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Dietary factors, gut microbiota, and serum trimethylamine-*N*-oxide associated with cardiovascular disease in the Hispanic Community Health Study/Study of Latinos

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ABSTRACT

Background: Trimethylamine-*N*-oxide (TMAO), a diet-derived and gut microbiota–related metabolite, is associated with cardiovascular disease (CVD). However, major dietary determinants and specific gut bacterial taxa related to TMAO remain to be identified in humans.

Objectives: We aimed to identify dietary and gut microbial factors associated with circulating TMAO.

Methods: This cross-sectional study included 3972 participants (57.3% women) aged 18–74 y from the Hispanic Community Health Study/Study of Latinos in the United States. Dietary information was collected by 24-h dietary recalls at baseline interview (2008–2011), and baseline serum TMAO and its precursors were measured by an untargeted approach. Gut microbiome was profiled by shotgun metagenomic sequencing in a subset of participants ($n = 626$) during a follow-up visit (2016–2018). Logistic and linear regression were used to examine associations of inverse-normalized metabolites with prevalent CVD, dietary intake, and bacterial species, respectively, after adjustment for sociodemographic, behavioral, and clinical factors.

Results: TMAO was positively associated with prevalent CVD (case number = 279; OR = 1.34; 95% CI: 1.17, 1.54, per 1-SD). Fish ($P = 1.26 \times 10^{-17}$), red meat ($P = 3.33 \times 10^{-16}$), and egg ($P = 3.89 \times 10^{-5}$) intakes were top dietary factors positively associated with TMAO. We identified 9 gut bacterial species significantly associated with TMAO (false discovery rate <0.05). All 4 species positively associated with TMAO belong to the order *Clostridiales*, of which 3 might have homologous genes encoding carnitine monooxygenase, an enzyme converting carnitine to trimethylamine (TMA). The red meat–TMAO association was more pronounced in participants with higher abundances of these 4 species compared with those with lower abundance ($P_{interaction} = 0.013$), but such microbial modification was not observed for fish–TMAO or egg–TMAO associations.

Conclusion: In US Hispanics/Latinos, fish, red meat, and egg intakes are major dietary factors associated with serum TMAO. The identified potential TMA-producing gut microbiota and microbial modification on the red meat–TMAO association support microbial TMA production from dietary carnitine, whereas the fish–TMAO association is independent of gut microbiota. *Am J Clin Nutr* 2021;113:1503–1514.

Keywords: cardiovascular disease, diet, gut microbiota, trimethylamine-*N*-oxide, Hispanic Americans

Introduction

Trimethylamine-*N*-oxide (TMAO), a potential diet-derived and gut microbiota (GMB)–related metabolite, has been associated with an increased risk of cardiovascular disease (CVD) independent of traditional risk factors in numerous clinic-based studies (1–3). Environmental and host factors, such as GMB,

dietary intake, and host genetics, are thought to independently and jointly influence circulating concentrations of TMAO (4), but data from human population-based studies are still limited. Identifying potential drivers for alterations in circulating TMAO could have preventive and therapeutic implications for CVD.

Dietary trimethylamine (TMA)-containing nutrients, including choline, phosphatidylcholine, carnitine, and betaine, abundant in animal foods such as red meat (e.g., beef and pork) and eggs, can be converted to TMAO in humans through a series of physiological processes (1, 5). Specifically, TMA is liberated from TMA-containing nutrients by intestinal bacteria (4), passively absorbed into the circulation system, and then oxidized to TMAO by flavin monooxygenases in the liver (1). In addition to TMAO produced from dietary precursors, preformed TMAO can be absorbed in a manner not involving gut microbes in humans (6) and animals (7). A human feeding study has shown that fish (cod fillet) contains a high concentration of TMAO, and that circulating TMAO was elevated within 15 min of fish consumption (6). However, human population studies assessing the relation between dietary factors and TMAO have been inconclusive (8–11), potentially due to differences in dietary habits, GMB, or host genetic background across populations. Major dietary determinants of circulating TMAO remain open for investigation in human populations.

As to the role of GMB in modulating circulating TMAO, it has been found that antibiotics can suppress blood concentrations of TMAO in mice (1) and humans (2). Two recent studies using 16S rRNA gene amplicon sequencing (16S) data identified several GMB features (e.g., genera from the orders *Clostridiales*, *Bifidobacteriales*, and *Bacteroidales*) associated with circulating TMAO (12, 13). In addition, previous studies have identified that choline TMA-lyase (choline utilization, CutC) and its activator CutD (14), and a 2-component Rieske-type oxygenase/reductase (carnitine monooxygenase, CntA/B) (15), can synthesize TMA from choline and carnitine, respectively. It has been suggested that CutC might be carried by *Clostridium* XIVa strains and *Eubacterium* sp. strain AB3007, and CntA might be carried by *Escherichia coli* (16). However, the specific gut bacterial taxa that can metabolize the TMA-containing nutrients into TMA and thus elevate host TMAO concentrations remain to be identified in human populations. Moreover, the potential interaction between GMB and dietary factors (e.g., red meat intake) on circulating TMAO concentrations also remains unexplored in human populations.

In this study of US Hispanics/Latinos, we aimed to: *1*) examine associations of serum TMAO and precursor metabolites (choline, carnitine, and betaine), with prevalent CVD; *2*) examine associations of dietary factors with serum concentrations of TMAO and precursor metabolites; and *3*) identify GMB features associated with serum TMAO.

Methods

Study design and population

The Hispanic Community Health Study/Study of Latinos (HCHS/SOL) is a prospective community-based cohort study of 16,415 Hispanic/Latino adults aged 18–74 y at recruitment who were living in 4 US urban areas (Bronx, NY; Chicago, IL; Miami, FL; and San Diego, CA). A 2-stage area probability sample design was used to recruit participants, which has been previously described (17, 18). A comprehensive set of interviews and a clinical examination with fasting blood draw were conducted by trained and certified staff at in-person clinic visits from 2008 to 2011. The HCHS/SOL Gut Origins of Latino Diabetes (GOLD) ancillary study was conducted during 2016–2018 to investigate the role of GMB composition in the risk of diabetes and other health outcomes, with a total of 3057 participants enrolled from the HCHS/SOL approximately concurrent with the second in-person visit period from 2014 to 2017 (19). The study was approved by the institutional review boards of corresponding site institutions. Written informed consent was obtained from all participants.

Ascertainment of cardiometabolic diseases

Cardiometabolic diseases were ascertained using information collected at the 2008–2011 examination. Diabetes was defined as either current use of antidiabetic medications, or fasting glucose ≥126 mg/dL, 2-h oral-glucose-tolerance test plasma glucose ≥200 mg/dL, or glycated hemoglobin ≥6.5% (20). Hypertension was defined as systolic/diastolic blood pressure ≥140/90 mmHg or currently taking antihypertensive medications. Dyslipidemia was defined as serum LDL cholesterol ≥160 mg/dL, HDL cholesterol <40 mg/dL, or triglycerides ≥200 mg/dL or currently taking antihyperlipidemic medications (21). CVD was ascertained according to self-reported physician

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Supplemental Tables 1–7 and Supplemental Figures 1–4 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/.

