Association of Uremic Solutes With Cardiovascular Death in Diabetic Kidney Disease

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Rationale & Objective: Cardiovascular disease (CVD) is a major cause of mortality among people with diabetic kidney disease (DKD). The pathophysiology is inadequately explained by traditional CVD risk factors. The uremic solutes trimethylamine-*N*-oxide (TMAO) and asymmetric and symmetric dimethylarginine (ADMA, SDMA) have been linked to CVD in kidney failure with replacement therapy (KFRT), but data are limited in populations with diabetes and less severe kidney disease.

Study Design: Observational cohort.

Settings & Participants: Random subcohort of 555 REGARDS (Reasons for Geographic and Racial Differences in Stroke) study participants with diabetes and estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2 at study entry.

Exposure: ADMA, SDMA, and TMAO assayed by liquid chromatography–mass spectrometry in plasma and urine.

Outcome: Cardiovascular mortality (primary outcome); all-cause mortality and incident KFRT (secondary outcomes).

Analytical Approach: Plasma concentrations and ratios of urine to plasma concentrations of ADMA, SDMA, and TMAO were tested for association with outcomes. Adjusted Cox regression models were fitted and hazard ratios of outcomes calculated per standard deviation and per doubling, and as interquartile comparisons.

Results: The mean baseline eGFR was 44 mL/min/1.73 m^2. Cardiovascular death, overall mortality, and KFRT occurred in 120, 285, and 89 participants, respectively, during a mean 6.2 years of follow-up. Higher plasma ADMA and SDMA (HRs of 1.20 and 1.28 per 1-SD greater concentration), and lower ratios of urine to plasma concentrations of ADMA, SDMA, and TMAO (HRs per halving of 1.53, 1.69, and 1.38) were associated with cardiovascular mortality. Higher plasma concentrations of ADMA, SDMA, and TMAO (HRs of 1.31, 1.42, and 1.13 per 1-SD greater concentration) and lower urine to plasma ratios of ADMA, SDMA, and TMAO (HRs per halving of 1.34, 1.37, and 1.26) were associated with all-cause mortality. Higher plasma ADMA and SDMA were associated with incident KFRT by categorical comparisons (HRs of 2.75 and 2.96, comparing quartile 4 to quartile 1), but not in continuous analyses.

Limitations: Single cohort, restricted to patients with diabetes and eGFR < 60 mL/min/1.73 m^2, potential residual confounding by GFR, no dietary information.

Conclusions: Higher plasma concentrations and lower ratios of urine to plasma concentrations of uremic solutes were independently associated with cardiovascular and all-cause mortality in DKD. Associations of ratios of urine to plasma concentrations with mortality suggest a connection between renal uremic solute clearance and CVD pathogenesis.

Visual Abstract online

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Cardiovascular disease (CVD) is a major cause of morbidity and mortality among people with chronic kidney disease (CKD),[1,2] particularly for those with diabetes as the underlying cause of CKD.[3] Although diabetic kidney disease (DKD) and CVD share some common risk factors, the pathogenesis of CVD in the context of DKD is incompletely understood. This is compounded by the lack of accurate biomarkers associated with these outcomes. Traditional risk factors for CVD (age, sex, diabetes duration, total cholesterol, high-density lipoprotein [HDL] cholesterol, smoking, systolic blood pressure, hypertensive therapy)[4] are not as strongly associated with CVD in those with CKD compared with the general population.[5,6] A number of low-molecular-weight organic metabolites accumulate in CKD (collectively termed "uremic solutes").[7-10] Several uremic solutes have been associated with CVD in people with kidney failure with replacement therapy (KFRT),[11] but data are more limited for people with less severe CKD. Our study focused on the prognostic value of 3 specific uremic solutes—asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and trimethylamine-N-oxide (TMAO)—in people with diabetes and CKD.

