Plasma Concentrations of Trimethylamine-N-Oxide, Choline, and Betaine in Patients With Moderate to Advanced Chronic Kidney Disease and Their Relation to Cardiovascular and Renal Outcomes

Rima Obeid,[*] Husain Awwad,[*] Gunnar Henrik Heine,[†'‡] Insa E. Emrich,[§] Danilo Fliser,[‡] Adam M. Zawada,[‡] and Jürgen Geisel[*]

Objectives: Trimethylamine N-oxide (TMAO) is a gut bacteria-mediated liver metabolite of dietary betaine, choline, and carnitine, which is excreted by glomerular filtration. We studied whether TMAO is excreted by cardiovascular disease (CVD) in patients with chronic kidney disease (CKD).

Methods: Among 478 patients with CKD stage G2 (n = 104), G3a (n = 163), G3b (n = 123), and G4 (n = 88), we studied the association between fasting plasma concentrations of TMAO, choline, or betaine at baseline and kidney function, prevalent CVD, and future renal outcomes during a mean follow-up of 5.1 years.

Results: Decreased glomerular filtration rate was associated with higher plasma concentrations of TMAO, choline, and betaine. Baseline concentrations of TMAO were higher in participants with preexisting CVD compared to those without CVD (8.4 [10.1] vs. 7.8 [8.0] μmol/L; $P = .047$), but the difference was not significant after adjusting for confounders. During the follow-up, 147 participants experienced CVD or died, and 144 reached the predefined renal endpoint. In the adjusted regression analyses, TMAO or choline concentrations in the upper three quartiles (vs. the lowest quartile) were not associated with any of the study's clinical endpoints. In contrast, the adjusted hazard ratio of plasma betaine in the highest quartile versus the lowest quartile was 2.14 (1.32, 3.47) for the CVD endpoint and 1.64 (1.00, 2.67) for the renal endpoint.

Conclusions: Elevated plasma TMAO concentrations were explained by impaired kidney function. Elevated plasma concentrations of betaine, but not those of TMAO or choline, constituted a risk factor for adverse outcomes. TMAO might not be an appropriate target to reduce CVD or renal outcomes in patients with preexisting CKD.

Keywords: betaine; cardiovascular disease; choline; chronic kidney disease; gut bacteria; trimethylamine N-oxide

Introduction

CHRONIC KIDNEY DISEASE (CKD) is highly prevalent (9.1% of the global population) and constitutes a predominant risk factor for cardiovascular disease (CVD) and mortality.[1] Between 1990 and 2017, the contribution of CKD to the overall mortality raised in rank in comparison to other risk factors.[1] Therefore, identification and treatment of risk factors at earlier stages of CKD remain important.

Trimethylamine N-oxide (TMAO) (MW = 75.11 g/mol) is a water-soluble osmolyte that is excreted in urine by glomerular filtration.[2] TMAO is larger than urea

[*]*Department of Clinical Chemistry and Laboratory Medicine, Saarland University Hospital, Homburg, Germany.*

[†]*Agaplesion Markus Hospital, Medical Clinic II, Frankfurt am Main, Germany.*

[‡]*Department of Internal Medicine IV–Nephrology and Hypertension, Saarland University Hospital and Saarland University Faculty of Medicine, Homburg, Germany.*

[§]*Department of Internal Medicine III - Cardiology, Angiology and Intensive Care Medicine, Saarland University Medical Center, Homburg, Germany.*

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Address correspondence to Rima Obeid, Saarland University Hospital and Saarland University Faculty of Medicine, Germany. E-mail: rima.obeid@uks.eu

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(molecular weight = 60.06 g/mol) and smaller than creatinine (molecular weight = 113.12 g/mol). In contrast to urea, TMAO appears to prevent protein denaturation, especially at high concentrations of urea.[3] Liver flavin monooxygenase (FMO3) converts trimethylamine (TMA) to TMAO. TMA originates through the effect of TMA-metabolizing gut bacteria on dietary betaine, choline, and carnitine. Elevated plasma concentrations of TMAO have shown associations with impaired kidney function,[4–6] type 2 diabetes,[7,8] CVD,[9–12] and mortality in patients on continuous renal replacement therapy.[9,13] TMAO is also associated with established risk factors for CVD such as obesity, dyslipidemia, and inflammation.[4,14]

Dietary intake of TMAO precursors and degree of enrichment in TMA-metabolizing gut bacteria explain a small part of circulating TMAO variations.[15,16] Plasma TMAO concentrations were 22-fold higher in patients on continuous renal replacement therapy compared to control subjects, while urinary TMAO (mmol/mol creatinine) did not differ according to renal function.[17] These results suggest that TMAO accumulation in diseases that affect the kidney is due to the inability to eliminate this metabolite instead of a higher production rate from dietary precursors. Gruppen et al. suggested that the estimated glomerular filtration rate (eGFR) could mediate the association between TMAO and mortality explaining 15% of the overall effect.[18]

