

Serum Trimethylamine-*N*-Oxide is Elevated in CKD and Correlates with Coronary Atherosclerosis Burden

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ABSTRACT

Trimethylamine-*N*-oxide (TMAO) was recently identified as a promoter of atherosclerosis. Patients with CKD exhibit accelerated development of atherosclerosis; however, no studies have explored the relationship between TMAO and atherosclerosis formation in this group. This study measured serum concentrations and urinary excretion of TMAO in a CKD cohort ($n=104$), identified the effect of renal transplant on serum TMAO concentration in a subset of these patients ($n=6$), and explored the cross-sectional relationship between serum TMAO and coronary atherosclerosis burden in a separate CKD cohort ($n=220$) undergoing coronary angiography. Additional exploratory analyses examined the relationship between baseline serum TMAO and long-term survival after coronary angiography. Serum TMAO concentrations demonstrated a strong inverse association with eGFR ($r^2=0.31$, $P<0.001$). TMAO concentrations were markedly higher in patients receiving dialysis (median [interquartile range], $94.4 \mu\text{M}$ [54.8–133.0 μM]) for dialysis-dependent patients versus $3.3 \mu\text{M}$ [3.1–6.0 μM] for healthy controls; $P<0.001$); whereas renal transplantation resulted in substantial reductions in TMAO concentrations (median [min–max] $71.2 \mu\text{M}$ [29.2–189.7 μM] pretransplant versus $11.4 \mu\text{M}$ [8.9–20.2 μM] post-transplant; $P=0.03$). TMAO concentration was an independent predictor for coronary atherosclerosis burden ($P=0.02$) and predicted long-term mortality independent of traditional cardiac risk factors (hazard ratio, 1.26 per 10 μM increment in TMAO concentration; 95% confidence interval, 1.13 to 1.40; $P<0.001$). In conclusion, serum TMAO concentrations substantially increase with decrements in kidney function, and this effect is reversed by renal transplantation. Increased TMAO concentrations correlate with coronary atherosclerosis burden and may associate with long-term mortality in patients with CKD undergoing coronary angiography.

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Patients with CKD have a high prevalence of cardiovascular comorbidities, which primarily contributes to the exceedingly high mortality in this group.^{1,2} For example, the 5-year survival for ESRD patients receiving dialysis is approximately 35%, with >50% of the mortality in this group resulting directly from cardiovascular causes.¹ It is well established that CKD patients exhibit a disproportionate burden of atherosclerosis as compared with individuals having normal kidney function.^{2–5} Furthermore, a higher prevalence of traditional risk factors for the development of atherosclerosis, such as hypertension, diabetes and hyperlipidemia, only partially

accounts for the accelerated atherosclerosis in CKD patients, leading to the hypothesis that unique risk factors must be present in this population.^{6,7}

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Trimethylamine-*N*-oxide (TMAO) is a circulating organic compound produced by the metabolism of dietary L-carnitine and choline,^{8,9} which was recently found to directly induce atherosclerosis in rodents. Moreover, high serum TMAO concentrations have been observed to strongly associate with incident cardiovascular events in humans with preserved kidney function,^{8–11} providing solid clinical evidence to support a link between TMAO and cardiovascular pathology. Both L-carnitine and choline are metabolized by intestinal bacteria to trimethylamine, a metabolite which is absorbed from the intestine and subsequently oxidized via hepatic flavin monooxygenase enzymes to form TMAO.¹² Under normal physiologic conditions, circulating TMAO is rapidly cleared from the bloodstream, almost exclusively by urinary excretion.^{13,14}

To date, there are very few published reports on TMAO metabolism in patients with CKD,^{15–17} and the association between TMAO concentrations and atherosclerosis burden in this group has not been investigated. Given that TMAO clearance is largely dependent on the renal excretion of this compound, and that prior studies have demonstrated TMAO to induce atherosclerotic plaque formation in rodents, we postulated that TMAO concentrations would be elevated in patients with CKD and be associated with the accelerated atherosclerosis observed in this population. Thus, in the current study we gathered several independent cohorts to accomplish the following goals: (1) to determine the relationship between serum TMAO concentrations and eGFR in a population of patients with CKD; (2) to examine changes in serum TMAO after renal transplantation to define the modifiability of TMAO concentrations following the restoration of renal function; and (3) to investigate the association of TMAO concentrations with atherosclerosis burden among CKD patients undergoing coronary angiography. Additionally, as an exploratory aim, we examined the association of baseline TMAO concentrations with long-term mortality in patients undergoing coronary angiography. Collectively, these studies could delineate the association of TMAO with kidney function and establish a foundation for TMAO as a novel promoter and modifiable risk factor for atherosclerosis formation in patients with CKD.

