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Associations of trimethylamine-N-oxide (TMAO) with nutritional and inflammatory biomarkers and cardiovascular outcomes in patients new to dialysis

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Abstract

Background—Trimethylamine-N-oxide (TMAO) is a product of metabolism of phosphatidylcholine (lecithin) and carnitine by the intestinal microbiome. Elevated serum concentrations of TMAO have been linked to adverse cardiovascular outcomes in the general population. We examined correlates of serum TMAO and the relations among serum TMAO concentrations, all-cause mortality and cardiovascular mortality and hospitalizations in a nationally derived cohort of patients new to hemodialysis (HD).

Methods—We quantified serum TMAO by liquid chromatography and online tandem mass spectrometry and assessed nutritional and cardiovascular risk factors in 235 patients receiving hemodialysis and measured TMAO in pooled serum from healthy controls. We analyzed time to death and time to cardiovascular death or hospitalization using Cox proportional hazards regression.

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Results—Serum TMAO concentrations (median 43, (25th – 75th percentile 28–67 μM/L) were elevated compared to persons with normal or near normal kidney function (1.41 ± 0.49 μM/L). TMAO was directly correlated with serum albumin (Spearman rank correlation 0.24, 95% CI 0.12, 0.35; P < 0.001), prealbumin (0.19, 95% CI 0.07, 0.31; P =0.003), and creatinine (0.21, 95% CI 0.08, 0.33; P =0.002), and inversely correlated with log CRP (−0.18, 95% CI −0.30, – 0.06; P =0.005). Higher serum concentrations of TMAO were not significantly associated with time to death (0.84, CI 0.65, 1.09 P=0.19) or time to cardiovascular hospitalization or cardiovascular death (0.88, CI 0.57, 1.35 P =0.55).

Conclusions—Serum TMAO concentrations were markedly elevated and correlated directly with biochemical markers of nutritional status and inversely with markers of inflammation in patients receiving hemodialysis. There was no significant association between serum TMAO concentrations and all-cause mortality or cardiovascular death or hospitalizations. In patients receiving dialysis – in contrast to the general population – adverse vascular effects of TMAO may be counterbalanced by associations with nutritional or inflammatory status.

Background

Meat consumption has been epidemiologically linked to cardiovascular disease. The traditional view that this association is mediated by fatty acid consumption has recently been questioned[1]. Foods rich in carnitine, choline and lecithin, including meat and eggs, have been found to be a source of trimethyl amine (TMA)[1], which is synthesized by gut microbiotia[2] and then oxidized to trimethyl amino oxide (TMAO) in the liver by flavin monooxygenase 3 (FMO3)[3]. TMAO has been demonstrated to augment macrophage scavenger activity and down-regulate bile acid synthetic enzymes Cyp7a1 and Cyp27a1, important but terminal components of reverse cholesterol transport[1]. Higher serum TMAO concentrations have been associated with higher risk of cardiovascular events in the general population[4]. TMA is elevated in patients with chronic kidney disease (CKD) and is responsible in part for uremic fetor[5]. TMAO has also been demonstrated to be significantly elevated in patients receiving dialysis relative to persons with normal or near normal kidney function and to be removed by dialysis[6]. More recently TMAO has been found to be high among patients with CKD not requiring dialysis, and higher concentrations in that population were associated with mortality and progressive loss of kidney function[7]. Alterations in gut permeability or the contents of the microbiome offer potential pathways for higher cardiovascular risk in the ESRD population[8,9], but TMAO is also excreted in the urine[2,10–12], suggesting that impaired kidney function could contribute to higher levels in this population. The increased level of TMAO, its water solubility, and its strong association with cardiovascular disease among patients without kidney disease make it a potential target for therapy in patients receiving dialysis. Herein, we aimed to determine clinical correlates of, and outcomes associated with, the putative cardiovascular risk marker TMAO in a national cohort of patients new to dialysis.