A recent meta-analysis has shown that higher circulating TMAO concentrations were associated with the risk of all-cause mortality among patients with CKD.[4] In contrast, the association between TMAO and the risk of CVD-specific mortality was inconclusive, and there was evidence for publication bias, suggesting that studies with negative results are less likely to be published.[4] TMAO has been suggested to act as thrombogenic, proatherogenic, or uremic toxin.[19] This is not in line with human studies showing no association between TMAO and carotid atherosclerosis,[20] thromboembolism, or biomarkers of thrombosis.[21] Moreover, higher choline intake (a dietary precursor of TMAO) was associated with lower systolic blood pressure and a lower odds ratio for hypertension in the National Health and Nutrition Examination Survey) study.[22] A study in a hypertensive rat's model suggested that TMAO could have protective effects on the heart by increasing urine volume, suggesting that TMAO may have a diuretic effect.[23] Since the elevation of plasma concentrations of TMAO is strongly related to the decline in renal function, it is debatable whether TMAO is an independent risk factor for CVD (with a causal role) or just a marker of impaired renal function or increased FMO3 activity.

Among 478 patients with CKD stages G2 through G4, we hypothesized that plasma concentrations of TMAO, choline, and betaine are associated with glomerular filtration rate and prevalent CVD. Moreover, we hypothesized that the circulating concentration of these biomarkers may predict future cardiovascular or renal outcomes including death during a mean (standard deviation [SD]) follow-up period of 5.1 years.

Methods

Subjects and Design

Participants in the CARE FOR HOMe cohort study were recruited between 2008 and 2015 from the outpatient clinic at the Department of Internal Medicine, the division of nephrology. The study aimed to identify novel risk factors for cardiovascular events and CKD progression.[24,25]

The definition of CKD was according to Kidney Disease: Improving Global Outcomes guidelines. eGFR was computed by the Modification of Diet in Renal Disease 4 equation. The definition of the CKD stage requires, in addition to having an eGFR in the range of 60-90 mL/min/1.73 m^2, additional evidence for chronic renal dysfunction. Each of the following findings was considered as an evidence of CKD: the presence of proteinuria >300 mg/g; the presence of albuminuria defined as >17 mg/g (in men) and >25 mg/g (in women); persisted glomerular hematuria; elevated plasma cystatin C concentrations (>1.05 mg/L); elevated plasma concentrations of creatinine (>1.2 mg/dL for men and >0.9 mg/dL for women); or renal disease confirmed by a biopsy or tubulopathy.

Adults with eGFR between 15 and 89 mL/min/1.73 m^2 were eligible. Exclusion criteria were using immune suppressive drugs, acute infections [C-reactive protein (CRP) above 50 mg/L or use of systemic antibiotics], cancer, acute renal dysfunction (defined as an increase of plasma creatinine by > 50% within 4 weeks prior to baseline), and pregnancy.[24]

The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) for medical research involving human subjects. Written informed consent was obtained from each patient included in the study.

Medical History

A standardized questionnaire was used to collect information on smoking habits (active smoker or none smoker), history of diabetes mellitus, medications, family history of premature cardiovascular events, and other cardiovascular comorbidities such as history of myocardial infarction, coronary artery angioplasty, stenting or bypass surgery, major stroke, carotid endarterectomy or stenting, nontraumatic lower extremity amputation or lower limb artery bypass surgery, angioplasty or stenting. Diabetes was defined as self-reported diabetes mellitus, a current use of glucose-lowering drugs, or a fasting blood glucose level of at least 126 mg/dL (7.0 mmol/L). Measurements of blood pressure, heart rate, and body weight and height were conducted during the baseline visit at the study center.

Blood Collection and Biomarker Measurements

Fasting blood samples (12 hours) were collected during the baseline visit. Several aliquots of ethylenediaminetetraacetic acid (EDTA) and heparin-plasma in addition to serum were prepared. Plasma concentrations of cystatin C, troponin T, amino-terminal pro-brain natriuretic peptide, creatinine (traceable to isotope dilution mass spectrometry), glucose, lipids, and CRP, in addition to a complete blood count, were performed at the Central Laboratory of the Hospital. HbA1c and insulin were measured in patients with diabetes. The remaining samples were stored at −70°C for measurements of advanced biomarkers. Additional blood samples that were collected during a second follow-up after 3 years were available from a subgroup of 95 patients for TMAO assay.