RESULTS

Cross-Sectional Assessment of Serum TMAO Concentrations in CKD

To evaluate whether circulating TMAO concentrations are elevated in CKD, we performed a cross-sectional study of serum TMAO concentrations in a group of patients ($n=104$) with variable degrees of kidney function, ranging from normal function to end-stage disease (Figure 1). We observed a graded elevation of serum TMAO concentrations with advancing CKD stage, with median concentrations in dialysis-dependent patients with ESRD being roughly 30-fold higher than in controls ($94.4 \mu\text{M}$ for dialysis-dependent patients versus $3.3 \mu\text{M}$ for controls, $P<0.001$). Simple linear regression analysis

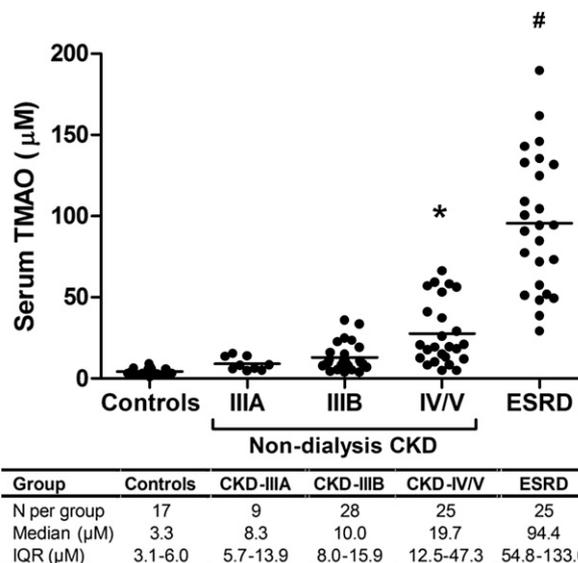


Figure 1. Cross-sectional analysis of serum TMAO concentrations by CKD stage. Serum TMAO concentrations were measured in a cohort of patients ($n=104$) with varying severity of kidney disease, ranging from normal kidney function (controls) to dialysis-dependent ESRD. Patients were grouped according to CKD staging: controls (eGFR ≥ 60 ml/min per 1.73 m^2), stage IIIA (eGFR 45–59 ml/min per 1.73 m^2), stage IIIB (eGFR 30–44 ml/min per 1.73 m^2), stage IV/V (eGFR <30 and not dialysis-dependent), and ESRD (receiving chronic hemodialysis therapy) * $P<0.05$; # $P<0.001$ compared with control group.

of CKD patients with residual kidney function revealed a strong inverse relationship between eGFR and serum TMAO concentrations (Figure 2A, $r^2=0.31$, $P<0.001$). The urine TMAO:Cr ratio and the fractional urinary excretion of TMAO (FE_{TMAO}) were equivalent in CKD and control patients (Figures 2, B and C, respectively; TMAO:Cr= 43.2 ± 29.7 for CKD group versus 31.6 ± 15.8 for controls, $P=0.20$; $\text{FE}_{\text{TMAO}}=70.3 \pm 20.7\%$ for CKD group versus $73.2 \pm 20.6\%$ for control group, $P=0.66$). Similarly, an analysis of FE_{TMAO} and TMAO:Cr when stratified by CKD stage revealed no apparent difference in the median values for these urinary parameters compared with control patients (Supplemental Figure 1). Demographic characteristics of this cohort are summarized in Supplemental Table 1.

Impact of Renal Transplantation on Serum TMAO Concentrations

To investigate whether extreme elevations of serum TMAO observed in advanced CKD were reversible with restoration of kidney function, we evaluated serum TMAO concentrations prior to and 3 months after successful renal transplantation in six patients with advanced-stage kidney disease (one patient with eGFR of $11 \text{ ml/min per } 1.73 \text{ m}^2$ and five dialysis-dependent patients with ESRD). All patients demonstrated a marked reduction in serum TMAO following transplantation, with a median pretransplant TMAO concentration of $71.3 \mu\text{M}$ and post-

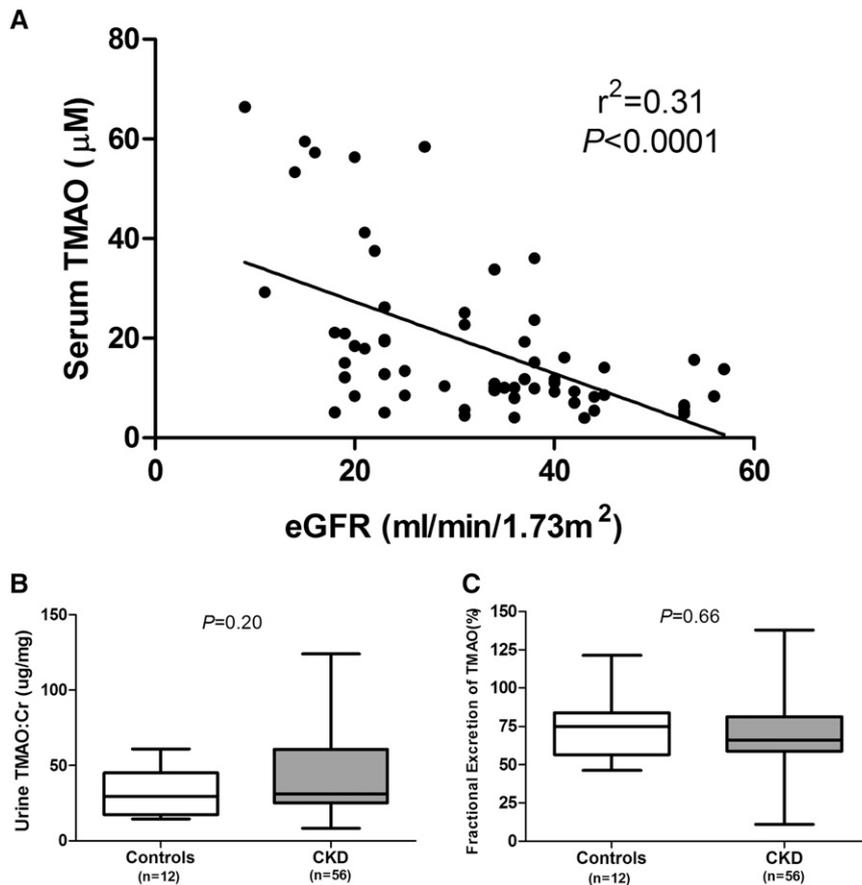


Figure 2. Relationship between serum and urine TMAO concentrations in CKD. (A) Linear regression analysis depicting the relationship between serum TMAO and eGFR in CKD patients from a cross-sectional cohort (excluding patients with ESRD). (B) Comparison of the spot urine TMAO to creatinine ratio (TMAO:Cr) in controls and CKD patients with residual kidney function. (C) Comparison of the spot fractional excretion of TMAO (FE_{TMAO}) in controls and patients with CKD with residual kidney function (box plots depict median + interquartile range and whiskers illustrate minimum-maximum values).

transplant TMAO concentration of $11.4 \mu\text{M}$ for the group (Figure 3, $P=0.03$).

