08
HOMA-IR	0.08 (0.03)	0.005*	0.16 (0.08)	0.06	0.26 (0.10)	0.01*	−0.34 (0.06)	<0.001*
HDL cholesterol	−1.34 (0.84)	0.11	−14.30 (2.34)	<0.001*	−10.35 (2.62)	<0.001*	0.27 (1.76)	0.88
LDL cholesterol	−1.35 (1.79)	0.45	9.52 (5.06)	0.06	−4.75 (6.19)	0.44	0.57 (3.77)	0.88
TGs	0.01 (0.02)	0.46	0.13 (0.05)	0.005*	0.18 (0.06)	0.001*	−0.17 (0.03)	<0.001*
SBP	0.01 (0.62)	0.98	3.96 (1.74)	0.02	−1.47 (2.08)	0.48	−2.33 (1.30)	0.07
DBP	−0.43 (0.38)	0.25	1.04 (1.06)	0.33	−1.34 (1.26)	0.29	−2.15 (0.79)	0.01*

[1] Associations of TMAO, choline, and betaine with biomarkers were assessed in $n = 1653$ participants, whereas carnitine and biomarker associations were assessed in $n = 1371$ participants. TMAO, choline, carnitine, betaine, CRP, HOMA-IR, and TGs were log transformed. Parameters derived from multivariable linear regression models adjusted for age, sex, race-ethnicity, physical activity, percentage body fat, and aspirin use. *Adjusted $P < 0.05$ after correcting for the false discovery rate by the Benjamini–Hochberg procedure. CRP, C-reactive protein; DBP, diastolic blood pressure; SBP, systolic blood pressure; TG, triglyceride; TMAO, trimethylamine *N*-oxide.

TABLE 3 Associations of gut microbiome community structure with plasma TMAO, choline, carnitine, and betaine in the Adiposity Phenotype Study[1]

	Unweighted UniFrac		Weighted UniFrac	
	R^2	P	R^2	P
TMAO	0.0009	0.007	0.0008	0.151
Choline	0.0007	0.184	0.0004	0.569
Carnitine	0.0007	0.491	0.0007	0.389
Betaine	0.0009	0.012	0.0013	0.049

[1]Associations of TMAO, choline, and betaine with biomarkers were assessed in $n = 1653$ participants, whereas carnitine and biomarker associations were assessed in $n = 1371$ participants. R^2 and P values estimated using permutational multivariate ANOVA. All models adjusted for age, sex, race-ethnicity, physical activity, percentage body fat, and laboratory batch. TMAO, trimethylamine *N*-oxide.

found that choline showed a significant association or suggestive trend with nearly all markers and indicated risk to adverse health outcomes, and that TMAO was associated with increased insulin resistance. We also showed that TMAO was associated with abundance of several fecal bacterial genera, as well as *M. smithii*.

Although the exact effects of TMAO in the human body have yet to be elucidated, its precursors are known to play critical roles in health. Carnitine is involved in the oxidation of fatty acids (39), whereas choline is an essential nutrient necessary for production of acetylcholine, as well as for phospholipids in cell membranes (40). Betaine, which can be obtained directly from the diet as well as by metabolism of choline, serves as a methyl donor for the conversion of homocysteine to methionine (41). These compounds may also contribute to cardiometabolic disease risk. Our results for plasma choline and betaine in particular are comparable with previous epidemiologic studies, which have found unfavorable cardiometabolic risk profiles for higher concentrations of choline and lower concentrations of betaine across multiple populations (42–44).

These consistent associations across studies with components of metabolic syndrome, along with insulin resistance in our study, suggest a potential role of these compounds in diabetes. Indeed, both dietary phosphatidylcholine intake and plasma TMAO have been linked to significantly increased type 2 diabetes risk (5, 45). There has also been support in animal models; Gao et al. (11) showed that dietary supplementation of mice with TMAO led to impaired glucose tolerance, alterations in hepatic insulin signaling pathways, and promotion of adipose tissue inflammation. In addition, insulin suppressed expression of *FMO3* in vitro, and knockdown of the enzyme in insulin-resistant mice suppressed forkhead box O1 (FOXO1), a key transcription factor involved in the regulation of insulin signaling (46). It is also possible that associations between TMAO and diabetes are driven in part by changes in the gut microbiome (47), as we found several TMAO-associated genera to be related to HOMA-IR.

Interestingly, we did not find TMAO to be associated with any other disease biomarker, even though a variety of studies have linked TMAO with CVD outcomes (9, 48). However, many of these studies were conducted in participants with other diseases or patient populations undergoing medical procedures such as hemodialysis and cardiac catheterization. Thus, these associations may in part be influenced by these health outcomes or other confounding factors. Although our participants were older adults, they were relatively healthy with no serious health conditions and were not on dialysis or undergoing insulin treatment. Additional studies in healthier populations are needed and may also show divergent results, as was seen in a prospective cohort study of healthy adults, which did not find associations for TMAO with either coronary artery calcium or carotid intima-media thickness (49).

How TMAO is obtained (i.e., directly from food or derived from precursors) could also affect these associations. For example, fish is a major source of TMAO even though fish consumption has been shown to reduce risk of CVD (50). Given the higher fish consumption among Japanese Americans and Native Hawaiians (23), dietary intake of fish in our study was greater than in some previous studies conducted primarily in white and black populations (23, 51). We found similar associations when further adjusting for food sources of TMAO, although this may not have fully captured the circulating concentrations of other derived compounds that could affect disease risk.