Plasma concentrations of TMAO, choline, and betaine were measured according to established methods using ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).[17,26] For the TMAO assay, 300 μL of an internal standard mixture of 10 μmol/L of d9-TMA (CDN Isotopes, Quebec, Canada) and d9-TMAO (Cambridge Isotop Laboratories, Inc., MA, USA) prepared in methanol/acetonitrile (15:85) and 0.2% formic acid were added to 100 μL EDTA-plasma or standards. The mix was vortexed and centrifuged (10,000 × g for 5 minutes at room temperature), and the supernatant was transferred to maximum recovery vials (Waters Corporation, Milford, MA, USA). TMA hydrochloride and TMAO (Sigma-Aldrich, Munich, Germany) were used to prepare the standard curve (range 0-100 μmol/L). Samples were measured on UPLC® Acquity coupled to a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). We used Acquity UPLC BEH HILIC column [100 mm × 2.1 mm (i.d.); 1.7 μm particle size] with an Acquity HILIC VanGuard pre-column [(5 mm × 2.1 mm (i.d.); 1.7 μm particle size] and a 0.2 μm in-line filter (Waters Corporation, Milford, MA, USA). The detection was performed on a triple quadrupole mass spectrometer (MicroMass Quattro Premier XE tandem quadrupole mass spectrometer, Waters Corporation, Milford, MA, USA) in a positive electrospray ionization mode.[17] The coefficients of variation for TMAO in quality control samples at concentrations of 10.0 μmol/L and 80.0 μmol/L were both <5%.

Concentrations of choline and betaine were measured in 100 μL EDTA-plasma samples following a protein precipitation step by using 300 μL of acetonitrile containing an internal standard mix of d9-betaine and d9-choline (both from Sigma-Aldrich).[26] After vortexing, samples were centrifuged for 5 minutes at 10,000 × g (10,900 rpm) at room temperature and the supernatant was transferred to maximum recovery vials to be measured on the UPLC-MS/MS. To separate and measure the analytes, we used an Acquity UPLC BEH HILIC column (100 mm × 2.1 mm (i.d.); 1.7 μm particle size), an Acquity HILIC VanGuard pre-column (5 mm × 2.1 mm [i.d.]; 1.7 μm particle size), and a 0.2 μm in-line filter (Waters Corporation).[26] The coefficients of variations for plasma betaine (at 75.0 μmol/L and 7.5 μmol/L) and choline (at 45.0 μmol/L and 4.5 μmol/L) were <8%.

Study Specific Exposures and Outcomes

Plasma concentration of TMAO was the primary exposure of the present investigation, while plasma concentrations of choline and betaine were secondary exposures. The primary outcomes for this study were: 1) cardiovascular events and all-cause mortality combined; and 2) worsening of renal function (defined as a persistent drop of eGFR ≥50% or progression to continuous need for renal replacement therapy) and all-cause mortality combined. The primary outcomes included all-cause mortality to increase the number of events and thus the precision of the estimates. The secondary outcomes in the present study were: continuous need for renal replacement therapy, all-cause death, cardiovascular events and cardiovascular death combined, and heart failure combined with all-cause death.

A yearly on-site clinical and laboratory investigation was performed to evaluate the general health and progression of the CKD. Patients who were unable to attend one or more of the follow-up visits were asked to fill out a study questionnaire during a follow-up phone interview. Medical reports and results of laboratory investigations were obtained from family physicians, nephrologists, or hospital reports to verify CKD progression and cardiovascular events.

The study recruited 579 patients with CKD stages G2 to G4. Three patients were lost to follow-up due to changing their country of residence, and a fourth patient declined participation in the study. The 4 patients are not included in the present analysis. EDTA-plasma samples for measurement of TMAO, choline, and betaine concentrations were available from 478 participants at baseline and 95 patients at a follow-up visit after 3 years (Supplemental Figure 1, Study Flow Diagram). The mean (SD) follow-up time in the present cohort was 5.1 (2.1) years.

Statistical Analyses

We used the one-sample Kolmogorov-Smirnov test and Q-Q plots to study the distribution of the continuous variables. Data on plasma concentrations of TMAO, choline, betaine, creatinine, urea, CRP, urinary albumin to creatinine ratio, and cystatin C were not normally distributed, and a natural logarithm of the values was used for statistical tests that assume normal distribution of the data. Data are shown as mean (SD) for continuous variables and absolute (n) and relative frequencies (%) for categorical variables.

The Chi-square test was used to compare categorical variable between 2 independent groups. The one-way

analysis of variance (ANOVA) test was used to compare the log-transformed data of circulating TMAO, choline, and betaine between independent groups. The significantly different groups were identified by applying a post-hoc Bonferroni assuming homogeneity of variances between the groups (as tested with Levene's test, $P > .05$). General linear model analysis was used to compare the mean of TMAO, choline and betaine concentrations between two factor variables (i.e., patients with a CVD event and those without an event) while adjusting for the covariates.