Correlation between Serum TMAO Concentration and Coronary Atherosclerosis in CKD

To investigate the correlation of elevated serum TMAO and atherosclerosis in a population with CKD, we measured serum TMAO concentrations in 220 study participants with impaired kidney function (ranging from stage IIIA CKD to dialysis-dependent ESRD) who underwent coronary angiography and long-term mortality follow-up as part of the Diabetes Genome Project (DGP).^{18,19} Table 1 summarizes the baseline characteristics of these study participants, stratified by tertiles of TMAO concentration. Similar to the findings in our cross-sectional cohort, individuals with higher serum TMAO concentrations in the DGP cohort exhibited lower eGFR. The highest TMAO tertile contained a greater proportion of males, a

higher prevalence of diabetes, and a higher prevalence of peripheral vascular disease. There was no significant association of TMAO tertiles with age, race, tobacco use, hypertension, history of myocardial infarction, prior coronary stenting or bypass grafting, congestive heart failure, cholesterol, triglycerides, C-reactive protein, albumin, or medication use (statins, angiotensin converting enzyme-inhibitors, angiotensin receptor blockers, or β -blockers).

Individuals in the highest TMAO tertile demonstrated a greater coronary atherosclerotic burden, as defined by a higher mean modified Gensini score, compared with participants in the lower TMAO tertiles (7.4 ± 5.1 versus 5.9 ± 4.0 for tertile 1, $P=0.11$ for intertertile trend). When serum TMAO was treated as a continuous variable, a statistically significant correlation with the modified Gensini score was observed ($r=0.17$, $P=0.02$). Similarly, after developing a predictive model including traditional atherosclerotic risk factors, TMAO remained a significant predictor of Gensini score ($P=0.02$).

Because only a subset of this cohort had urine samples collected ($n=98$), we were unable to fully assess the effect of proteinuria on the relationship between TMAO and atherosclerosis burden. However, in an additional subanalysis to determine the correlation between the urine protein-to-creatinine ratio and Gensini scoring, there was no evidence of a correlation between these parameters ($r=0.10$, $P=0.33$).

Association between TMAO and Long-term Mortality following Coronary Angiography

Figure 4 illustrates the survival curves for study participants divided by TMAO tertile. The 4-year survival for individuals with the highest TMAO concentrations (tertile 3) was 64.6%, compared with 77.6% for tertile 2 and 81.1% for tertile 1 (Figure 4). When comparing survival across TMAO tertiles, individuals in the highest tertile exhibited a greater 4-year mortality compared with individuals in lower tertiles; however, this association was not statistically significant (unadjusted hazard ratio of 1.95 [95% confidence interval (95% CI), 0.91 to 4.17] compared with tertile 1, $P>0.05$, and 2.08 [95% CI, 0.95 to 4.57] compared with tertile 2, $P>0.05$, respectively). Conversely, when this relationship was examined considering TMAO as a continuous variable, TMAO was independently associated with 4-year mortality. There was a 19% increase in mortality for every $10 \mu\text{M}$ increment in serum TMAO (HR 1.19, 95% CI, 1.10 to 1.29, $P<0.001$) in

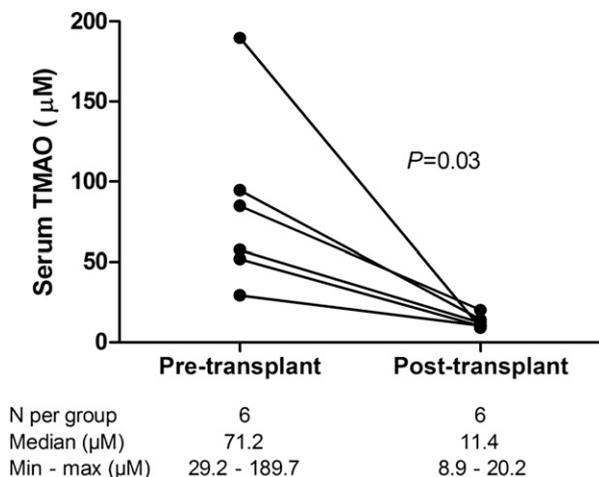


Figure 3. Characterization of serum TMAO changes following renal transplantation. Serum TMAO concentrations in six patients with ESRD prior to and three months following successful renal transplantation.

the unadjusted model. Similarly, in the multivariate prediction model TMAO remained a predictor of mortality (Table 2; HR 1.26, 95% CI, 1.13 to 1.40, $P < 0.001$).