METHODS

Study Design, Participants, and Data Collection

Design and Participants—The Comprehensive Dialysis Study (CDS) was a United States Renal Data System (USRDS) prospective cohort study of adults with end-stage renal disease (ESRD) who initiated hemodialysis or peritoneal dialysis between June 2005 and June 2007 in dialysis facilities throughout the US. The CDS was designed to examine associations among nutritional status, physical activity, and health-related quality of life in patients new to dialysis[13]. The CDS has been previously described in detail, including sampling of dialysis facilities, recruitment, and measures[13,14]. In brief, participants were successfully recruited from 297 (89%) of 335 selected dialysis facilities. Fifty six of 73 (77%) facilities subsampled to participate in the nutrition substudy agreed to participate and collected serum samples. Facilities were selected *a priori* by systematic probability sampling proportional to estimated size to participate in the nutrition substudy. Participants (n=266) provided serum samples at enrollment. We did not include 22 patients who were on peritoneal dialysis in our analyses because the small number of participants would not have allowed us to make valid inferences, and we were unable to obtain TMAO measurements on nine participants. Our analysis cohort included 235 participants receiving hemodialysis with baseline TMAO measurements. USRDS hospitalization data is available only for Medicare beneficiaries, so analyses of cardiovascular mortality or cardiovascular hospitalization included 152 patients.

The CDS was approved by the Institutional Review Boards of the University of California, San Francisco and Emory University. All patients provided informed consent for participation, including analysis of stored serum samples.

Data collection—We collected outcome data through linkage to the USRDS. Data on demographics, body composition (height and weight), dialysis modality and access, co-morbidities, and serum creatinine at dialysis initiation were collected from the Center for Medicare and Medicaid Services (CMS) Medical Evidence Form (CMS 2728) and a telephone interview administered by DataBanque Research Services (Pittsburgh, PA). Information on the date and cause of death was based on the ESRD Death Notification Form (CMS Form 2746). Cause-specific hospitalization was ascertained from the principal discharge diagnosis or from procedure codes, with hospitalization captured through Medicare claims (see Appendix for the International Classification of Diseases - 9 diagnosis and procedure codes used to ascertain cardiovascular hospitalizations of interest and codes selected from the CMS 2746 form to ascertain cardiovascular death).

Laboratory Methods—We quantitated TMAO using a Waters Acuity UPLC in tandem with an ABSciex 5600 Triple TOF mass spectrometer. A Waters Acuity BEH Amide column (2.1 i.d. ×150mm length, 1.8um particle size) with attached guard-column held at 45°C was used for separation. The mass spectrometer was operated using an electrospray source in positive mode with a voltage of 5.5kV, temperature of 300°C, source gas at 40 psi and curtain gas at 25 psi. Data were acquired both in full scan mode as well as in product ion mode with enhanced sensitivity for the products of native TMAO (76.1 $\rightarrow$ 58.1 Da) and the

deuterated internal standard (85.1 → 66.1 Da). A declustering potential of 80V and collision energy of 22V was used for the product ion scans. We performed data quantification in MultiQuant 2.1 (ABSciex) using peak area from product ions of TMAO against the known quantity of its deuterated internal standard.

We measured albumin, prealbumin, and C-reactive Protein (CRP) concentrations in duplicate on each serum sample using a Beckman Array 360 nephelometer (Beckman Instructions 015–248545–F; Beckman Instruments, Inc., Brea, CA). Coefficients of variation (CoV) albumin: interassay CoV, 8.3%; intra-assay CoV 0.2%; prealbumin: interassay CoV, 4.3%; intra-assay CoV, 1.1%; and CRP: interassay CoV, 9.2%; intra-assay CoV 3.6%. If the two measures on any given sample differed within the duplicates by >1%, we repeated the testing.

Control samples included two commercially available pooled normal control samples, one consisting of only male participants (Valley BioMedical Winchester, Virginia.) and one of unspecified sex (UTAK Laboratories Inc Valencia, CA), as well as an internal laboratory normal control standard.