Cox-regression analysis was used to compute the hazard ratio (HR) and the 95% confidence intervals (95% CI) for each of the clinical outcomes in relation to quartiles of baseline plasma concentrations of TMAO, choline and betaine. Cox-regression models included the primary or secondary clinical outcome (present or absent) and the time of follow-up as a time scale. Crude models included a single covariate that consists of quartile categories of the metabolites with the lowest quartile being the reference group.

The covariates were defined a priori based on previous knowledge. Cox-regression models were adjusted for age, systolic blood pressure, eGFR, cystatin C, urinary albumin to creatinine ratio, and body mass index (all as continuous variables), and sex (M, F), smoking (yes, no), and diabetes (yes, no) as categorical variables. Cystatin C and urinary albumin-to-creatinine ratio were used in the adjusted models as $\log_e$-transformed data. Data on blood pressure and urinary albumin to creatinine ratio were missing from two and one patients, respectively. Missing values were not imputed, and fully adjusted analyses included 376 participants.

Sensitivity analysis included Cox-regression models using the concentrations of each of the metabolites as a continuous variable ($\log_e$-transformed values). In addition, we run regression models with minimal adjustments (only for glomerular filtration rate [GFR] and cystatin C) to investigate whether adjustment for renal function was sufficient to abolish the associations. The renal function markers, creatinine-to-TMAO ratio and the urea-to-TMAO ratio were converted to z-scores (SD deviations from zero on a standard normal distribution). The z-scores were plotted according to the CKD stage to visualize the relative changes in plasma TMAO according to renal function.

In a subgroup of 95 patients, we studied test-retest reliability by computing the interclass correlation coefficient and (95% CI) between two independent TMAO measurements within 3 years using a two-way mixed effect model applied on the $\log_e$-transformed TMAO data. The paired t-test was used to study within-group differences of TMAO concentrations among 95 participants upon repeated measurements after 3 years.

The statistical analyses were conducted using version 29 of IBM® SPSS® Statistics package (SPSS Inc., Chicago, IL, USA). P values ≤ 0.05 were considered statistically significant.

Results

Population Characteristics

This study included 104 (21.7%) patients with CKD G2, 163 (34.0%) with CKD G3a, 123 (25.7%) with CKD G3b, and 88 (18.4%) with CKD G4. The mean age was 65.1 years (SD = 12.4 years). Baseline characteristics, disease conditions, risk factors, and prevalent diseases are shown in Table 1 and Supplemental Table 1. After a mean follow-up of 5.1 (SD = 2.1) years, 147 (30.7%) patients experienced new cardiovascular event including death of any cause, while 144 (30.1%) patients experienced halving of eGFR, progressed to continuous need for renal replacement therapy or died. The number of patients who developed secondary outcomes during the follow-up is shown in Supplemental Table 2.

Baseline Metabolites in Relation to Renal Function and Prevalent Cardiovascular Diseases

The mean (SD) of plasma TMAO concentrations at baseline was 13.1 (21.1) μmol/L in the whole group, and the concentrations showed a dose-response increase according to CKD stage: [29.2 (41.3) μmol/L in G4, 12.6 (11.8) μmol/L in G3b, 8.9 (9.2) μmol/L in G3a, and 6.5 (5.8) μmol/L in G2] (Table 2). More advanced CKD stage was also associated with higher plasma concentrations of betaine and choline (Table 2), but the magnitude of the difference in betaine and choline concentrations between G4 and G2 was less than that for TMAO (Table 2). The ratio of plasma creatinine (in μmol/L) or urea (in mmol/L) to plasma TMAO concentrations (in μmol/L) was lower at higher CKD stages. However, the mean z score of the ratio of creatinine to TMAO ($P = .092$ ANOVA test) and z score for the ratio of urea to TMAO ($P = .436$ ANOVA test) did not differ between the CKD stages (Supplemental Figure 2).

Baseline plasma concentrations of TMAO were slightly higher in participants with preexisting CVD (n = 147) compared to those without CVD (n = 331) [12.3 (21.3) μmol/L vs. 14.8 (20.7) μmol/L; $P = .047$]. Similarly, plasma choline and betaine were higher in patients with preexisting CVD than those who were free of events at baseline (Table 3). Participants with preexisting CVD were older than those who were free of cardiovascular events at baseline, and they differed in several risk factors such as blood pressure, plasma cystatin C, eGFR, and plasma creatinine (Table 3). The differences in plasma concentrations of TMAO, choline, and betaine according to the presence and absence of CVD at baseline were not significant after adjusting for age, sex, plasma concentrations of cystatin C, and urine albumin to creatinine ratio (Table 3).