DISCUSSION

To our knowledge, the current study is the first investigation to comprehensively define the association between serum TMAO concentrations and residual kidney function across a broad spectrum of GFR estimates and to demonstrate an independent association between TMAO and coronary atherosclerotic burden in a CKD cohort. In conjunction with a recent publication by Wang *et al.* suggesting TMAO to be a direct inducer of atherosclerosis in rodents,⁹ and additional studies in non-CKD human cohorts demonstrating an association with TMAO and adverse cardiovascular outcomes,^{8,10,11} we believe our current observations provide strong preliminary evidence to support TMAO as a nontraditional risk factor for atherosclerosis formation in patients with CKD.

Our observation of a direct inverse relationship between TMAO and kidney function supports and extends prior studies suggesting that TMAO clearance from the circulation is largely dependent on urinary excretion. However, the extent of TMAO elevation in advanced CKD stages was far greater than originally anticipated. The median serum TMAO concentration in ESRD individuals was 94.4 µM, roughly 30-fold higher than in our control group with normal kidney function (Figure 1). This observation is particularly striking when compared with the non-CKD cohort reported by Tang *et al.*¹⁰ The median serum TMAO concentration in their cohort was 3.7 µM with an interquartile range of 2.4–6.2 µM. Even with this modest variation in serum TMAO, they observed a 2.54-fold increased risk of myocardial infarction, stroke, or death in the highest TMAO quartile compared with the lowest

quartile. In a more recent publication by this same group, serum TMAO concentrations were observed both to independently predict all-cause mortality in a nondialysis CKD cohort and to contribute directly to renal fibrosis in animal models.¹⁷ Of note, exploratory survival analyses in our cohort confirmed TMAO as a predictor of mortality by demonstrating a 26% increased risk of long-term mortality per 10 µM increase in serum TMAO, even after adjusting for other traditional cardiovascular risk factors (Table 2). Based on this estimate, it could be postulated that an ESRD population would be at substantial risk for cardiovascular mortality given the considerably elevated serum TMAO concentrations observed in this setting. However, because the DGP cohort contained very few patients with ESRD receiving dialysis, it is unclear if TMAO would remain an independent predictor of mortality endpoints if similar analyses were repeated in larger populations comprised solely of dialysis patients. Thus, future large-scale studies which are specifically designed to examine the predictive value of TMAO for cardiovascular and mortality outcomes in both CKD and ESRD populations will be necessary to determine the generalizability of our initial observations to these separate groups.

The generation of trimethylamine from dietary precursors occurs within the intestinal lumen and appears to be exclusively dependent on the presence of intestinal bacteria, as interventions to alter the composition of the gut microbiome have led to marked reductions in circulating TMAO concentrations in both rodents and humans with intact kidney function;^{9,10} however, it remains unclear which intestinal microbes are primarily responsible for this process. The role of the intestinal microbiome in the development of CKD comorbidities has been the topic of extensive biomedical research over the last decade, with numerous studies attempting to describe a pathophysiologic link between alterations in gut flora and various clinical observations in CKD, including cardiovascular disease.²⁰ Several previous studies have suggested a shift in the gut microbiome in CKD,^{21,22} as well as altered intestinal barrier function in this setting.^{23–26} Thus, it is plausible that an increased intestinal absorption of trimethylamine or hepatic synthesis of TMAO could contribute to the elevated TMAO concentrations in CKD.

It is noteworthy that we observed comparable urinary TMAO:Cr values between CKD and control patients (Figure 2). The urine TMAO concentration, when normalized to that of creatinine, is a measure of the TMAO excretion rate and, under steady-state conditions, this should be equal to the rate of TMAO generation. Our results therefore suggest that the intestinal absorption of trimethylamine and hepatic production of TMAO are largely unchanged in CKD, so the increments in serum TMAO in earlier CKD stages are likely driven by reductions in renal excretion. Furthermore, our observation that FE_{TMAO} is largely unchanged suggests that the major contributor to the reduction in TMAO excretion is reduced glomerular filtration of TMAO, while tubular reabsorption is largely unaffected. Our finding of a dramatic reduction in

Table 1. Baseline demographic characteristics stratified by TMAO tertile for CKD cohort undergoing atherosclerosis assessment by coronary angiography