Statistical methods

Baseline characteristics of our study cohort were described using proportions for categorical variables and either means and standard deviations or medians and 25th to 75th percentiles for continuous variables. We calculated Spearman's rank correlation coefficients among TMAO measurements and concurrent measurements of age, BMI, and other laboratory values.

We modeled time to death and time to cardiovascular death or cardiovascular hospitalization using Cox proportional hazards regression models with TMAO as the primary predictor of interest and covariates including age, sex, race, diabetes, BMI, CRP as an inflammatory marker, and prealbumin and albumin as nutritional markers. We conducted unadjusted and multivariable adjusted analyses. The pre-specified model for the Cox analysis of time to death included all the predictors detailed above. Because of the smaller number of participants and events for the analysis of time to CV death or hospitalization, all predictors were not evaluated at once. We first examined TMAO as a univariate predictor, and evaluated whether the hazard ratio for TMAO changed with addition of additional predictors individually or in groups of up to three. Our final model consisted of prealbumin, race and diabetes, which were all significant in univariate models. We ran additional models including each non-included predictor with this set of four. None of the additional predictors were significant, and the hazard ratios for TMAO remained stable. We conducted all analyses using SAS 9.2 (Cary, NC, USA)

RESULTS

Patient characteristics and correlates of TMAO

The mean age of our cohort of 235 patients on hemodialysis was 62±14 years (mean ± SD), 28% were black, and 59% had diabetes mellitus. TMAO values appeared non-normally distributed, i.e., skewed to the right (Figure 1) and were markedly elevated (mean 50±32

(Median 43, 25th –75th percentile – (28–67 μM/L)) (Table 1) when compared to our pooled control samples (1.41±0.49 μM/L). TMAO was directly correlated with albumin (Spearman rank correlation 0.24 [95% CI 0.11, 0.35]; p <0.001), prealbumin (0.19 [0.07, 0.31]; p =0.003), and creatinine (0.21 [0.08, 0.33]; p =0.002), and inversely with log CRP (–0.18 [–0.30, –0.06]; p=0.005). There were no significant associations between TMAO and age or body mass index (BMI) (Table 2).

Positive control samples using commercial blood plasma as well as NIST SRM 1950 standardized blood plasma yielded TMAO levels similar to TMAO levels published previously[2,4]. Reproducibility of the assays was better than 5% relative standard deviation using these standard blood plasma samples under the extraction conditions detailed above.

Association of TMAO with all-cause mortality

In the Cox model for mortality, 235 patients were followed from enrollment date through December 31, 2011 for a median follow-up time of 4.0 (25th – 75th percentile 1.8, 5.9) years, and 132 (56%) died. Median time to death was 2.5 (25th – 75th percentile 1.6, 4.6) years. The reasons for censoring included 38 (16%) for transplant, 2 (0.9%) for loss to follow-up, and 63 (27%) for study end.

Contrary to our hypothesis, higher serum concentrations of TMAO were not significantly associated with higher mortality either by univariate or multivariable Cox model analysis (Tables 3 and 4). In univariate analysis (Table 3), log TMAO was not significantly associated with death (HR 0.84, 95% [0.65, 1.09] per one unit (p=0.19)). When analyzed by quartiles, patients in the highest quartile of TMAO had a forty percent lower hazard of mortality (HR 0.61, 95% [0.38, 0.97]; p=0.04) than those in the lowest quartile (Table 3).

In multivariable analyses, the adjusted association between serum TMAO and mortality was in the hypothesized direction, but not statistically significant with a wide confidence interval (HR 1.14, [95% CI 0.67 – 1.93]; p=0.62, comparing the fourth to the first quartile) (Table 4). Older age, non-Black race, and lower serum prealbumin concentrations were associated with higher mortality (Table 4).