SDMA and ADMA arise from the metabolism of proteins with methylated arginine residues.[12] ADMA is metabolized in the kidney; both ADMA and SDMA are cleared by the kidneys and accumulate with decreasing glomerular filtration rate (GFR).[13-15] Investigations of potential associations of ADMA and SDMA with CVD have given inconsistent results in the general population, populations with CKD with or without diabetes, and in those with KFRT.[16-23]

PLAIN-LANGUAGE SUMMARY

For diseases such as chronic kidney disease (CKD), which progress over many years, it would be helpful to identify factors early in the disease course that are associated with complications or disease progression. These biomarkers could ultimately be relevant to the disease pathophysiology or as prediction tools to identify patients who need the most attention. For CKD patients, premature cardiovascular disease is by far the most common cause of death. In this study of diabetic patients with modest CKD, we investigated the association with cardiovascular mortality of 3 biomarkers that are derived from gut bacteria and that have previously been shown to be elevated in patients on dialysis. We found a strong association between 1 of these biomarkers, asymmetric dimethylarginine, and cardiovascular and all-cause mortality.

TMAO is derived from the metabolism of compounds with a quaternary ammonia structure, such as choline or carnitine, by intestinal flora.[24-26] The resulting trimethylamine is absorbed and oxidized in the liver to TMAO, which is cleared primarily by filtration and secretion in the kidney.[27,28] Extremely high TMAO levels have been detected in patients with KFRT,[27,29] though less is known about concentrations in CKD patients,[30] especially in high-risk populations with diabetes. In prior studies, plasma TMAO levels were associated with CVD events in the general population, even after adjusting for traditional risk factors including estimated GFR (eGFR).[24-26]

Despite some precedent to implicate ADMA, SDMA, and TMAO as biomarkers for CVD in the context of DKD, there has been insufficient validation for clinical utilization. In the current study, we assayed plasma and urine for ADMA, SDMA, and TMAO in a random subcohort of participants with diabetes and baseline eGFR < 60 mL/min/1.73 m^2 from the REGARDS (Reasons for Geographic and Racial Differences in Stroke) cohort study. These measures permitted study of associations with plasma concentrations as well as urine to plasma solute ratios with the primary outcome (cardiovascular mortality) and the secondary outcomes of all-cause mortality and incident KFRT.

Methods

Study Population

The REGARDS study is a prospective cohort study designed to investigate regional differences in stroke incidence among Black and White adults ≥45 years of age. The study design has been reported previously.[31] Briefly, participants were recruited from the 48 contiguous United States and Washington, DC. The study oversampled for Black people, and those residing in the stroke belt region of the southeastern United States. Trained interviewers collected sociodemographic, CVD risk factors, and use of antihypertensive, antiglycemic, and cholesterol-lowering medication information by phone interviews. Trained personnel then conducted an in-home study visit that included an electrocardiogram, height, weight and blood pressure measurements, medication reconciliation, and blood and urine sample collection.

Between January 2003 and October 2007, 30,239 individuals (42% Black, 55% women) were enrolled. Participants or their proxies were then contacted by telephone every 6 months to assess outcomes. Among this sample, 1,145 had diabetes and eGFR <60 mL/min/1.73 m^2 at baseline. From this group, we randomly selected a subcohort of 600 REGARDS participants, of whom 555 had plasma and urine samples available at baseline (Fig 1). The baseline characteristics for included and excluded participants are shown in Table S1. Urine albumin and urinary albumin-creatinine ratio (UACR) were not significantly different between the included and excluded groups ($P = 0.6$ and $P = 0.5$, respectively, by nonparametric median tests). The REGARDS study protocol was approved by the institutional review boards from each participating institution, and all participants provided informed consent.

Materials

Deuterated trimethylamine *N*-oxide (TMAO-d9) and deuterated asymmetric dimethylarginine (ADMA-d7) were purchased from Cambridge Isotope Labs. Deuterated symmetric dimethylarginine (SDMA-d6) was purchased from Toronto Research Chemicals (Toronto, ON). High-performance liquid chromatography (HPLC)–grade solvents were purchased from Thermo Fisher Scientific. All other reagents were purchased from Sigma-Aldrich or Thermo Fisher.