	Total Cohort (n=220)	TMAO Tertiles			P Value
		Tertile 1 0.63 to <5.58 μM (n=73)	Tertile 2 5.58 to <9.26 μM (n=73)	Tertile 3 9.26–163.03 μM (n=74)	
Demographics					
Age (yr)	69.7 \pm 10.3	70.3 \pm 8.9	71.2 \pm 8.6	67.7 \pm 12.8	0.11
Gender					0.05
Male	94 (42.7%)	23 (31.5%)	33 (45.2%)	38 (51.4%)	
Female	126 (57.3%)	50 (68.5%)	40 (54.8%)	36 (48.6%)	
Race					0.19
Caucasian	200 (90.9%)	70 (95.9%)	67 (91.8%)	63 (85.1%)	
African American	16 (7.3%)	3 (4.1%)	5 (6.8%)	8 (10.8%)	
Other	4 (1.8%)	0 (0.0%)	1 (1.4%)	3 (4.1%)	
Hypertension	214 (97.3%)	71 (97.3%)	70 (95.9%)	73 (98.6%)	0.54
Current smoker	15 (6.8%)	5 (6.8%)	4 (5.5%)	6 (8.1%)	0.94
Body mass index	30.6 \pm 7.0	30.4 \pm 7.1	30.3 \pm 7.5	31.2 \pm 6.5	0.71
Diabetes	141 (64.1%)	39 (53.4%)	46 (63.0%)	56 (75.7%)	0.02
History of CVD	55 (25.0%)	17 (23.3%)	18 (24.7%)	20 (27.0%)	0.87
History of PVD	73 (33.2%)	17 (23.3%)	22 (30.1%)	34 (45.9%)	0.01
History of CHF	69 (31.4%)	16 (21.9%)	28 (38.4%)	25 (33.8%)	0.09
History of MI	81 (36.8%)	27 (37.0%)	25 (34.2%)	29 (39.2%)	0.82
History of PCI	107 (48.9%)	28 (38.9%)	38 (52.1%)	41 (55.4%)	0.11
History of CABG	57 (25.9%)	19 (26.0%)	19 (26.0%)	19 (25.7%)	0.99
Laboratory parameters					
TMAO (μM)	6.9 (4.8–10.9)	4.0 (3.2–4.8)	6.9 (6.0–7.7)	17.6 (10.9–31.3)	
eGFR (ml/min per 1.73 m ²)	40.3 \pm 13.9	45.2 \pm 9.8	44.3 \pm 9.6	31.4 \pm 16.7	<0.001
CKD stage					<0.001
IIIA	89 (40.5%)	41 (56.2%)	33 (45.2%)	15 (20.3%)	
IIIB	89 (40.5%)	26 (35.6%)	35 (47.9%)	28 (37.8%)	
IV	20 (9.1%)	4 (5.5%)	5 (6.8%)	11 (14.9%)	
V	2 (0.9%)	1 (1.4%)	0 (0.0%)	1 (1.4%)	
ESRD	20 (9.1%)	1 (1.4%)	0 (0.0%)	19 (25.7%)	
Cholesterol (mg/dl)	161.2 \pm 42.2	168.0 \pm 40.8	162.9 \pm 40.5	152.8 \pm 44.4	0.09
Triglycerides (mg/dl)	153.2 \pm 94.9	150.3 \pm 109.6	158.2 \pm 92.1	151.3 \pm 82.4	0.86
Albumin (mg/dl)	3.6 \pm 0.6	3.6 \pm 0.4	3.6 \pm 0.5	3.5 \pm 0.8	0.25
C-reactive protein (mg/dl)	12.4 \pm 25.2	10.3 \pm 16.7	13.8 \pm 31.3	13.1 \pm 25.5	0.67
Medication use					
Statins	163 (74.1%)	61 (83.6%)	50 (68.5%)	52 (70.3%)	0.08
Beta-blockers	173 (78.6%)	61 (83.6%)	52 (71.2%)	60 (81.1%)	0.16
ACE-inhibitors	107 (48.6%)	36 (49.3%)	38 (52.1%)	33 (44.6%)	0.66
ARB	43 (19.5%)	14 (19.2%)	19 (26.0%)	10 (13.5%)	0.16
Atherosclerosis quantification					
Diseased vessels >70%					0.03
0	35 (15.9%)	12 (16.4%)	15 (20.5%)	8 (10.8%)	
1	95 (43.2%)	36 (49.3%)	27 (37.0%)	32 (43.2%)	
2	39 (17.7%)	17 (23.3%)	13 (17.8%)	9 (12.2%)	
3	51 (23.2%)	8 (11.0%)	18 (24.7%)	25 (33.8%)	
Multivessel disease >70%	90 (40.9%)	25 (34.2%)	31 (42.5%)	34 (45.9%)	0.33
Modified Gensini score	6.7 \pm 4.6	5.9 \pm 4.0	6.8 \pm 4.4	7.4 \pm 5.1	0.11

Continuous variables presented as median (interquartile range) or mean \pm SD. CVD, cerebrovascular disease; PVD, peripheral vascular disease; MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blockers.

serum TMAO following renal transplantation (Figure 3) provides strong evidence for reversibility of TMAO elevations in advanced CKD, and may partially explain the significant survival benefit associated with the restoration of kidney

function in patients with renal transplants.^{27,28} Nonetheless, it is clear that further studies exploring the physiology of TMAO generation and metabolism are warranted to more thoroughly define the etiology of TMAO elevations in CKD.

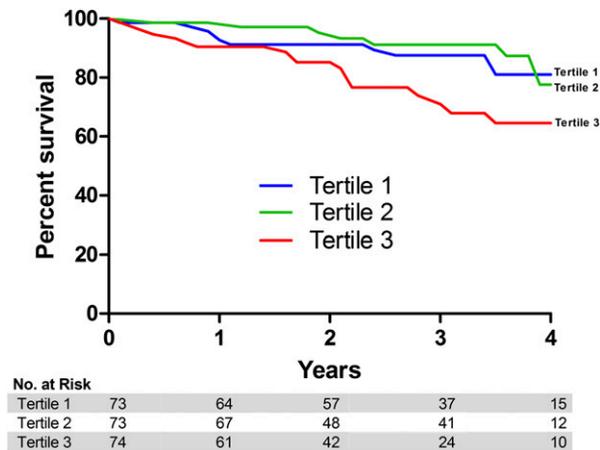


Figure 4. Survival curve for TMAO tertiles in a longitudinal cohort. Kaplan–Meier survival analysis based on TMAO tertiles for 220 patients with CKD enrolled in the Diabetes Genome Project study.