Association of TMAO with cardiovascular death or hospitalization

In the Cox model for time to cardiovascular death or first cardiovascular hospitalization, 152 Medicare beneficiaries were followed from enrollment date through December 31, 2011 for a median follow-up time of 2.5 (IQR 1.4, 5.0) years. There were 48 (32%) cardiovascular deaths or hospitalizations during follow-up with median time to event of 1.7 (IQR 0.67, 2.5) years. The presence of diabetes mellitus was associated with higher risk (HR 2.20 CI 1.14 – 4.40 P = 0.02), and prealbumin was associated with lower risk (HR 0.96 CI 0.92 – 0.99) per mg/dL, P = 0.01) of cardiovascular death or hospitalization when each were used as single predictors for analysis of risk. Higher log CRP was associated with higher risk but failed to reach statistical significance (HR 1.19 95% CI 0.93 – 1.52, P = 0.17). TMAO was not statistically significantly associated with cardiovascular death or hospitalizations, either as a continuous variable or by quartile (Table 3).

The relation between TMAO and time to cardiovascular death or hospitalization was not statistically significant in multivariable models after adjusting for race, diabetes, and prealbumin (Table 5). Adjusted hazard ratios comparing the forth quartile with the first quartile had large confidence intervals ranging from 60% lower risk of cardiovascular death or hospitalization to a 2-fold higher risk.

DISCUSSION

We found that serum TMAO concentrations were markedly higher in our cohort of patients receiving hemodialysis compared with control serum we obtained and compared with levels previously reported in the literature among healthy individuals[1,2,4].

TMAO was directly correlated with albumin and prealbumin and inversely correlated with CRP. In our cohort, serum TMAO concentrations were not statistically significantly associated with all-cause mortality or cardiovascular mortality or hospitalization.

The levels of TMAO that we have reported are lower than those reported in a smaller cohort of patients receiving dialysis previously reported by Bain et al.[6]. The assays used in our laboratory and in use by others not exploring patients with dialysis dependent kidney disease[1,2,4,7] differ from that used by Bain et al.[6] in that we used liquid mass spectroscopy utilizing liquid extraction and internal standards instead of gas chromatography – mass spectroscopy (GC-MS) with solid phase microextraction[6], a method that could not add an internal standard. Their results from healthy individuals were approximately an order of magnitude higher than we report here from normal controls as well as values previously reported by others[1,2,4,7] using liquid chromatography. Thus, because of these fundamental methodological differences, the levels of TMAO reported here should not be compared to previous studies of patients with ESRD[6].

TMAO has recently been described as a risk factor for cardiovascular disease and has been shown in animal models to up regulate macrophage receptors associated with atherosclerosis[2], providing a link between dietary nutrients, the gut microbiome, and vascular injury. TMAO has been found to decrease expression of a number of bile acid transport genes in the liver (Cyp7a1 and Cyp27a1) adversely affecting cholesterol elimination[1] blocking reverse cholesterol transport through this mechanism, providing another potential link to vascular disease. Bile acids are also increased in CKD and ESRD, although no disruption in the Cyp7a1 pathway has been identified as the mechanism in ESRD[15,16]. Although mortality risk in the general population is also associated with precursors of TMA, such as L-carnitine[1], when TMAO and L-carnitine are considered together, only TMAO is associated with higher risk. Patients on dialysis have been reported to have functional L-carnitine deficiency[17–19]. Despite the potential for L-carnitine conversion to TMAO, supplemental L-carnitine has been studied for use in intradialytic hypotension, left ventricular dysfunction and to correct or ameliorate resistance to erythropoietin[17–19]. A recently published meta-analysis examined the results of a total of 49 trials on L-carnitine in patients on dialysis that enrolled 1734 patients, finding a reduction in LDL cholesterol and CRP, but no increase or decrease in cardiovascular risk. Each of the

studies were relatively small, but the doses of L-carnitine administered were well in excess of what would be encountered nutritionally[20] with no increase in adverse events noted.