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Abbreviations used: CDCR, cerebrovascular disease or carotid revascularization; CHD, coronary heart disease; *Cnt*, carnitine monooxygenase; *Cut*, choline utilization; CVD, cardiovascular disease; FDR, false discovery rate; GMB, gut microbiota; GOLD, Gut Origins of Latino Diabetes; HCHS/SOL, Hispanic Community Health Study/Study of Latinos; NCBI, National Center for Biotechnology Information; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

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diagnosis of coronary heart disease (CHD) (including a history of a cardiac event or procedure, or electrocardiographic evidence of myocardial infarction observed) or cerebrovascular disease or carotid revascularization (CDCR) (history of stroke, mini-stroke, or transient ischemic attack, or balloon angioplasty or surgery to the arteries in the neck) (21).

Assessment of diet and other covariates

Dietary information was collected by two 24-h dietary recalls as described previously (22, 23). The first recall was performed by in-person interviews during the 2008–2011 examination, and the second recall was conducted via telephone ~30 d after the first interview. Participants estimated portion sizes with the use of food models (for the in-person interviews) or a food-amount booklet (for the telephone interviews). Foods and nutrients were analyzed using the multiple-pass methods of the Nutrition Data System for Research software (version 11) from the Nutrition Coordinating Center at the University of Minnesota (24). The present analyses mainly focused on 15 major food groups, and 36 major macronutrients and micronutrients were also examined in secondary analyses. Participants who had ≥1 set of 24-h dietary recall data (97% had the first 24-h dietary recall) were included in the current study. Because circulating TMAO concentrations increased over a short time in response to animal source foods (6), we used the first 24-h dietary recall data, which were concurrent with fasting blood draw in terms of the collection time in the main analyses. For those who had no data from the first 24-h dietary recall, data from the second 24-h dietary recall were used.

Sociodemographic and behavioral characteristics of participants, including Hispanic/Latino background, education attainment, annual household income, smoking, and alcohol consumption, in addition to medical and family histories, were collected using structured questionnaires (17, 18). Physical activity was measured using the Global Physical Activity Questionnaire and the metabolic equivalent-hours/day was derived (25). BMI was computed as measured weight (in kilograms) divided by measured height (in meters) squared.

Serum metabolite measurement

Metabolomic profiling was performed on serum specimens collected during the 2008–2011 examination from 3972 participants randomly selected from the entire study population, using an untargeted LC-MS approach based on the discoveryHD4 platform at Metabolon Inc. Details of sample extraction, separation, and MS analysis have been described elsewhere (26). Briefly, this approach utilized a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. Metabolite peaks were identified and confirmed using authentic reference standards. Metabolites were measured using AUC of the peaks. TMAO and 3 precursor metabolites (choline, carnitine, and betaine) were captured by this method and were included in the present analysis. The undetectable rates of these 4 metabolites were all <0.1%. Missing values of the 4 metabolites were imputed with half of the detectable minimum values. A rank-based inverse normal transformation was applied to the metabolite data before analyses (27).

Gut microbiome shotgun metagenomics sequencing

In the GOLD ancillary study, enrolled participants were provided with a stool collection kit. For each participant, a single fecal specimen was self-collected using a disposable paper inverted hat (Protocult collection device, ABC Medical Enterprises, Inc.). Detailed sample processing and DNA extraction procedures have been described previously (19). A shallow shotgun metagenomics sequencing, which has been recommended to obtain species-level taxonomic data as an alternative to 16S sequencing at around the same cost for large human microbiome studies where deep whole-metagenome shotgun sequencing can be cost-prohibitive (28), was performed on the Illumina NovaSeq platforms (29). Sequences were boosted using inference from reference genomes of likely genomic content. The adapters and barcode indices were processed following the iTru adapter protocol (30). Shallow shotgun data were left trimmed to remove low-quality bases that had a Phred quality score of ≤25 using prinseq-lite 0.20.4 (31). The quality controlled paired-end data were then concatenated and aligned against the National Center for Biotechnology Information (NCBI) RefSeq representative prokaryotic genome collection (release 82) (32) using default SHOGUN (28) settings. Samples with a coverage depth <100,000 reads per sample were excluded. Finally, the average reads per sample was 436K (range 100K–4122K). Of 3035 samples with sequencing data, 2764 samples passed all quality control metrics and were included in the present analyses. Bowtie2 (33) was used to build the SHOGUN assemblies. The reads that mapped to a single reference genome were labeled with the NCBI taxonomic annotation at the species level. Those reads that mapped to multiple reference genomes were labeled as the last common ancestor of each label according to the NCBI taxonomy (28). Cumulative sum scaling normalization was conducted for the species-level abundance of taxonomic units (34) before analyses.

Statistical analysis

We examined cross-sectional associations of TMAO and its precursor metabolites with prevalence of CVD and its constituent components, CHD and CDCR, in 3827 participants without cancer. For the analysis on dietary factors and serum concentrations of TMAO and its precursor metabolites, we further excluded participants with prevalent CVD ($n = 279$), with missing dietary information ($n = 28$), or with extreme dietary energy intake (>6000/<600 kcal/d in men, or >4000/<400 kcal/d in women) ($n = 54$), leaving 3466 participants (**Supplemental Figure 1**). Next, in a subgroup of participants ($n = 626$) who had both metabolite and GMB data, we examined the associations of gut microbial α-diversity and species abundance with TMAO concentrations (Supplemental Figure 1).

Characteristics of the study participants were presented according to quartile of TMAO as means ± SDs or percentages where appropriate. Participant characteristics were compared across the quartile of TMAO by using 1-factor ANOVA for continuous variables and χ^2 test for categorical variables. Associations of TMAO and its precursor metabolites with the prevalence of CVD, CHD, and CDCR were assessed using multivariable logistic regression models with adjustment for age,

TABLE 1 Characteristics of participants according to quartiles of serum TMAO concentration[1]