Plasma TMAO, Choline, and Betaine and Clinical Outcomes

We found generally higher crude HR for people with higher plasma TMAO concentrations to develop any of

Table 1. Clinical Characteristics of the 478 Participants With Chronic Kidney Disease Stages G2 to G4 at Baseline Visit

Characteristics	Mean (SD) or n (%)
Age, y	65.1 (12.4)
BMI, kg/m^2	30.3 (5.4)
Systolic blood pressure, mmHg	153 (24)
Diastolic blood pressure, mmHg	86 (13)
Women, n (%)	194 (40.5%)
Chronic kidney disease stage*, n (%)	
G2 (eGFR min-max. 60.2-88.4 mL/min/1.73 m^2)	104 (21.7%)
G3a (eGFR min-max. 45.1-59.9 mL/min/1.73 m^2)	163 (34.0%)
G3b (eGFR min-max. 30.0-44.8 mL/min/1.73 m^2)	123 (25.7%)
G4 (eGFR min-max. 17.2-29.8 mL/min/1.73 m^2)	88 (18.4%)
Smoker, n (%)	49 (10.2%)
Diabetes mellitus, n (%)	181 (37.8%)
History of any cardiovascular disease, n (%)	147 (30.7%)
History of coronary artery disease, n (%)	103 (21.5%)
History of cerebrovascular disease, n (%)	45 (9.4%)
Peripheral arterial disease, n (%)	34 (9.0%)

BMI, body mass index; eGFR, estimated glomerular filtration rate; SD, standard deviation.

*Chronic kidney disease stage is according to Kidney Disease: Improving Global Outcomes guidelines.

the study outcomes (Table 4 and Supplemental Table 3). However, the associations were abolished after adjustment for the covariates (Table 4). Similarly, the adjusted analysis showed no significant associations of TMAO as a continuous variable with any of the study outcomes. Adjustment for eGFR and cystatin C was sufficient to abolish the associations between baseline TMAO concentrations and the risk of all of the study outcomes (data not shown).

Plasma concentrations of choline were not associated with any of the study outcomes in the adjusted analyses (Table 4 and Supplemental Table 4). In contrast, the adjusted HR (95% CI) for CVD including death in the upper quartile of plasma betaine (≥43.3 μmol/L) compared to the lowest quartile (<26.2 μmol/L) was 2.14 (95% CI: 1.32, 3.47). Analysis using betaine as a continuous variable confirmed the associations between plasma betaine and CVD including all-cause mortality [HR = 1.72 (1.12, 2.63) for each 1 unit increase in logarithm plasma betaine] (Table 4). The adjusted analyses showed that plasma betaine was associated with the secondary outcomes of the study (all-cause mortality, CVD including death of CVD, and heart failure including death of any cause) (Supplemental Table 5).

Repeated Measurements of Plasma TMAO Concentrations During the Follow-Up

In a subgroup of 95 patients with repeated measurements of fasting plasma TMAO, the mean (SD) concentrations were 11.6 (9.5) μmol/L at baseline and 14.2 (15.9) μmol/L after 3 years ($P = .221$ for paired differences according to t-test) (Supplemental Figure 3). The interclass correlation coefficient (and 95% CI) were 0.673 and (0.510, 0.782), $P < .001$.

Discussion

Declining eGFR shows a dose-response association with adverse clinical outcomes,[27] and at the same time, it shows a strong association with plasma concentrations of TMAO. The high test-retest reliability of TMAO over 3 years suggests that a single measurement of fasting plasma TMAO may be representative of the TMAO status of a person over a long period of time. Plasma concentrations of TMAO, choline, and betaine were not associated with prevalent CVD at baseline after adjustment for confounders. In the longitudinal study, the adjusted analysis showed that the concentrations of TMAO and choline were not associated with any of the study outcomes. In contrast, plasma betaine (molecular weight 117.15 g/mol) > 43.2 μmol/L (upper quartile) showed consistent associations with a roughly 2-fold higher risk for multiple outcomes compared to when betaine was <26.2 μmol/L (lowest quartile).

Our data on mean concentrations of TMAO, choline and betaine correspond well with earlier studies on patients with CKD.[28,29] Missailidis et al. reported that higher TMAO concentrations (>32.2 μmol/L vs. < 32.2 μmol/L) were associated with an adjusted HR of 4.32 (1.32-14.2) for all-cause mortality among 179 patients with CKD G3 to G5 followed for 5 years (51 patients or 28% died during this time).[29] In our multivariate adjusted model, we found no association between TMAO and mortality, despite the fact that our study included 96 cases of mortality out of 478 participants (20% died within 5 years), which has a higher power to detect possible associations than the study of Missailidis et al.[29] In addition, we found no evidence that TMAO could contribute to future renal function impairment, which was postulated in a previous study.[18] The Prevention of Renal and Vascular End Stage Disease study included 5,469 participants and reported 322 death cases during the 8.3 years follow-up period.[18] No overall association between TMAO and all-cause mortality was found in a multivariate adjusted model in that study (HR, 1.15; 95% CI, 0.81-1.64; $P = .22$ after adjustment for urinary albumin excretion and eGFR).[18] However, in the subgroup with eGFR <90 mL/min/1.73 m^2, TMAO was significantly associated with all-cause mortality in models adjusted for age, sex, and urinary albumin excretion, but not for GFR.[18] In two other large studies, the associations between