We did not observe a statistically significant association between serum TMAO concentrations and mortality or cardiovascular outcomes despite levels that were several-fold higher than values reported to be associated with the highest risk of adverse cardiovascular outcomes in the general population[4]. These results parallel those of other biomarkers, such as total and LDL cholesterol, which are either unassociated with risk or are associated with *lower* risk of adverse outcomes in patients receiving dialysis in contrast to associations observed in the general population. It is not entirely clear why risk factors, such as total[20] and LDL cholesterol[22,23] – and TMAO in our study – do not find a significant association with outcomes in dialysis patients. It is possible that by the time patients reach ESRD, damage to the vascular endothelium has reached a point where further exposure can no longer affect outcome. TMAO has been shown to cause injury in experimental models, at least in part, by interfering in reverse cholesterol transport. However, this effect is distal to sites that are impaired in ESRD, where there are multiple structural, functional and maturational abnormalities affecting HDL[24–26]. It is possible that reverse cholesterol transport is so significantly disrupted in patients receiving dialysis that additional downstream effects on bile acid transport contribute no additional risk. Alternatively, cholesterol and other injurious compounds may still be exerting deleterious effects on the vasculature, but competing risks from other factors associated with these biomarkers, such as inflammation and/or malnutrition, may overwhelm any deleterious effects. The correlations between TMAO and nutritional and inflammatory biomarkers we observed would be consistent with the latter possibility. We have previously shown that both serum albumin and creatinine concentrations are associated with measures of protein intake (normalized Protein Catabolic Rate (nPCR) and inflammation (CRP) cross-sectionally and longitudinally[27,28]. Similarly, prealbumin concentration was associated also with nPCR and inflammatory biomarkers[29].

The high levels of TMAO we detected also raise a third possibility – that any negative effects of TMAO on the vasculature occur when concentrations exceed levels observed among healthy individuals and do not increase further when levels rise to the extraordinary levels seen in this dialysis cohort. In this scenario, all patients would experience the negative effects associated with high TMAO, possibly contributing to the exceedingly high cardiovascular risk observed in this population.

Our study has several strengths. First, the cohort is drawn from a national sample and patients were treated in many facilities across the country. TMAO concentrations are significantly elevated in hemodialysis patients and clearly would provide a target to reduce cardiovascular risk, were one conferred by this oxidized amine.

However, our study has several limitations. First, given the modest sample size, we cannot conclude definitively that there is no association between the markedly elevated serum TMAO concentrations and mortality (or cardiovascular disease). In addition, we did not collect information on dietary intake or antibiotic use, which are known to affect serum

TMAO concentrations. Therefore we could not determine whether these factors were associated with serum TMAO concentrations in our population.