TABLE 4 Abundance of bacterial genera associated with plasma TMAO among 1653 participants in the Adiposity Phenotype Study[1]

Phylum; class; order; family; genus	Prevalence	β (SE)	P value	Q value
Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; *Prevotella 7*	0.54	0.992 (0.152)	<0.001	<0.001
Firmicutes; Negativicutes; Selenomonadales; Veillonellaceae; *Mitsuokella*	0.23	1.444 (0.232)	<0.001	<0.001
Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; *Prevotella 2*	0.58	0.937 (0.168)	<0.001	<0.001
Fusobacteria; Fusobacteriia; Fusobacteriales; Fusobacteriaceae; *Fusobacterium*	0.53	0.931 (0.189)	<0.001	<0.001
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Ruminococcaceae UCG-011*	0.39	−0.528 (0.120)	<0.001	<0.001
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Ruminococcaceae NK4A214* group	0.86	0.330 (0.083)	<0.001	0.002
Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; uncultured	0.27	1.196 (0.353)	0.001	0.014
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [*Ruminococcus*] *torques* group	1.00	0.085 (0.026)	0.001	0.016
Proteobacteria; Deltaroteobacteria; Desulfovibrionales; Desulfovibrionaceae; *Bilophila*	0.60	0.237 (0.072)	0.001	0.016
Proteobacteria; Deltaroteobacteria; Desulfovibrionales; Desulfovibrionaceae; *Desulfovibrio*	0.26	0.890 (0.268)	0.001	0.016
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [*Bacteroides*] *pectinophilus* group	0.33	0.555 (0.174)	0.001	0.018
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Eisenbergiella*	0.69	0.346 (0.114)	0.002	0.028
Proteobacteria; Alpharoteobacteria; Rhodospirillales; uncultured; gut metagenome	0.56	0.455 (0.155)	0.003	0.037

[1]Parameters derived from the count model from hurdle regression with a negative binomial distribution or standard negative binomial regression, adjusted for age, sex, race-ethnicity, physical activity, percentage body fat, laboratory batch, and total sample sequence count. All taxa with false discovery rate–adjusted $Q < 0.05$ are included in the table. Prevalence indicates proportion of participants with each genus. TMAO, trimethylamine *N*-oxide.

Because gut microbes are necessary for the conversion of choline, carnitine, and betaine into TMA (13, 15), we hypothesized that composition of the gut microbiome would be associated with TMAO concentrations. We found that TMAO and its precursors each explained ~0.1% of overall fecal microbiome variation, which closely mirrors the relative abundance of bacteria estimated to harbor TMA-producing genes based on a previous metagenomic analysis from the Human Microbiome Project (52). Furthermore, the association may be influenced by variation of other factors related to the human conversion of TMA to TMAO as well as excretion rates of TMAO.

We found associations between TMAO and several bacterial genera, including *Desulfovibrio*, in which the *cut* gene cluster was first discovered (20). *Prevotella* were among the genera that showed the strongest association. Baseline measures of 53 participants in a controlled feeding study showed that those with an enterotype enriched with *Prevotella* had higher plasma TMAO concentrations than those with an enterotype enriched with *Bacteroides* (13). In addition, choline supplementation in $ApoE^{-/-}$ mice increased concentrations of *Prevotella* (53). Three genera from the family Lachnospiraceae were also positively associated with TMAO in our study. Several Lachnospiraceae possess *cutC/D*, especially those in *Clostridium XlVa* (52). Lachnospiraceae was also more abundant among high-TMAO producers in a crossover feeding trial of healthy young men (12). None of the genera we identified are known to have *cntA/B*, which may reflect the fact that the gene cluster is less prevalent in gut bacteria (15, 52).

Archaea also play a role in determining TMAO concentrations. Members of the Methanomassiliicoccales order can use TMA to produce methane through the trimethylamine methyltransferase (*mttB*) gene (22, 54). This has been shown experimentally, as the strain *Methanomassiliicoccus luminyensis B10* was able to reduce TMA and H_2 for methanogenesis (22). Although *M. smithii* does not possess the *mttB* gene, it has been shown to reduce plasma TMAO concentrations in mice (55). Our results lend support to a role of *M. smithii* in TMAO formation, as we found the methanogen to be inversely associated with TMAO. Additional studies are needed to better understand how *M. smithii* and other archaea affect TMAO production.

Our study has several strengths and limitations. First, we had an ethnically diverse and relatively healthy study population, whereas many of the TMAO studies to date have been limited to white and/or black participants with metabolic disease or undergoing various medical procedures. Second, we were also able to assess associations of disease biomarkers not only with TMAO, but also choline, carnitine, and betaine. Third, this is one of few population-based studies of TMAO and the gut microbiome, and is, to our knowledge, the largest to date. A limitation of our study was the cross-sectional design, which limited inference of causality between TMAO and its precursors and disease risk biomarkers. As with all observational studies, there is potential for residual confounding, although our well-characterized study population allowed for adjustment of risk factors in biomarker and microbiome analyses. We also used 16S rRNA gene data, which did not allow us to directly examine bacterial functional genes; however, several of the genera we found to be associated with TMAO include species that possess the *cut* genes, which suggests an important role for this gene cluster.

In summary, our findings lend support to a possible role of TMAO and its precursors, particularly choline, in cardiometabolic risk, as well as associations between TMAO and fecal microbiota. Longitudinal studies of TMAO and its precursors in disease risk are needed, along with additional population-based studies of TMAO and the gut microbiome.

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