Table 2. Predictors of all-cause mortality

Predictor Variable	Hazard Ratio (95% CI)	P Value
TMAO (per 10 μ M increase)	1.26 (1.13–1.40)	<0.001
Age (per 10 year increase)	1.76 (1.17–2.64)	<0.01
C-reactive protein (per 1 mg/dL increase)	1.014 (1.01–1.02)	<0.001
History of congestive heart failure	2.68 (1.35–5.29)	<0.01
History of peripheral vascular disease	2.56 (1.23–5.32)	0.01

Covariates available to the model were: TMAO, eGFR, diabetes, hypertension, race, gender, age, body mass index, CKD stage, history of percutaneous intervention, triglycerides, cholesterol, history of coronary artery bypass grafting, history of myocardial infarction, history of cerebrovascular accident, history of peripheral vascular disease, history of congestive heart failure, and smoking status.

While the observed association between serum TMAO concentrations and coronary atherosclerosis in our CKD cohort is consistent with prior observations in animals and non-CKD human populations, the mechanism whereby TMAO potentiates atherosclerosis development remains poorly defined. The prevailing hypothesis in the literature is that TMAO promotes an imbalance in cholesterol uptake and efflux from macrophages, leading to an abundance of pro-atherogenic foam cells migrating to the arterial wall.^{8,9} Current evidence suggests that this could occur by either promoting macrophage expression of cholesterol scavenger receptors, CD36 and SR-A1, or by restricting reverse cholesterol transport out of these cells. Interestingly, numerous studies have suggested enhanced scavenger receptor expression on cells of the monocyte/macrophage lineage, along with defects in macrophage cholesterol efflux, in humans with CKD.^{29–33} Furthermore, studies in *ApoE*^{−/−} mice, a rodent model of atherosclerosis, demonstrate that induction of kidney disease in this model enhances macrophage cholesterol accumulation independent of alterations in plasma cholesterol concentrations.³⁴

This investigation has both important strengths and limitations. The strengths of this study include: (1) the use of several unrelated CKD cohorts exhibiting a broad range of eGFR measurements for the exploration and validation of the relationship between serum TMAO and eGFR; (2) the extensive characterization of the coronary atherosclerosis burden in all study participants in the DGP cohort using a standardized method of atherosclerosis quantification; (3) the long (4-year) follow-up for mortality endpoints; and (4) the demonstration of short-term reversibility of high serum TMAO concentrations following renal transplantation. Potential limitations of this work include: (1) a lack of ethnic diversity (90% Caucasian) which may affect the generalizability of our findings to other populations with CKD; (2) limited inclusion of outcomes data from patients with ESRD, which are the subset of patients with CKD with the highest concentrations of TMAO and at the greatest risk for adverse cardiovascular outcomes; (3) the relatively small number of mortality endpoints and lack of data on cause of death in the DGP cohort preventing us from obtaining a more nuanced understanding of the relationship between TMAO and cardiovascular mortality; and (4) the potential for confounding by unmeasured risk factors, such as proteinuria.

The current investigation advances our understanding of cardiovascular disease in CKD by providing a novel mechanistic link to explain the presence of accelerated atherosclerosis in patients with reduced kidney function. As such, our study demonstrates a strong inverse relationship between circulating concentrations of TMAO, a novel atherogenic compound, and residual kidney function in patients with CKD, and defines TMAO as an independent predictor of coronary atherosclerosis in this population. Based on evidence that TMAO production is dependent on dietary precursors and the intestinal microbiome, TMAO may represent a novel modifiable risk factor and therapeutic target for reducing cardiovascular mortality in patients with CKD.

CONCISE METHODS

Study Design

We assembled data from five cohorts to accomplish our goals in this investigation. A cross-sectional analysis of TMAO serum and urine concentrations across CKD stages was performed using stored patient samples from three separate prospective studies: (1) Impact of Vitamin D Therapies on Monocyte Function in CKD (clinicaltrials.gov ID: NCT01222234); (2) Differential Effects of Ergocalciferol and Cholecalciferol Therapies in CKD (clinicaltrials.gov no. NCT01835691); and (3) Impact of Exercise on Cognitive Function in ESRD (clinicaltrials.gov no. NCT02145702). Only samples from the baseline study visits (prior to study interventions) were used for these cross-sectional analyses. To assess changes in TMAO following successful treatment of ESRD with renal transplantation, we used stored serum samples from the Cognitive Impairment and Imaging Correlates in ESRD study (clinicaltrials.gov no. NCT01883349). TMAO was measured in samples collected from the baseline and 3-month post-transplant visits.

The cross-sectional association of TMAO concentrations with the burden of coronary atherosclerosis and its association with subsequent survival were conducted using stored serum samples and clinical data collected from individuals with CKD (eGFR < 60 ml/min per 1.73 m²) who participated in the Diabetes Genome Project (clinicaltrials.gov no. NCT00428961).^{18,19} Briefly, consecutive patients undergoing coronary angiography were invited, prior to their procedure, to participate in a prospective registry of genetic factors and biomarkers associated with atherosclerosis and prognosis. For the current study, patients were stratified by CKD stage and a subset of patients from each stage (IIIA–V, or ESRD) was randomly selected for analysis of TMAO concentrations. Glomerular filtration rate was estimated via the CKD-EPI equation as previously described,³⁵ and CKD stages were defined according to the Kidney Disease Outcomes Quality Initiative guidelines.³⁶ Study participants underwent quantitative coronary angiography at Saint Luke's Hospital Imaging Core Lab,³⁷ and atherosclerotic burden was quantified using the modified Gensini score.³⁸ Four-year survival was assessed through the Social Security Death Master file. All of these studies were approved by the Institutional Review Boards at either the University of Kansas Medical Center (KUMC) or St. Luke's Health System, and complied with the Declaration of Helsinki. All participants in these studies provided written informed consent for study participation. Because all patient data were de-identified for the *post-hoc* analyses of TMAO in stored biologic samples, a human subjects research exemption was granted by the KUMC Institutional Review Board for this work.