Sample Preparation

Urine and blood were collected at study entry and centrifuged; aliquots were barcoded and stored at −80°C at

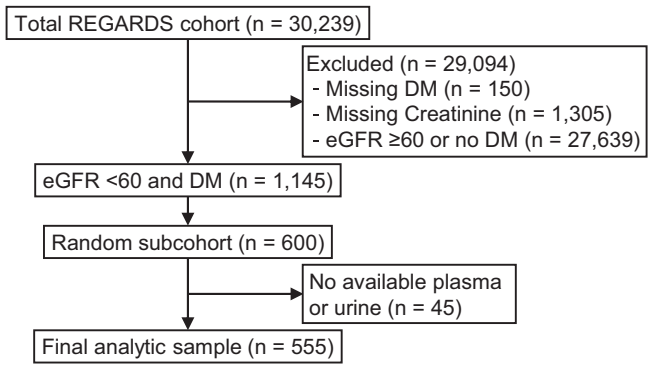

Figure 1. Sampling of REGARDS population to achieve study subcohort. Among 30,239 REGARDS participants, a total of 1,145 had eGFR <60 mL/min/1.73 m^2 and DM at study entry, and a subcohort of 600 individuals was randomly selected from those participants. Abbreviations: DM; diabetes mellitus; eGFR, estimated glomerular filtration rate.

the REGARDS biorepository at the University of Vermont. Samples were shipped on dry ice to Case Western Reserve University, where they were maintained at −80°C until biomarker assays were performed. Freeze/thaw experiments demonstrated a high degree of correlation across a broad concentration range (Table S2). To 20 μL of plasma or 20 μL diluted urine samples (1:20 in HPLC-grade water), 80 μL of internal standard mixture (containing 10 μM TMAO-d9, 1 μM of ADMA-d7, and 1 μM of SDMA-d6) in methanol was added, according to published methods.[27] Protein in the samples was precipitated by vortex mixing for 2 minutes, and then the supernatant was recovered after centrifugation (20,000*g*, 4 °C, 10 minutes).

Liquid Chromatography–Mass Spectrometry Analysis

Five microliters of supernatant was injected into a Luna Silica column (2 × 150 mm, 5 μm silica, 00F-4274-B0; Phenomenex). The liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis employed a Shimadzu Prominence LC system coupled to an API 4000 Q-TRAP mass spectrometer (AB Sciex). Binary flow was generated to resolve the analytes by using mobile phases A (0.1% propionic acid in H_2O) and B (0.1% acetic acid in methanol) at 0.2 mL/min flow rate. The analytes TMAO, TMAO-d9, SDMA, SDMA-d6, ADMA, and ADMA-d7 were monitored using electrospray ionization in positive-ion mode with multiple reaction monitoring of precursor and characteristic product ion transitions of m/z 76.0 → 59.0 amu, 85.0 → 66.0 amu, 203.1 → 172.0 amu, 209.2 → 70.0 amu, 203.1 → 70.0 amu, and 210.1 → 77.2 amu, respectively. The parameters for the ion monitoring were as follows: ionization voltage, 5.5 kV; ion source temperature 650 °C; curtain gas, 40; GS1, 40; GS2, 55; CAD gas, 4; DP, 50; CE, 25.0 volts; CXP, 11; EP, 10. Nitrogen (99.95% purity) was used as the only gas.

Calibration curves were generated using TMAO, SDMA, and ADMA standards at 6 different concentrations, to which internal standards (TMAO-d9, SDMA-d6, and ADMA-d7) were added. Curves were analyzed by linear regression and 1/x weighting, using Analyst software (version 1.6) for TMAO and SDMA. Because of an overlapping peak with ADMA, MultiQuant SignalFinder software (version 3.0) was used to achieve superior peak modeling and ADMA quantification.

Quality control samples of 3 different concentrations were run with each assay batch. The coefficients of variation (CVs) for the interassay quality control samples and intra-assay calibration curves were consistently <15%. The mean CVs for blind duplicate plasma TMAO, SDMA, and ADMA values were 8.85%, 11.62%, and 6.71%, respectively. Prior studies in individuals with similar baseline eGFR values yielded similar plasma ADMA, SDMA, and TMAO levels.[14,20,30,32] For urine assays, the mean blind duplicate CVs were 5.84%, 6.89%, and 7.38% for TMAO, SDMA, and ADMA, respectively.