	Quartiles of TMAO				
	Q1 ($n = 957$)	Q2 ($n = 957$)	Q3 ($n = 957$)	Q4 ($n = 956$)	*P*
Age, y	41.3 ± 13.9	45.0 ± 13.2	47.2 ± 13.6	48.4 ± 13.5	<0.001
Female	592 (61.9)	557 (58.2)	539 (56.3)	476 (49.8)	<0.001
Education, y					
1–10	319 (33.4)	335 (35.1)	348 (36.4)	363 (38.0)	0.56
11–12	256 (26.8)	253 (26.5)	249 (26.0)	241 (25.2)	
>13	380 (39.8)	367 (38.4)	359 (37.6)	351 (36.8)	
Household yearly income, US$					
≤20,000	577 (60.3)	607 (63.4)	619 (64.7)	610 (63.8)	0.37
20,001–50,000	323 (33.8)	292 (30.5)	278 (29.0)	297 (31.1)	
>50,000	57 (6.0)	58 (6.1)	60 (6.3)	49 (5.1)	
Background					
Dominican	122 (12.8)	96 (10.1)	98 (10.3)	63 (6.6)	0.004
Central American	84 (8.8)	91 (9.5)	104 (10.9)	109 (11.4)	
Cuban	147 (15.4)	159 (16.7)	178 (18.6)	156 (16.3)	
Mexican	359 (37.7)	332 (34.8)	329 (34.4)	365 (38.2)	
Puerto Rican	148 (15.5)	190 (19.9)	157 (16.4)	180 (18.8)	
South American	61 (6.4)	52 (5.5)	58 (6.1)	57 (6.0)	
Other/more than one	32 (3.4)	34 (3.6)	32 (3.3)	25 (2.6)	
Smoking					
Never	615 (64.3)	584 (61.0)	534 (55.8)	507 (53.1)	<0.001
Former	161 (16.8)	182 (19.0)	196 (20.5)	213 (22.3)	
Current	180 (18.8)	191 (20.0)	227 (23.7)	234 (24.5)	
Alcohol consumption, g/d	3.3 ± 16.0	2.7 ± 12.1	3.7 ± 15.7	4.2 ± 16.0	0.09
BMI, kg/m^2	29.0 ± 6.1	29.7 ± 6.1	30.2 ± 6.1	30.1 ± 5.8	<0.001
Physical activity, MET-min/d	638.9 ± 935.2	625.9 ± 974.2	625.4 ± 954.6	659.8 ± 1059.2	0.66
Total energy intake, kcal/d	1978.5 ± 993.3	1977.0 ± 966.5	2023.2 ± 1030.1	2069.2 ± 1119.1	0.03
Diabetes	138 (14.4)	141 (14.7)	201 (21.0)	267 (27.9)	<0.001
Hypertension	201 (21.0)	239 (25.0)	301 (31.5)	333 (34.8)	<0.001
Dyslipidemia	343 (35.8)	365 (38.2)	377 (39.4)	418 (43.8)	0.004

[1]Data are mean ± SD, or *n* (%). *P* values were calculated from the 1-factor ANOVA for continuous variables and χ^2 test for categorical variables. MET, metabolic equivalent task; Q, quartile; TMAO, trimethylamine-*N*-oxide.

sex, BMI, study field center, Hispanic/Latino background, education, yearly household income, smoking, alcohol consumption, physical activity, and total energy intake. Associations of dietary factors (including food groups and nutrients) with TMAO and its precursor metabolites were assessed using linear regression models, with controlling for the aforementioned covariates plus diabetes, hypertension, and dyslipidemia. To reduce potential influences of extreme values, quintiles were created for all dietary intake variables. For the food groups with a percentage of nonconsumers >20%, nonconsumers were coded as 0, and the other consumers were coded as 1–4 by quartiles. Finally, the continuous quintile ranks (ranged 0–4) were used in the analyses (**Supplemental Table 1**).

The gut microbial α-diversity and species abundance were examined for associations with serum TMAO using linear regression, after controlling for the aforementioned covariates in addition to use of antibiotics and probiotics. Next, we explored potential effect modification on diet–metabolite associations by gut microbial species. We examined associations between food groups (i.e., fish, red meat, eggs) and TMAO concentrations stratified by abundance of microbial species (i.e., above compared with below median) that were significantly and positively associated with TMAO in our study, because these bacteria might have the potential to produce TMAO and thus might modify the associations of red meat and egg intake with TMAO. In addition, we also calculated a GMB score (range 0–4) based on the abundance of the 4 microbial species positively associated with TMAO (less than median value = 0; and equal to or greater than median value = 1) to represent an overall GMB feature positively associated with TMAO. We then examined associations of food groups with TMAO across 3 levels of the GMB score (0, low GMB score; 1–2, medium GMB score; and 3–4, high GMB score). The interactions between food groups and GMB score on serum TMAO concentrations were tested by including the respective interaction terms in the models (e.g., red meat intake × GMB score). To examine the joint effect of red meat intake and the GMB score on serum TMAO, red meat intake was further categorized as tertiles instead of quintiles to ensure adequate sample sizes in each of 9 subgroups (red meat intake tertiles × 3 GMB score categories). The Benjamini–Hochberg false discovery rate (FDR) method was used for multiple testing correction. All analyses were performed using R version 3.6.0 (https://www.r-project.org/).

Results

Participant characteristics

Characteristics of participants according to quartile of serum TMAO are presented in **Table 1**. Participants with a higher

concentration of TMAO were older and less likely to be women, were more likely to be current smokers, had higher BMI and higher dietary energy intake on average, and were more likely to have diabetes, hypertension, and dyslipidemia, compared with those with a lower concentration of TMAO.

TMAO metabolites and CVD

As expected, the 4 metabolites showed a modest-to-moderate correlation with each other except for the lack of correlation between choline and carnitine (**Supplemental Figure 2**). Serum TMAO was associated with elevated odds of CVD (OR per SD increment: 1.34; 95% CI: 1.17, 1.54), CHD (OR = 1.31; 95% CI: 1.13, 1.53), and CDCR (OR = 1.38; 95% CI: 1.10, 1.74) (model 2 in **Table 2**). There was no significant heterogeneity in these associations across different Hispanic/Latino subgroups (all *P*-interaction >0.05; **Supplemental Figure 3**). In addition, serum betaine, but not choline or carnitine, was also associated with elevated odds of CVD and CDCR (Table 2).

Diet and TMAO metabolites

We next assessed associations of dietary factors with serum concentrations of TMAO and its precursor metabolites. After multivariable adjustment for sociodemographic and behavioral factors plus medical history, higher intakes of red meat ($P = 6.74 \times 10^{-21}$), fish ($P = 9.24 \times 10^{-17}$), and eggs ($P = 5.11 \times 10^{-4}$) were strongly associated with a higher concentration of TMAO, whereas intakes of poultry ($P = 5.78 \times 10^{-8}$), dairy ($P = 5.10 \times 10^{-5}$), and processed meat ($P = 0.001$) were inversely associated with serum TMAO (**Figure 1**A; **Supplemental Table 2**). In addition, egg and refined grain intakes were positively associated with serum choline ($P = 1.37 \times 10^{-7}$ and 0.002, respectively) and betaine ($P = 6.59 \times 10^{-6}$ and 0.003, respectively). Red meat intake was positively associated with serum carnitine ($P = 1.85 \times 10^{-4}$). The associations of red meat, fish, and egg intakes with serum TMAO were generally consistent and no significant heterogeneity was observed across Hispanic/Latino subgroups (all *P*-interaction >0.05, **Supplemental Table 3**). In sensitivity analyses, we also examined associations of red meat, fish, and egg intakes with serum metabolite concentrations using dietary data combined from the two 24-h dietary recalls (average of 2 recalls) or from the second 24-h dietary recall alone. As expected, the associations were relatively weaker using data based on combined dietary recalls or the second 24-h dietary recall compared with those using the first dietary recall (**Supplemental Table 4**). In addition, we also examined associations of TMAO and related metabolites with prevalent CVD after further adjustment for intakes of food groups positively associated with TMAO (i.e., red meat, fish, and eggs), and the associations were only slightly attenuated (model 3 in Table 2).