Table 2. Concentrations of TMAO and Related Metabolites in Addition to Renal Function Markers According to CKD Stages G2 to G4

Marker	All n = 478	CKD G2 n = 104	CKD G3a n = 163	CKD G3b n = 123	CKD G4 n = 88	*P**
eGFR, ml/min/1.73 m^2	48.9 (16.0)	Range 59.1-86.5	Range 44.0-58.9	Range 30.0-43.8	Range 15.1-29.8	-
Plasma creatinine, μmol/L	139.3 (56.6)	92.2 (12.5)	111.6 (17.1)†	147.6 (23.9)†‡	234.9 (51.1)†‡§	<.001
Plasma cystatin C, mg/L	1.6 (0.6)	1.1 (0.2)	1.4 (0.2)†	1.8 (0.5)†‡	2.5 (0.4)†‡§	<.001
Plasma urea, mmol/L	10.8 (5.4)	6.6 (1.7)	8.4 (2.4)†	12.2 (4.7)†‡	18.1 (5.3)†‡§	<.001
Urine albumin (in mg) to creatinine (in gr) ratio	3.3 (8.3)	2.2 (6.9)	2.1 (4.7)	3.4 (9.1)	6.9 (12.2)†‡§	<.001
Plasma TMAO, μmol/L	13.1 (21.1)	6.5 (5.8)	8.9 (9.2)†	12.6 (11.8)†‡	29.2 (41.3)†‡§	<.001
Plasma choline, μmol/L	12.3 (3.9)	9.3 (2.0)	11.3 (3.4)†	13.2 (3.3)†‡	15.6 (4.4)†‡§	<.001
Plasma betaine, μmol/L	36.6 (16.5)	35.4 (11.8)	35.2 (18.6)	40.5 (17.3)‡	35.1 (15.2)§	.006
Plasma creatinine/TMAO ratio (both in μmol/L)	18.4 (14.6)	20.6 (13.1)	19.3 (12.3)	17.3 (10.5)	15.9 (22.7)†‡	<.001
Plasma urea/TMAO ratio (mmol/L/μmol/L)	1.39 (0.19)	1.44 (0.96)	1.45 (1.04)	1.40 (1.06)	1.20 (1.77)†‡§	.003

Data are shown as mean (SD). CKD stage is according to Kidney Disease: Improving Global Outcomes guidelines.
CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; SD, standard deviation; TMAO, trimethylamine N-oxide.
**P* values for between-group comparisons are according to ANOVA test applied on the $\log_e$-transformed data.
†*P* values < .05 compared to G2 (post-hoc Bonferroni test).
‡*P* values < .05 compared to G3a (post-hoc Bonferroni test).
§*P* values < .05 compared to G3b (post-hoc Bonferroni test).

TMAO and mortality were attenuated when eGFR was added to other covariates in the multivariate model.[30,31] Plasma TMAO was associated with a higher risk of death among elderly people with eGFR <45 mL/min/1.73 m^2 (in stratified analysis)[30] or death due to kidney failure (only in analysis not adjusting for eGFR).[31] Therefore, the associations between TMAO and clinical outcomes could be explained by residual confounding mainly due to kidney function.

It is not known whether TMAO elevation in patients with CKD is solely explained by impaired glomerular filtration of this metabolite. Dietary intake of TMAO precursors is not likely to differ according to the CKD stage. Patients with different stages of CKD may differ in the

Table 3. Plasma Concentrations of TMAO, Choline, and Betaine and Selected CVD Risk Factors or Markers According to the Presence and the Absence of Established Cardiovascular Disease at Baseline Visit