Practical applications

Diets rich in carnitine and phosphatidylcholine; red meats and eggs for example, have the potential to increase the risk of cardiovascular disease by interaction with bacteria in the gut and serving as a precursor for trimethyl amine oxide (TMAO). While elevated serum TMAO concentrations are associated with higher cardiovascular risk in patients who are not receiving dialysis, we could not find evidence linking TMAO concentrations with mortality or cardiovascular risk in patients new to dialysis. For now, there is no specific rationale to restrict from the diet foods rich in TMAO precursors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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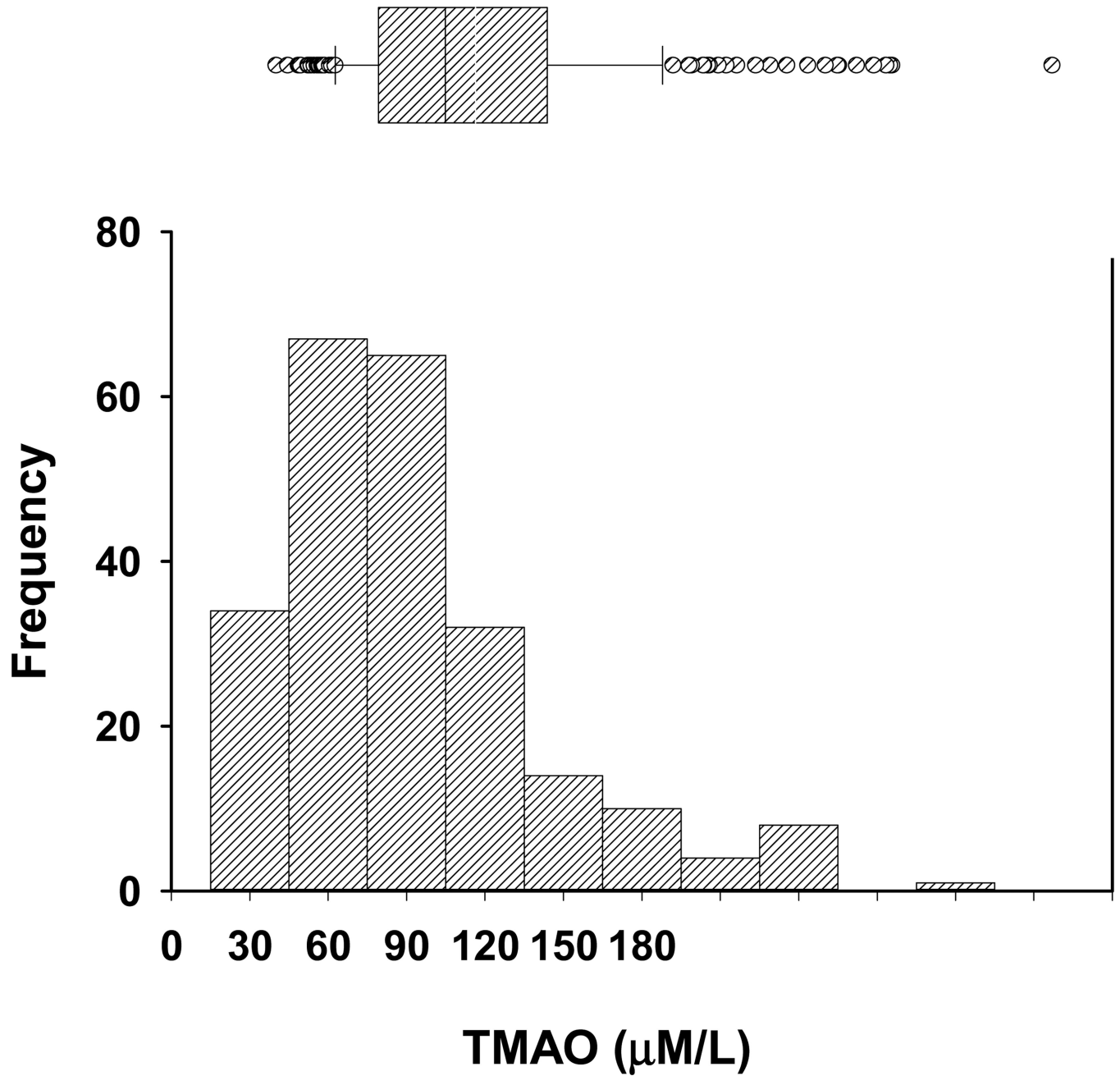**Figure 1. Distribution of trimethylamine N Oxide levels in hemodialysis patients**
Distribution of trimethylamine N Oxide (TMAO) by decile (lower figure) mean ± 2 standard deviations (upper figure) in 235 hemodialysis patients.

Table 1

Baseline Demographic Characteristics of analyzed cohort (N=235)[*]

Characteristic	Value
Age, y	61.8 ± 14.2
Sex, male	130 (55.3%)
Race, black	65 (27.7%)
BMI, kg/m^2	29.7 ± 7.28
Albumin, g/dl	3.47 ± 0.48
Prealbumin, mg/dl	30.7 ± 9.02
TMAO, μM/L	43 (27.5–66.6)
Creatinine, mg/dl	7.15 ± 3.86
CRP, mg/L	7.5 (3.95–13.0)
Atherosclerosis	85 (36.2%)
Congestive heart failure	79 (33.6%)
Diabetes Mellitus	138 (58.7%)

[*] Values are mean ± sd or median (interquartile range). Categorical data are shown as N (%).