We then included all 15 food groups in 1 model (mutual adjustment) to assess independent relations of food groups with TMAO and its precursor metabolites. Red meat, fish, and egg intakes remained strongly and positively associated with serum TMAO concentrations. As shown in Figure 1B, the ascending trends of TMAO were significant as intakes of fish ($P = 1.26 \times 10^{-17}$), red meat ($P = 3.33 \times 10^{-16}$), and eggs ($P = 3.89 \times 10^{-5}$) increased. In addition, red meat intake was positively associated with serum carnitine ($P = 2.17 \times 10^{-4}$), and egg intake was positively associated with serum choline ($P = 2.26 \times 10^{-7}$) and betaine ($P = 8.66 \times 10^{-6}$), whereas fish intake was not associated with any of these TMAO precursors (all $P > 0.05$).

Results for dietary nutrients in relation to metabolites were generally in agreement with those for food groups (Supplemental Table 2). Dietary intakes of total protein, animal protein, thiamin, vitamin B-12, calcium, zinc, and selenium, which were largely from animal source foods, were strongly associated with a higher concentration of TMAO after the multivariable adjustment (all FDR-adjusted $P \leq 0.011$). In addition, dietary EPA and DHA, which were mainly from fish intake, were positively associated with serum TMAO (both FDR-adjusted $P \leq 0.003$). As expected, dietary choline was positively correlated with serum concentrations of TMAO (FDR-adjusted $P < 0.001$) and choline (FDR-adjusted $P < 0.001$).

GMB and TMAO

We firstly assessed the relation between 3 different measures of microbiome α-diversity (i.e., Chao1, Shannon, and Simpson) and serum concentrations of TMAO, and did not find significant associations (**Supplemental Table 5**).

Among the 339 identified gut microbial species (average reads ≥10 and prevalence ≥15%), 9 were significantly associated with serum TMAO concentrations after adjusting for age, sex, BMI, study field center, Hispanic/Latino background, education, family income, smoking, alcohol consumption, total energy intake, physical activity, diabetes, hypertension, dyslipidemia, and antibiotic and probiotic use (FDR-adjusted $P < 0.05$; **Figure 2**, **Supplemental Table 6**). Four of the 9 species, belonging to the order *Clostridiales* (i.e., *Oscillibacter* sp. ER4, *Oscillibacter* sp. 1-3, *Intestinimonas butyriciproducens*, and *Pseudoflavonifractor capillosus*), were positively associated with serum TMAO, whereas the other 5 species (i.e., *Megasphaera micronuciformis*, *Streptococcus mitis*, *Alloscardovia omnicolens*, *Bifidobacterium saguini*, and *Haemophilus influenzae*) were inversely associated with serum TMAO. We did not find significant associations of these 9 species with fish, red meat, or egg intake.

We then focused on 4 species that were positively associated with TMAO and might have the potential to produce TMA, hypothesizing that they might modify the associations of TMAO with red meat and egg intake. As shown in **Figure 3**A, there was a significant interaction between red meat intake and the GMB score based on these 4 species on serum TMAO (*P*-interaction = 0.013), with a stronger association between red meat intake and serum TMAO in participants with a higher GMB score compared with those with a lower score. Higher red meat intake was significantly associated with higher serum TMAO only in participants with a high GMB score, but not in those with a low or medium score (Figure 3B). No such microbial modification was observed on the associations of fish or egg intake with TMAO (Figure 3A). Similar results were observed when we analyzed each of these 4 species individually (**Supplemental Figure 4**). Higher red meat intake was significantly associated with higher serum TMAO only in participants with higher abundance of these species (above median).

TABLE 2 Associations of serum TMAO and its precursor metabolites with prevalent cardiovascular disease[1]