Risk Factors or Markers	Participants With CKD Without CVD at Baseline (n = 331)	Participants With CKD and Established CVD at Baseline (n = 147)	*P**	*P*†
Age, y	62.7 (12.9)	70.4 (9.2)	<.001	-
Male/female, n	192/139	92/55	.365‡	
BMI, kg/m^2	30.2 (5.6)	30.3 (4.9)	.905	
Systolic blood pressure, mmHg	152 (23)	157 (27)	.043	
Diastolic blood pressure, mmHg	88 (12)	83 (13)	<.001	
Plasma NT-proBNP, pg/mL	514 (1425)	1285 (2285)	<.001	
eGFR, ml/min/1.73 m^2	47.5 (16.5)	42.2 (14.2)	.001	
Plasma creatinine, μmol/L	136.4 (57.6)	139.3 (56.6)	.087	
Plasma cystatin C, mg/L	1.6 (0.6)	1.7 (0.6)	.024	
Urine albumin (in mg) to creatinine (in gr) ratio	3.8 (9.4)	2.2 (5.0)	.399	
Plasma TMAO, μmol/L	12.3 (21.3)	14.8 (20.7)	.047	.723
Plasma choline, μmol/L	11.9 (3.7)	13.0 (3.9)	.005	.120
Plasma betaine, μmol/L	35.5 (16.1)	39.0 (17.1)	.028	.124

BMI, body mass index; CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; NT-proBNP, amino-terminal pro-brain natriuretic peptide; SD, standard deviation; TMAO, trimethylamine N-oxide.
Data are mean (SD) if not otherwise specified.
*Between-group differences in continuous variables were tested using ANOVA test applied on the $\log_e$-transformed values.
†General Linear Model (GLM) analysis was used to compare the concentrations of TMAO, choline, and betaine between the groups while adjusting for the following covariates: sex (M or F), age, and plasma concentrations of cystatin C and creatinine at baseline.
‡The *P* value for the difference in sex distribution is according to the Chi-square test.

Table 4. Cox-Regression Analyses for the Association Between Baseline Plasma Concentrations of TMAO, Choline, or Betaine and Primary Cardiovascular and Renal Outcomes Including All-Cause Mortality Among 478 Patients With CKD Stages G2 to G4

	Q1 (Reference)	Q2	Q3	Q4	
	HR (95% CI)* for the Quartiles				HR (95% CI)†
TMAO, μmol/L (range)	**0.9-4.8**	**4.9-8.0**	**8.1-13.1**	**13.2-284.7**	TMAO as continuous variable
Cardiovascular event including death of any cause					
Crude model	HR = 1.00	1.73 (0.95, 3.15)	2.21 (1.25, 3.92)	4.20 (2.45, 7.19)	1.56 (1.31, 1.86)
n event/total	17/119	29/120	39/120	62/119	147/478
Fully adjusted model‡	-	1.09 (0.58, 2.03)	1.04 (0.56, 1.93)	1.22 (0.61, 2.46)	1.03 (0.79, 1.34)
Halving of eGFR, continuous need for renal replacement therapy, or death of any cause					
Crude model	HR = 1.00	1.40 (0.72, 2.72)	2.66 (1.47, 4.80)	5.39 (3.07, 9.45)	1.82 (1.54, 2.16)
n event/total	16/119	21/120	41/120	66/119	144/478
Fully adjusted model‡	-	0.79 (0.39, 1.59)	0.80 (0.41, 1.57)	0.71 (0.33, 1.51)	0.91 (0.69, 1.18)
Choline, μmol/L (range)	**4.8-9.3**	**9.4-11.6**	**11.7-14.1**	**14.2-29.2**	Choline as continuous variable
Cardiovascular event including death of any cause					
Crude model	HR = 1.00	1.23 (0.71, 2.12)	1.46 (0.87, 2.45)	2.75 (1.71, 4.42)	3.74 (2.22, 6.31)
n event/total	24/117	28/121	35/120	60/120	147/478
Fully adjusted model‡	-	1.01 (0.58, 1.76)	0.86 (0.48, 1.52)	1.11 (0.63, 1.97)	1.20 (0.63, 2.29)
Halving of eGFR, continuous need for renal replacement therapy, or death of any cause					
Crude model	HR = 1.00	1.23 (0.71, 2.12)	1.46 (0.87, 2.45)	2.75 (1.71, 4.42)	3.74 (2.22, 6.31)
n event/total	18/117	22/121	33/120	71/120	144/478
Fully adjusted model‡	-	1.04 (0.54, 1.98)	1.00 (0.53, 1.89)	1.39 (0.76, 2.54)	1.29 (0.67, 2.46)
Betaine, μmol/L (range)	**6.3-26.1**	**26.2-33.7**	**33.8-43.2**	**43.3-188.5**	Betaine as continuous variable
Cardiovascular event including death of any cause					
Crude model	HR = 1.00	1.26 (0.78, 2.04)	1.02 (0.62, 1.69)	1.88 (1.20, 2.93)	1.82 (1.23, 2.69)
n event/total	32/120	35/119	30/120	50/119	147/478
Fully adjusted model‡	-	1.29 (0.78, 2.15)	0.99 (0.59, 1.66)	2.14 (1.32, 3.47)	1.92 (1.26, 2.94)
Halving of eGFR, continuous need for renal replacement therapy, or death of any cause					
Crude model	HR = 1.00	1.06 (0.65, 1.71)	1.03 (0.64, 1.67)	1.39 (0.88, 2.18)	1.58 (1.04, 2.41)
n event/total	34/120	34/119	34/120	42/119	144/478
Fully adjusted model‡	-	1.29 (0.77, 2.14)	1.20 (0.73, 1.97)	1.64 (1.00, 2.67)	1.72 (1.12, 2.63)

CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; TMAO, trimethylamine N-oxide.