Laboratory Analyses

Serum and urine TMAO concentrations were measured by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) using heated electrospray ionization (positive mode) and selected reaction monitoring as previously described.³⁹ Briefly, the UHPLC-MS/MS system consisted of an Accela autosampler, Accela UHPLC binary pump, and a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Serum or urine (50 μ L) was combined with an internal standard (200 μ L of *d*9-TMAO at 0.5 μ g/mL) in methanol to precipitate protein. After centrifugation 20 μ L of supernatant was diluted with 100 μ L of 75:25 acetonitrile:methanol and 10 μ L of this mixture was injected onto the UHPLC-MS/MS system. The mobile phases used for the analysis were 10 mM ammonium formate pH 3.5 (mobile phase A) and acetonitrile (mobile phase B), delivered in a gradient at a flow rate of 400 μ L/min. The analytical column was a Waters Acquity BEH amide 50 \times 2.1, 1.7 μ m held at 10°C. The selected reaction monitoring scheme followed transitions of the [M+H]⁺ precursor to selected product ions with the following values: *m/z* 76.1 \rightarrow 58.1 for TMAO, *m/z* 85.1 \rightarrow 66.2 for *d*9-TMAO. The standard curve used for plasma samples ranged from 0.010 to 5.00 μ g/mL (0.13–66.6 μ M), while the standard curve used for urine samples ranged from 1.00 to 150 μ g/mL (13.3–1,997 μ M). Samples that were above the upper limit of quantification were diluted in phosphate-buffered saline to within the assay range. The within-run and between-run precision (percent coefficient of variation) was <7.5%. Recovery of TMAO spiked into human plasma and urine ranged from 93.0% to 103% at three different concentrations in both matrices.

For the studies based at KUMC, serum creatinine was measured on an AU5800 autoanalyzer (Beckman Coulter, Brea, CA) and urine

creatinine was measured using a colorimetric urine detection kit (Arbor Assays, Ann Arbor, MI). For the DGP cohort, serum creatinine, albumin, hs-CRP and lipid measurements were performed on a Vitros 5600 autoanalyzer (Ortho Clinical Diagnostics, Raritan, NJ). The urinary TMAO:creatinine ratio was presented as micrograms of TMAO per mg of creatinine, and urinary FE_{TMAO} was calculated by the following formula: [(urine TMAO in μ g/ml / serum TMAO in μ g/ml) / (urine creatinine in mg/dl / serum creatinine in mg/dl)] \times 100.

Statistical Analyses

Between-group comparisons for the cross-sectional evaluation of TMAO concentrations across CKD stages was performed using one-way ANOVA. The relationship between TMAO and eGFR was determined by simple linear regression analysis. Comparison of urine TMAO parameters between CKD patients and controls was performed utilizing unpaired two-sided Student's *t* test, while the change in TMAO following renal transplantation was evaluated by paired two-sided Student's *t* test. The population from the Diabetes Genome Project was divided into tertiles of TMAO and baseline characteristics displayed. Continuous variables were compared using one-way ANOVA and discrete variables compared using chi-squared test or Fisher's exact test, as appropriate. The modified Gensini score demonstrated a non-normal distribution; thus, log transformation was performed and all goodness-of-fit tests (Kolmogorov–Smirnov, Cramer–van Mises and Anderson–Darling) supported the lognormal distribution as a good model. Correlations between the log Gensini Score and TMAO were tested using a Pearson correlation coefficient. A regression model was developed to predict the log Gensini score using a stepwise selection method with 0.20 to enter the model and 0.05 to stay in the model. The variables that were allowed to enter the model included TMAO, eGFR, diabetes, hypertension, race, gender, age, body mass index, CKD stage, history of percutaneous intervention, triglycerides, cholesterol, history of coronary artery bypass grafting, history of myocardial infarction, history of cerebrovascular accident, history of peripheral vascular disease, history of congestive heart failure, and smoking status. To determine the effect of TMAO on survival, an unadjusted Cox proportional hazards model was developed with TMAO as a continuous variable. A model to predict survival was developed using Cox proportional hazards and having an entry criteria of 0.20 and a stay criteria of 0.05. The same covariates utilized in the Gensini score model were available to enter the survival model. Unadjusted Kaplan–Meier curves were used to depict survival for the TMAO tertiles.

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DISCLOSURES

None.

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See related editorial, "Trimethylamine N-Oxide as a Novel Therapeutic Target in CKD," on pages 8–10.

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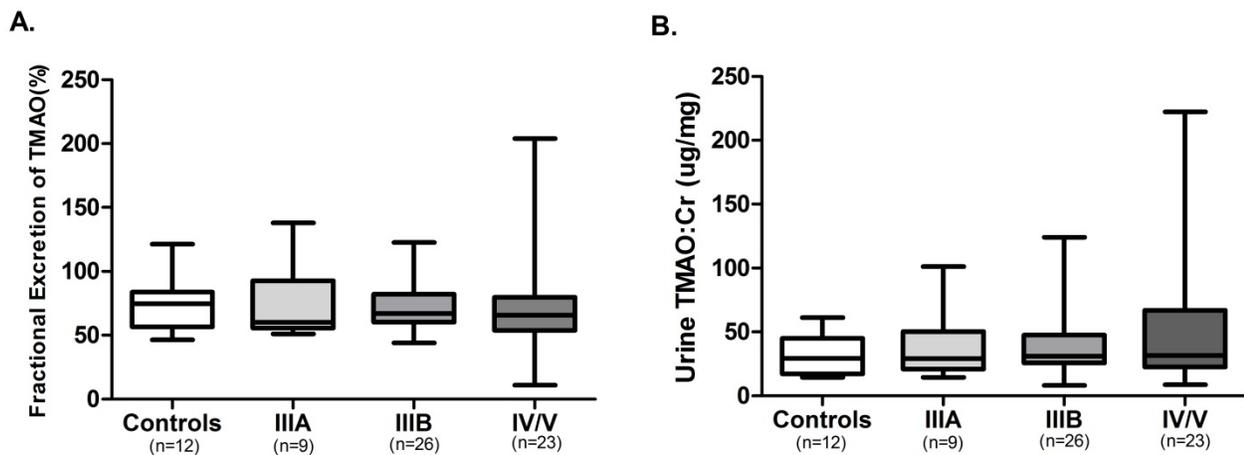
SUPPLEMENTAL DATA