	Q1	Q2	Q3	Q4	*P* for trend	Per 1-SD increment
Cardiovascular disease						
TMAO						
Cases/participants	41/957	49/957	82/957	107/956		279/3824
Model 1	1.00 (reference)	1.21 (0.79, 1.85)	2.09 (1.43, 3.11)	2.82 (1.96, 4.13)	<0.001	1.53 (1.35, 1.73)
Model 2	1.00 (reference)	0.89 (0.57, 1.39)	1.41 (0.94, 2.12)	1.80 (1.23, 2.69)	<0.001	1.34 (1.17, 1.54)
Model 3	1.00 (reference)	0.89 (0.57, 1.39)	1.38 (0.93, 2.10)	1.78 (1.20, 2.68)	<0.001	1.34 (1.17, 1.55)
Choline						
Cases/participants	52/957	61/957	76/957	90/956		279/3824
Model 1	1.00 (reference)	1.18 (0.81, 1.74)	1.50 (1.05, 2.17)	1.81 (1.27, 2.59)	<0.001	1.34 (1.18, 1.51)
Model 2	1.00 (reference)	0.94 (0.63, 1.40)	1.03 (0.70, 1.52)	0.97 (0.66, 1.44)	0.97	1.07 (0.94, 1.23)
Model 3	1.00 (reference)	0.95 (0.63, 1.42)	1.02 (0.69, 1.51)	0.95 (0.64, 1.41)	0.88	1.06 (0.92, 1.21)
Carnitine						
Cases/participants	66/957	64/957	66/957	83/956		279/3824
Model 1	1.00 (reference)	0.97 (0.68, 1.38)	1.00 (0.70, 1.43)	1.28 (0.92, 1.80)	0.14	1.12 (0.99, 1.27)
Model 2	1.00 (reference)	0.88 (0.60, 1.29)	0.88 (0.61, 1.28)	0.97 (0.67, 1.40)	0.91	1.00 (0.88, 1.14)
Model 3	1.00 (reference)	0.88 (0.60, 1.29)	0.90 (0.61, 1.31)	0.98 (0.68, 1.42)	0.98	1.01 (0.89, 1.15)
Betaine						
Cases/participants	55/957	72/957	65/957	87/956		279/3824
Model 1	1.00 (reference)	1.33 (0.93, 1.92)	1.20 (0.83, 1.74)	1.64 (1.16, 2.34)	0.01	1.18 (1.05, 1.34)
Model 2	1.00 (reference)	1.39 (0.95, 2.04)	1.33 (0.90, 1.97)	1.64 (1.12, 2.41)	0.02	1.16 (1.01, 1.33)
Model 3	1.00 (reference)	1.38 (0.94, 2.03)	1.32 (0.89, 1.97)	1.59 (1.08, 2.35)	0.03	1.15 (1.00, 1.32)
Coronary heart disease						
TMAO						
Cases/participants	31/957	38/957	61/957	82/956		212/3824
Model 1	1.00 (reference)	1.24 (0.76, 2.01)	2.03 (1.32, 3.20)	2.80 (1.86, 4.34)	<0.001	1.52 (1.32, 1.75)
Model 2	1.00 (reference)	0.92 (0.56, 1.53)	1.34 (0.85, 2.15)	1.76 (1.14, 2.78)	0.001	1.31 (1.13, 1.53)
Model 3	1.00 (reference)	0.92 (0.56, 1.53)	1.35 (0.86, 2.17)	1.76 (1.13, 2.80)	0.002	1.32 (1.13, 1.55)
Choline						
Cases/participants	40/957	49/957	55/957	68/956		212/3824
Model 1	1.00 (reference)	1.24 (0.81, 1.91)	1.40 (0.92, 2.13)	1.76 (1.18, 2.64)	0.005	1.32 (1.15, 1.52)
Model 2	1.00 (reference)	0.98 (0.63, 1.53)	0.95 (0.62, 1.49)	0.92 (0.59, 1.42)	0.67	1.04 (0.90, 1.21)
Model 3	1.00 (reference)	0.97 (0.62, 1.52)	0.94 (0.61, 1.47)	0.88 (0.57, 1.37)	0.53	1.03 (0.88, 1.20)
Carnitine						
Cases/participants	58/957	48/957	48/957	58/956		212/3824
Model 1	1.00 (reference)	0.82 (0.55, 1.21)	0.82 (0.55, 1.21)	1.00 (0.69, 1.46)	1.00	1.03 (0.90, 1.18)
Model 2	1.00 (reference)	0.71 (0.47, 1.07)	0.70 (0.46, 1.05)	0.72 (0.48, 1.07)	0.13	0.91 (0.78, 1.05)
Model 3	1.00 (reference)	0.72 (0.47, 1.09)	0.70 (0.46, 1.07)	0.73 (0.49, 1.10)	0.17	0.92 (0.79, 1.06)
Betaine						
Cases/participants	43/957	54/957	51/957	64/956		212/3824
Model 1	1.00 (reference)	1.27 (0.84, 1.93)	1.20 (0.79, 1.82)	1.53 (1.03, 2.28)	0.06	1.15 (1.00, 1.33)
Model 2	1.00 (reference)	1.29 (0.84, 1.99)	1.25 (0.80, 1.94)	1.44 (0.94, 2.23)	0.13	1.11 (0.95, 1.29)
Model 3	1.00 (reference)	1.24 (0.81, 1.92)	1.23 (0.79, 1.92)	1.42 (0.92, 2.20)	0.15	1.10 (0.95, 1.29)
Cerebrovascular disease or carotid revascularization						
TMAO						
Cases/participants	12/957	17/957	31/957	37/956		97/3824
Model 1	1.00 (reference)	1.42 (0.68, 3.07)	2.64 (1.38, 5.37)	3.17 (1.69, 6.38)	<0.001	1.53 (1.25, 1.88)
Model 2	1.00 (reference)	1.09 (0.51, 2.39)	1.80 (0.92, 3.75)	2.16 (1.12, 4.46)	0.007	1.38 (1.10, 1.74)
Model 3	1.00 (reference)	1.11 (0.52, 2.43)	1.75 (0.89, 3.67)	2.16 (1.10, 4.50)	0.01	1.38 (1.09, 1.75)
Choline						
Cases/participants	15/957	19/957	29/957	34/956		97/3824
Model 1	1.00 (reference)	1.27 (0.64, 2.56)	1.96 (1.06, 3.78)	2.32 (1.28, 4.40)	0.002	1.44 (1.17, 1.76)
Model 2	1.00 (reference)	1.04 (0.52, 2.13)	1.40 (0.73, 2.76)	1.36 (0.72, 2.68)	0.25	1.20 (0.96, 1.49)
Model 3	1.00 (reference)	1.10 (0.54, 2.29)	1.43 (0.74, 2.89)	1.37 (0.71, 2.77)	0.27	1.19 (0.95, 1.48)
Carnitine						
Cases/participants	18/957	27/957	21/957	31/956		97/3824
Model 1	1.00 (reference)	1.51 (0.83, 2.81)	1.17 (0.62, 2.23)	1.75 (0.98, 3.21)	0.13	1.19 (0.97, 1.46)
Model 2	1.00 (reference)	1.7 (0.91, 3.26)	1.23 (0.63, 2.43)	1.69 (0.91, 3.23)	0.24	1.14 (0.92, 1.41)
Model 3	1.00 (reference)	1.67 (0.89, 3.23)	1.26 (0.64, 2.48)	1.66 (0.89, 3.20)	0.25	1.14 (0.92, 1.41)
Betaine						
Cases/participants	18/957	26/957	21/957	32/956		97/3824
Model 1	1.00 (reference)	1.46 (0.80, 2.72)	1.17 (0.62, 2.23)	1.81 (1.02, 3.30)	0.09	1.21 (0.99, 1.48)
Model 2	1.00 (reference)	1.62 (0.87, 3.10)	1.54 (0.79, 3.03)	2.16 (1.16, 4.15)	0.03	1.27 (1.01, 1.58)
Model 3	1.00 (reference)	1.72 (0.91, 3.34)	1.58 (0.81, 3.16)	2.04 (1.07, 4.00)	0.05	1.23 (0.99, 1.55)

[1]Data are ORs (95% CIs) from logistic regression models. Model 1 was the crude model; model 2 was adjusted for age, sex, BMI, study field center, Hispanic/Latino background, physical activity, alcohol consumption, smoking, education, yearly household income, and total energy intake; and model 3 was further adjusted for intakes of fish, red meat, and eggs. Q, quartile; TMAO, trimethylamine-*N*-oxide.