Abbreviations: BMI, body mass index; TMAO, trimethyl amino oxide; CRP, C-reactive protein.

Table 2

Correlations between TMAO and clinical predictors (N=235)[*]

Variable	Spearman Rank Correlation	95% Confidence Interval	P-Value
BMI	−0.02	−0.15 to 0.11	0.76
Albumin	0.24	0.11 to 0.35	<0.001
Prealbumin	0.19	0.07 to 0.31	0.003
Log CRP	−0.18	−0.30 to −0.06	0.005
Creatinine	0.21	0.08 to 0.33	0.002
Age	−0.09	−0.22 to 0.04	0.17

[*] Abbreviations: BMI, body mass index; TMAO, trimethyl amine N oxide; CRP, C-reactive protein.

Table 3

Univariate associations of TMAO with outcomes

TMAO variable	HR (95% CI)	p-value
All-cause mortality		
Continuous, log-transformed	0.84 (0.65 – 1.09)	0.19
Quartiles		
Q1: 27.5	1.00	
Q2:> 27.5 – 43.0	0.69 (0.38 – 1.11)	0.12
Q3: >43.0 – 66.6	0.69 (0.42 – 1.11)	0.12
Q4: >66.6 – 184	0.61 (0.38 – 0.97)	0.04
Cardiovascular death or hospitalization		
Continuous, log-transformed	0.88 (0.57 – 1.35)	0.55
Quartiles		
Q1: 26.8	1.00	
Q2: >26.8 – 42.0	0.93 (0.43 – 2.00)	0.86
Q3: >42.0 – 62.0	0.86 (0.39 – 1.92)	0.71
Q4: >62.0	0.71 (0.32 – 1.59)	0.40

Table 4

Multivariable predictors of all-cause mortality.

Variable	HR (95% CI)	p-value
TMAO, μM/L		
Q1: 27.5	1.00	
Q2: >27.5 – 43.0	0.86 (0.53 – 1.40)	0.54
Q3: >43.0 – 66.6	1.20 (0.71 – 2.00)	0.50
Q4: >66.6 – 184	1.14 (0.67 – 1.93)	0.62
Age, per 10 years	1.46 (1.25 – 1.70)	<0.001
Sex, female	1.07 (0.74 – 1.54)	0.73
Race, black	0.54 (0.33 – 0.87)	0.01
BMI, kg/m^2		
<20	0.75 (0.20 – 2.90)	0.68
20 – 24.99	1.00	
25 – 29.99	1.07 (0.68 – 1.70)	0.77
30	0.82 (0.50 – 1.36)	0.45
Albumin, g/dL	0.74 (0.45 – 1.22)	0.66
Prealbumin, mg/dL	0.94 (0.91 – 0.97)	<0.001
Log CRP	1.04 (0.87 – 1.24)	0.66
Diabetes mellitus	0.97 (0.66 – 1.43)	0.87

Table 5

Multivariable predictors of cardiovascular death or hospitalization.

Variable	HR (95% CI)	p-value
TMAO, µM/L		
Q1: 27.5	1.00	
Q2: >27.5 – 43.0	1.37 (0.61 – 3.10)	0.44
Q3: >43.0 – 66.6	1.23 (0.53 – 2.80)	0.63
Q4: >66.6 – 184	0.92 (0.40 – 2.10)	0.84
Race, black	0.49 (0.23 – 1.04)	0.06
Prealbumin, mg/dL	0.96 (0.92 – 0.99)	0.02
Diabetes mellitus	2.10 (1.05 – 4.10)	0.04