Supplemental Table 1. Demographics of cohort utilized for cross-sectional comparison of TMAO concentrations among CKD stages

	Total Cohort (n=104)	Controls (n=17)	CKD-IIIA (n=9)	CKD-IIIB (n=28)	CKD-IV/V (n=25)	ESRD (n=25)	P-value
Demographics							
Age (years)	57.6 ± 14.2	44.1 ± 14.6	53.4 ± 7.4	57.5 ± 13.8	62.3 ± 13.3	63.9 ± 10.2	< 0.001
Sex							0.015
Female	52 (50.0%)	12 (70.6%)	3 (33.3%)	16 (57.1%)	15 (60.0%)	6 (24.0%)	
Male	52 (50.0%)	5 (29.4%)	6 (66.7%)	12 (42.9%)	10 (40.0%)	19 (76.0%)	
Race							0.751
African-American	24 (23.1%)	3 (17.6%)	3 (33.3%)	7 (25.0%)	6 (24.0%)	5 (20.0%)	
Caucasian	71 (68.3%)	13 (76.5%)	6 (66.7%)	19 (67.9%)	18 (72.0%)	15 (60.0%)	
Other	9 (8.7%)	1 (5.9%)	0 (0.0%)	2 (7.1%)	1 (4.0%)	5 (20.0%)	
Hypertension							< 0.001
No	26 (25.0%)	12 (70.6%)	4 (44.4%)	6 (21.4%)	3 (12.0%)	1 (4.0%)	
Yes	78 (75.0%)	5 (29.4%)	5 (55.6%)	22 (78.6%)	22 (88.0%)	24 (96.0%)	
Smoking History	54 (52.4%)	4 (23.5%)	6 (66.7%)	16 (57.1%)	14 (56.0%)	14 (58.3%)	0.131
BMI	31.3 ± 7.0	29.1 ± 6.6	36.5 ± 6.5	33.2 ± 7.2	30.9 ± 7.7	29.1 ± 4.9	0.023
Diabetes	39 (37.9%)	2 (11.8%)	6 (66.7%)	11 (39.3%)	10 (40.0%)	10 (41.7%)	0.071
History of CAD	31 (30.1%)	2 (11.8%)	1 (11.1%)	10 (35.7%)	7 (28.0%)	11 (45.8%)	0.119
History of CHF	14 (13.6%)	0 (0.0%)	3 (33.3%)	4 (14.3%)	4 (16.0%)	3 (12.5%)	0.181
History of PCI or CABG	19 (18.4%)	2 (11.8%)	2 (22.2%)	6 (21.4%)	4 (16.0%)	5 (20.8%)	0.924
Etiology of CKD		N/A					< 0.001
DM	40 (46.5%)	-	6 (66.7%)	14 (50.0%)	11 (44.0%)	9 (37.5%)	
Glomerular	6 (7.0%)	-	0 (0.0%)	1 (3.6%)	1 (4.0%)	4 (16.7%)	
Hypertension/CV	15 (17.4%)	-	1 (11.1%)	6 (21.4%)	4 (16.0%)	4 (16.7%)	
PKD	11 (12.8%)	-	2 (22.2%)	3 (10.7%)	5 (20.0%)	1 (4.2%)	
Urologic	9 (10.5%)	-	0 (0.0%)	1 (3.6%)	2 (8.0%)	6 (25.0%)	
Other	5 (5.8%)	-	0 (0.0%)	3 (10.7%)	2 (8.0%)	0 (0.0%)	
Laboratory Parameters							
eGFR (ml/min/1.73m ²)	42.5 ± 27.1	96.8 ± 27.3	53.7 ± 8.4	39.3 ± 6.3	20.5 ± 5.0	N/A	< 0.001

Continuous variables presented as mean ± standard deviation

Abbreviations: CAD, coronary artery disease; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; TMAO, trimethylamine-N-oxide; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; CKD, chronic kidney disease; CV, cardiovascular; DM, diabetes mellitus; PKD, polycystic kidney disease; CHF, congestive heart failure



Supplemental Figure 1. FE_{TMAO} and TMAO:Cr Values Stratified by CKD Stage

(A) Comparison of the spot fractional excretion of TMAO (FE_{TMAO}) in controls and CKD patients with residual kidney function after stratification by CKD stage. **(B)** Comparison of the spot urine TMAO to creatinine ratio (TMAO:Cr) in controls and CKD patients with residual kidney function after stratification by CKD stage. (box plots depict median + interquartile range and whiskers illustrate minimum-maximum values; $P=NS$ for all statistical comparisons).