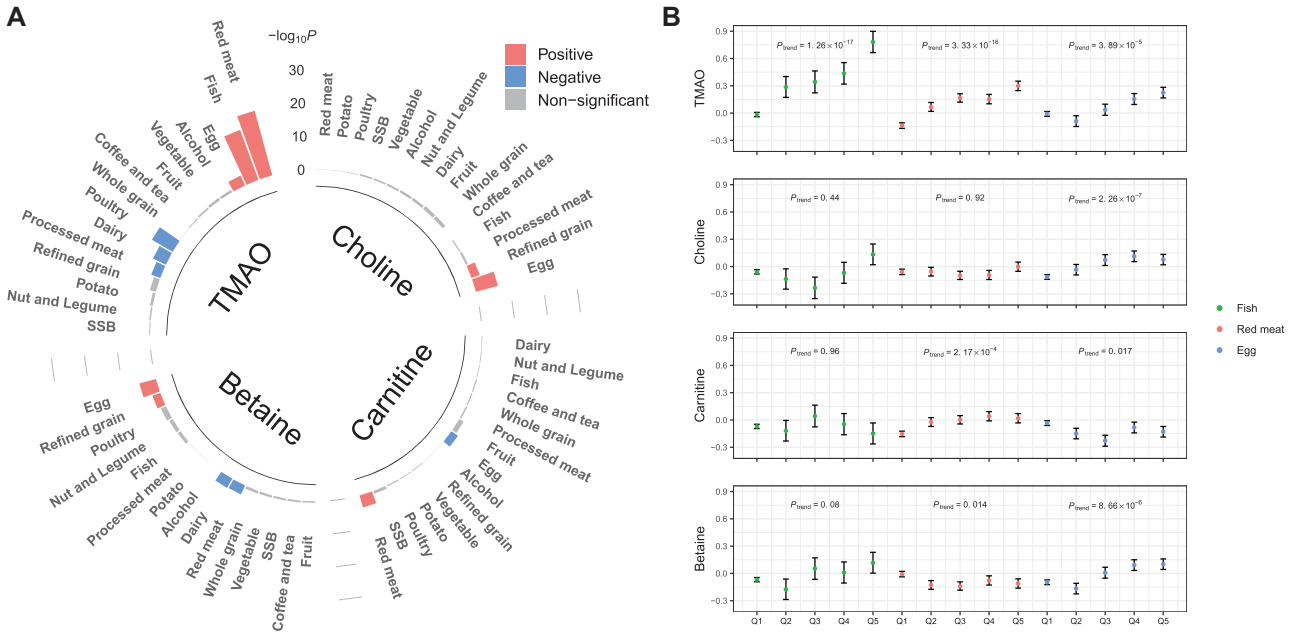

FIGURE 1 Associations of food groups with serum TMAO and its precursor metabolites. (A) Associations ($-\log_{10}P$) of 15 food groups with serum TMAO and its precursor metabolites in 3466 individuals. *P* values were estimated from linear regression after adjustment for age, sex, BMI, study field center, Hispanic/Latino background, education, yearly household income, smoking, alcohol consumption, total energy intake, physical activity, diabetes, hypertension, and dyslipidemia. Red bars indicate positive associations (FDR-adjusted $P < 0.05$), blue bars indicate inverse associations (FDR-adjusted $P < 0.05$), and gray bars indicate nonsignificance (FDR-adjusted $P \geq 0.05$). (B) Circulating concentrations of TMAO and its precursor metabolites (inverse normal transformation) across quintiles of fish, red meat, and egg intakes in 3466 individuals. The dots and error bars are the means and SEs estimated from linear regression models, which included all 15 food groups in the same model after multivariable adjustment for age, sex, BMI, study field center, Hispanic/Latino background, education, yearly household income, smoking, alcohol consumption, total energy intake, physical activity, diabetes, hypertension, and dyslipidemia. Numbers of individuals were 3191, 71, 64, 67, and 73 across the quintiles of fish intake; 1708, 434, 474, 401, and 449 across the quintiles of red meat intake; and 2339, 288, 270, 282, and 287 across the quintiles of egg intake. FDR, false discovery rate; SSB, sugar-sweetened beverage; TMAO, trimethylamine-*N*-oxide.

Discussion

In a population-based representative sample of US Hispanic/Latino adults, we found that higher serum concentrations of TMAO, a diet-derived, gut microbial–related metabolite, were associated with a higher prevalence of CVD. Since 2011, it has been reported that elevated circulating TMAO is associated with an increased risk of major adverse cardiovascular events (including myocardial infarction, stroke, or death) (1, 2, 5). The relation between TMAO and CVD was examined in subsequent studies of different populations and confirmed in a meta-analysis of 19 clinical-based cohorts, which mainly included Caucasian and black participants (3). To the best of our knowledge, this is the first study to report a positive association between TMAO and prevalent CVD in US Hispanic/Latino adults of diverse backgrounds, though further studies with prospective data in this population are needed.

Our analyses demonstrated both GMB-dependent (e.g., red meat) and GMB-independent (e.g., fish) dietary sources of circulating TMAO in human populations. GMB-dependent TMAO, meaning the fraction that is produced by gut bacterial metabolism, was mainly from dietary choline and carnitine, both of which are abundant in eggs, liver, and a variety of meat (9, 35, 36). However, TMAO also naturally exists in seafood in a preformed state (37, 38). A randomized controlled trial confirmed that eggs had the highest content of choline, beef had the highest concentration of carnitine, whereas fish had 650 times more TMAO compared with eggs and beef (6). Along with another dietary intervention study, these reports found that circulating TMAO increased over a short time after consumption of fish (6, 39). A few observational studies conducted in general populations have examined the associations of food groups with TMAO and yielded various results (8–11, 40). For example, fish and red meat intakes were positively correlated with circulating TMAO in studies from Germany and Italy (10, 11), whereas another study from Germany found that consumption of dairy, but not meat, eggs, or fish, was positively associated with plasma TMAO (8). In addition, a study from China found that consumption of fish but not red meat was associated with elevated urinary TMAO (9). Our current study found that fish, red meat, and eggs were 3 major dietary determinants of serum TMAO in US Hispanics/Latinos; and that red meat and egg intakes, but not fish intake, were positively associated with serum concentrations of TMAO precursors. Our findings provide strong support for 2 suggested major pathways of TMAO in human circulation (6, 39).

Previous studies in mice and humans indicated that circulating TMAO from choline or carnitine was GMB dependent (1, 5). However, the specific taxa that might be associated with TMAO production have not been fully understood. Recently, several studies have explored the relation between gut bacterial taxa and circulating concentrations of TMAO in humans (5, 12, 13, 41, 42). For example, 3 intervention studies including 20 to 60 participants found that several genera (e.g., *Clostridium* clusters XIVa) belonging to the order *Clostridiales* were associated with elevated TMAO (5, 41, 42). Strains from *Clostridium* XIVa have