Crude regression models included TMAO, choline, or betaine concentrations, each entered in the model as quartiles (the lowest quartile was considered the reference group) or as a continuous variable (natural logarithm).

*The proportional change in hazard for Q2, Q3, and Q4 of the metabolite compared to the reference group (lowest quartile, Q1).

†The proportional change in hazard when the natural log of the metabolite increases by 1 unit.

‡The fully adjusted models included the exposure variable (TMAO, choline or betaine) in addition to the following covariates: eGFR, urine albumin to creatinine ratio (mg/g), plasma cystatin C concentrations, age, sex (M, F), smoking (yes, no), BMI, systolic blood pressure, and diabetes (yes, no).

composition of gut bacteria[32] and possibly the proportion of TMA-producing bacteria. Thus, adjustment for renal function may indirectly condition on differences in gut bacteria between the CKD stages.

A causal role of TMAO in diseases can be investigated by studying the effect of TMAO-lowering on CVD outcomes. Interventions with several antioxidants[33] or modifying gut bacteria[34] showed negligible effects on TMAO (also reviewed in[35]). Low-abundant TMA-producing bacteria appear to be part of the core gut community.[36] The diversity of genes encoding TMA-forming enzymes implies that whole consortia rather than single bacterial taxa need to be eliminated in order to restrict TMA production.[36] In animals, broad-spectrum antibiotics altered gut microbiota composition and reduced TMAO levels.[37] If TMAO is atherogenic, then it can be argued that patients treated with antibiotics may, in theory, have a lower risk of CVD. However, the use of antibiotics in human studies (macrolides and quinolones) has been shown to be associated with a higher risk of all-cause mortality, stroke, and probably also CVD mortality.[38] Collectively, these results may disqualify TMAO as a target for the prevention of CVD.

Like TMAO, betaine is an osmolyte, but it has a unique role in metabolism and body composition.[39] Analogous to creatinine that correlates with muscle mass and cystatin C that correlates with body fat mass,[40] plasma concentrations of betaine might mirror intracellular metabolic processes in muscles, liver, or fat tissues. Studies showed mostly no association between plasma betaine and adverse events.[30,41] Betaine intake showed both protective[42] and null associations[43,44] with a variety of CVD outcomes, suggesting that differences in the cohort characteristics and comorbidities could impact the association of plasma betaine with CVD.

The present study has strengths such as the large number of participants and the long follow-up time, in addition to collecting data on many risk factors. The limitations of the study are the lack of data on gut bacteria and TMAO concentrations in urine as possible determinants of plasma TMAO concentrations.

In conclusion, lower GFR was associated with higher plasma concentrations of TMAO, choline, and, to a lesser degree, betaine. Prevalent CVD at baseline visit was not associated with the concentrations of these metabolites after adjusting for renal function markers. Among patients with CKD stages G2 to G4 and after a mean follow-up of 5.1 years, baseline plasma TMAO and choline were not associated with adverse cardiovascular and renal outcomes in the adjusted analysis. In contrast, patients in the upper quartile of plasma betaine had a 2-fold higher risk for cardiovascular and renal events in the adjusted analysis than those in the lowest quartile. The strong dependency of plasma TMAO on renal function suggests that TMAO could serve as a prognostic marker rather than an etiological factor. Future studies may investigate the role of FMO3 in CVD among patients with CKD.

Practical Application

Sufficient dietary intake of choline is necessary to maintain normal body functions, especially liver functions and lipid metabolism. The choline gut-bacteria mediated metabolite, TMAO, is not an independent risk factor for adverse health outcomes among patients with CKD. Therefore, there is no evidence that choline intake from foods or food supplements should be restricted in this population.

CRediT Authorship Contribution Statement

Rima Obeid planned the present study, conducted data analyses, and drafted the manuscript. Insa E. Emrich and Adam M. Zawada collected the clinical data and biosamples. Gunnar Henrik Heine and Danilo Fliser planned and supervised the CARE FOR HOMe study. Jürgen Geisel planned and supervised the present study. Husain Awwad measured concentrations of TMAO, choline, and betaine in plasma. All authors provided critical input to the content of this publication.

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Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1053/j.jrn.2024.03.009.

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