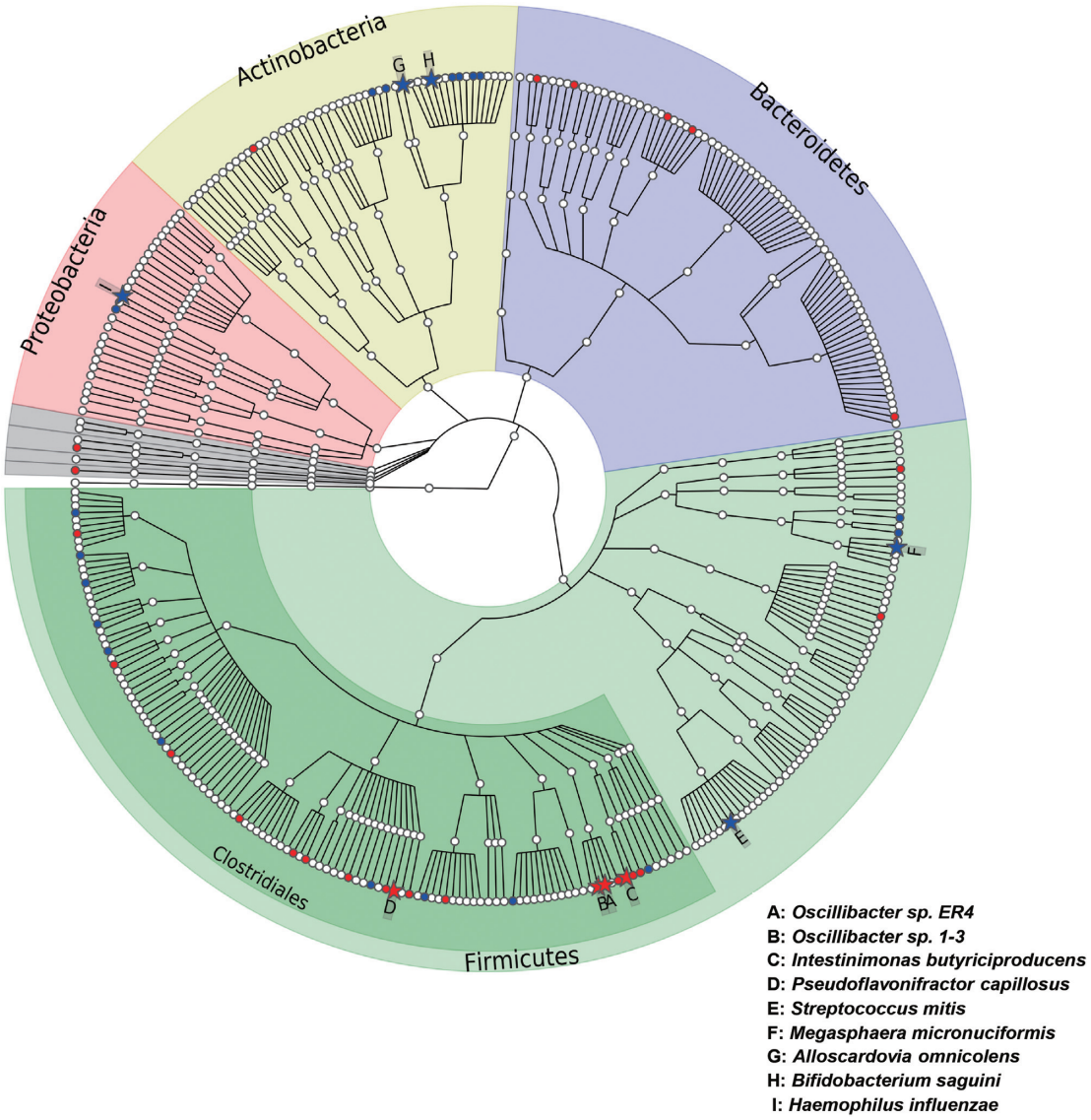

FIGURE 2 Phylogenetic tree of taxonomic features in association with serum TMAO concentrations. Species associated with TMAO at FDR-adjusted $P < 0.05$ were highlighted as solid stars and noted by capital letters, and species with a raw $P < 0.05$ were highlighted as solid circles. Solid stars or circles with red indicate positive associations whereas blue indicates inverse associations. *P* values were estimated from linear regression models in 626 individuals after controlling for age, sex, BMI, study field center, Hispanic/Latino background, physical activity, alcohol consumption, smoking, education, yearly household income, total energy intake, diabetes, hypertension, dyslipidemia, and use of antibiotics and probiotics. FDR, false discovery rate; TMAO, trimethylamine-*N*-oxide.

been suggested to possess a choline TMA-lyase gene (*cutC*) (16). Another 2 recent studies in general populations also found that genera belonging to the orders *Clostridiales*, *Bacteroidales*, or *Desulfovibrionales* were positively associated with circulating TMAO (12, 13). Partially consistent with previous results, the 4 gut microbial species that were positively associated with serum TMAO in our study all belong to the order *Clostridiales*. More specifically, 2 microbial species (i.e., *Oscillibacter* sp. ER4 and *Oscillibacter* sp. 1-3) identified in our study, belong to *Oscillibacter*, a genus that was previously reported to be associated with circulating TMAO concentrations (42, 43) and cerebrovascular disease (44). Moreover, our bacterial gene alignment analysis indicated that 3 of these 4 gut microbial species (*Oscillibacter* sp. 1-3, *Pseudoflavonifractor capillosus*, and *Intestinimonas butyriciproducens*) possess homologous genes encoding carnitine monooxygenase (CntA/B), an enzyme that converts carnitine to TMA (**Supplemental Table 7**). This suggests that these species might have the potential to produce TMA, though further analytical and experimental studies are needed to demonstrate the TMA-producing capability of these species.

The identified gut microbial species and their potential capability to produce TMA from carnitine were further supported by a significant microbial modification on the red meat–TMAO association observed in this study. This finding suggests that the positive association between red meat and circulating TMAO is

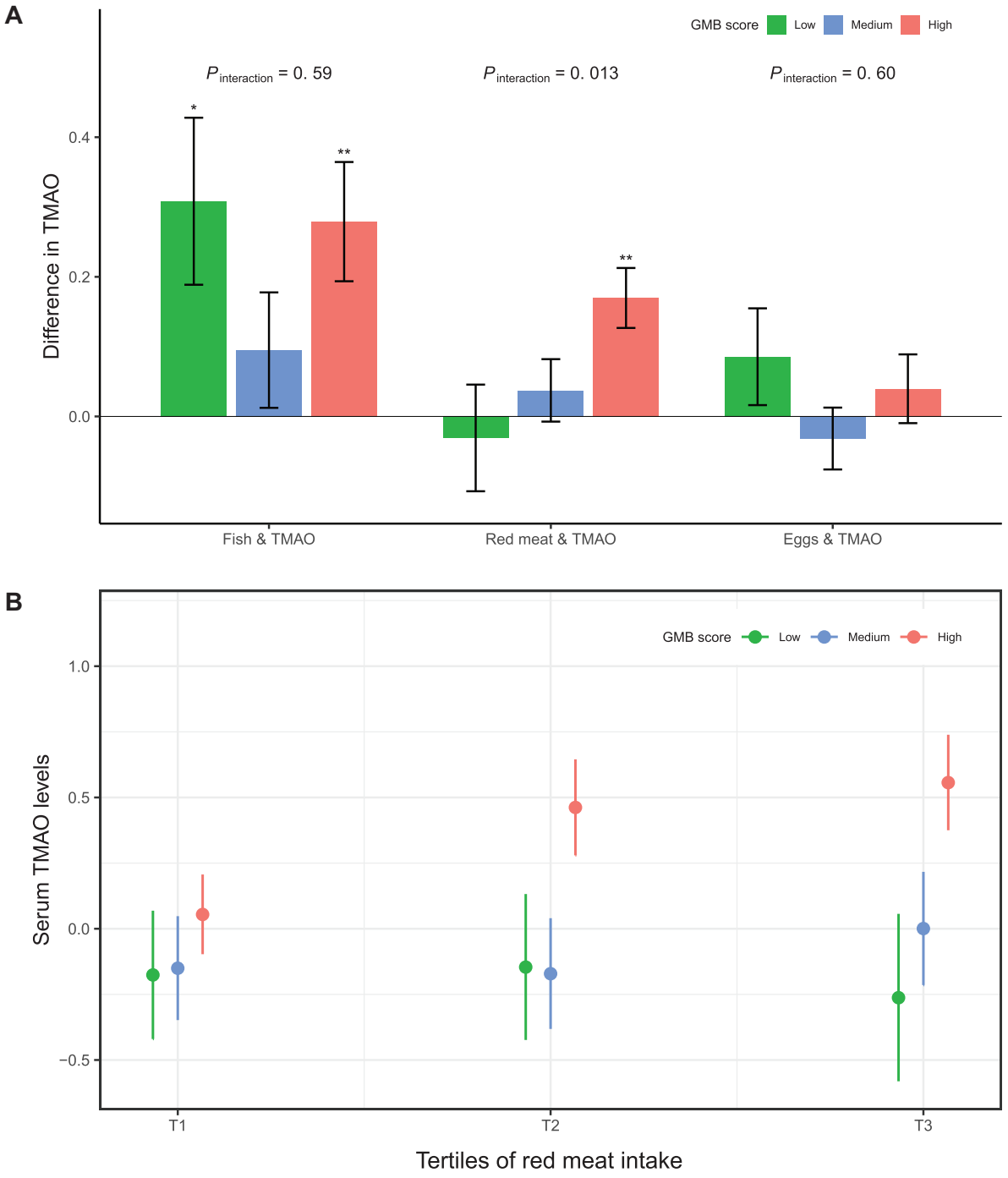

FIGURE 3 Associations of fish, red meat, and egg intake with TMAO according to the GMB score. (A) Data are effect size (β coefficients and SEs) of food intake (per quintile) on serum TMAO levels (inverse-normal transformed) estimated from linear regression after adjustment for age, sex, BMI, study field center, Hispanic/Latino background, physical activity, alcohol consumption, smoking, education, yearly household income, total energy intake, diabetes, hypertension, dyslipidemia, and use of antibiotics and probiotics. The GMB score (range 0–4) was calculated based on the abundance of the 4 microbial species positively associated with TMAO (less than median value = 0; equal to or greater than median value = 1): 0, low GMB score ($n = 137$); 1–2, medium GMB score ($n = 241$); and 3–4, high GMB score ($n = 248$). *,** Significant difference in TMAO concentration were estimated from multivariable linear regression models: * $P < 0.05$ and ** $P < 0.01$. *P*-interaction values were calculated by adding a multiplicative factor in the multivariable linear regression models. (B) TMAO levels (inverse-normal transformed) across tertiles (instead of quintiles to ensure adequate sample sizes) of red meat intake according to GMB score. Data are means and SEs estimated from linear regression after adjustment for age, sex, BMI, study field center, Hispanic/Latino background, physical activity, alcohol consumption, smoking, education, yearly household income, total energy intake, diabetes, hypertension, and dyslipidemia. *P*-trend values across tertiles of red meat intake were 0.83, 0.40, and 6.21×10^{-4} in the low, medium, and high GMB score groups, respectively. Numbers of individuals were 70, 119, and 126 across the low, medium, and high GMB score groups in the first tertile of red meat intake; 38, 64, and 52 across the 3 GMB score groups in the second tertile of red meat intake; and 28, 55, and 69 across the 3 GMB score groups in the third tertile of red meat intake, respectively. GMB, gut microbiota; TMAO, trimethylamine-*N*-oxide.

dependent on these gut bacterial taxa, which might contribute to the processing of carnitine from dietary red meat to TMA (10, 45). As expected, we did not find such microbial modification for the association between fish intake and serum TMAO, further supporting fish as a GMB-independent diet source of circulating TMAO (6, 39). We also did not find such microbial modification for the association between egg intake, a major dietary source of choline (10), and serum TMAO, which is in line with our bacterial gene alignment results, namely that these gut microbial species contain carnitine monooxygenase genes (*cntA/B*), but not choline TMA-lyase gene (*cutC*) (Supplemental Table 7). Consistently, a recent dietary intervention study also suggested that higher intake of dietary red meat could increase systemic TMAO concentrations through microbial TMA production from dietary carnitine, but not choline (45). Nevertheless, because the dose–response relation between egg consumption and circulating TMAO has been demonstrated in this and previous studies (2, 46), further studies are needed to clarify the gut microbial pathway linking dietary egg and choline consumption to microbial TMA and TMAO production in humans.

This study also identified a number of gut microbial species inversely associated with serum TMAO. Among them, it is noteworthy that *Bifidobacterium saguini* (FDR-adjusted $P < 0.05$) and several others (i.e., *B. longum*, *B. breve*, *B. gallinarum*, and *B. bifidum* with a raw $P < 0.05$ but did not pass FDR), belong to *Bifidobacterium*, a genus that has been widely reported to be inversely associated with circulating TMAO concentrations in humans (12, 47) and mice (48). In support of these findings, some members of *Bifidobacterium* and other gut microbiota, such as the *Streptococcaceae* family (*Streptococcus mitis*, inversely associated with serum TMAO in the current study, belongs to this family), have been found to convert TMAO to TMA in mice and pure culture (49), although the conversion rate was relatively low.

Several limitations of our study need to be acknowledged. A major limitation is that ascertainment of dietary intake and serum metabolites preceded sampling of GMB by a median of 7.2 y, although human GMB has been found to be notably stable over a long period (50). This time lag might bias the observed association between gut microbiota and serum TMAO. However, our results have strong biological plausibility, because several identified TMAO-associated species have the potential to produce TMA from carnitine. Due to the cross-sectional study design and its observational nature, our study is unable to make causal inference. We used an untargeted metabolomic approach that did not allow us to obtain absolute concentrations of serum metabolites, although this would not influence association results. Other limitations include uncontrolled [e.g., kidney function measures (51)] or unknown confounding factors, self-reported dietary recalls with inevitable measurement errors, and shallow shotgun sequencing data (28). Finally, the present study included US Hispanics/Latinos, who have a distinctive dietary pattern and GMB composition (19), and triadmixed genetic backgrounds; and hence, it should take caution to generalize our findings.

In summary, our study found a cross-sectional association between TMAO and CVD, and demonstrated that fish, red meat, and egg intakes were major dietary determinants of circulating TMAO concentrations in US Hispanics/Latinos. We identified several gut microbial species that might have the potential to produce TMA, positively associated with serum TMAO. Moreover, the association between red meat intake and serum TMAO could depend on these specific gut microbial species, supporting the essential role of gut microbiota in TMA and TMAO production from dietary carnitine. Our findings provide evidence from a human population study supporting diet-derived TMAO formation in the human circulation, in both a GMB-independent and GMB-dependent manner. This in turn might have important implications for future efforts to prevent CVD related to this pathway in human populations through diet and GMB modification.

The authors' responsibilities were as follows—ZM, G-CC, QQ: conceived the study; ZM, ZW: performed statistical analyses; ZM: drafted the manuscript; G-CC, ZW, QQ: critically revised the manuscript; MLD, RDB, RCK, QQ: collected the data and specimens from the HCHS/SOL participants and obtained funding; ZW, MU, YVB, GH, RSB, RK: did the gut microbial sequencing analysis; MU, RDB: did the processing of the HCHS/SOL fecal samples; BY, EB: did the metabolome profiling analysis; JL, JSW-N, LH, JC, YZ, RK: edited and reviewed the manuscript; QQ: is the guarantor of this work and had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; and all authors: read and approved the final manuscript. The authors report no conflicts of interest.

Data Availability

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

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