Outcomes of Interest

Definitions of the primary outcome (cardiovascular death) and the secondary outcome (all-cause mortality) were previously published.[33] Briefly, deaths were identified by report from next-of-kin and online sources (Social Security Death Index, National Death Index). Information from medical records, death certificates, and interviews with surviving family members was compiled and reviewed by physician adjudicators to determine cause of death, which was classified by *International Classification of Diseases, Ninth Revision* (ICD-9) or *International Classification of Diseases, Tenth Revision* (ICD-10) codes. Cardiovascular death was defined as death due to circulatory disease (I00-I19, I26-I59, I70-I99) or coronary heart disease (CHD) (I20-I25) or stroke (I60-I69).

Incident KFRT data were from the US Renal Data System (USRDS) through 2014.

Covariates of Interest

Age, sex, race, smoking history, and education level were determined by self-report. Body mass index (BMI) was determined using height and weight obtained at the baseline home visit. Blood pressure was defined as the average of 2 measures while seated and after a 5-minute rest. Medications for hypertension were obtained by self-report. History of CHD was defined as evidence of myocardial infarction on the baseline electrocardiograph, self-report of prior myocardial infarction, coronary artery bypass surgery, or emergent percutaneous coronary intervention. History of stroke was determined by self-report. The eGFR was determined from isotope-dilution mass spectrometry–traceable serum creatinine concentration measurements and the 2009 CKD-EPI creatinine equation.[34] Urine albumin concentration was measured on a BNII ProSpec nephelometer (Siemens AG), and urine creatinine concentration was measured by the Jaffé method (Roche/Hitachi). The UACR was expressed as mg/g.

Statistical Analyses

Descriptive statistics were used to characterize the study cohort across quartiles of plasma concentrations and urine to plasma uremic solute ratios. Ratios were used rather than fractional excretions because the latter require incorporation of serum creatinine data, which may induce collinearity with eGFR in the same models. Instead, we adjusted for urine creatinine (to account for differences in urine tonicity at the time of urine specimen sampling)[35] and eGFR as separate covariates in multivariable models. Age- and sex-adjusted Pearson partial correlations were used to examine associations of plasma and urine biomarkers with each other and with eGFR and UACR.[36] After confirming the assumption of proportionality of hazards, Cox regression models were used to estimate the hazard ratios (HRs) for incident cardiovascular death, all-cause mortality, and incident KFRT as a function of baseline biomarkers. The relationship between the plasma

biomarkers and the primary outcome, cardiovascular mortality, was linear (Fig S1). Model 1 adjusted for age, sex, race, education, and urine creatinine. Model 2 was further adjusted for BMI, systolic blood pressure, use of hypertension medications, smoking status, history of CHD, history of stroke, low-density lipoprotein (LDL), HDL, lipid-lowering medications, baseline urine albumin, and eGFR. Missing data were taken into account using multiple imputation with chained equations in all regression analyses.[37] The resulting estimates were combined using Rubin's rules to account for the variability in the imputation procedure.[38]

In both models 1 and 2, plasma biomarkers and urine to plasma biomarker ratios were analyzed in quartiles (median values represented by the cutoff between quartiles 2 and 3), with the lowest plasma and highest urine to plasma ratio quartiles serving as the reference groups. Urine to plasma biomarker ratio data were also analyzed on a continuous scale after $\log_2$ transformation (ie, per 2-fold lower value). Because the plasma biomarker interquartile ranges were generally less than half the median, regression analyses were conducted using continuous function (based on standard deviation). A 2-tailed P value was determined with IBM SPSS version 26 software (IBM Corp), and $P < 0.05$ was considered statistically significant.

Results

Characteristics of the Study Population

Among the 555 participants with diabetes and eGFR <60 mL/min/1.73 m^2 in the cohort, the mean age was 70 years, 53% were Black, and 53% were women. The prevalence of hypertension, CHD, and stroke was 88%, 42%, and 16%, respectively. The mean eGFR at baseline was 44 ± 12 (SD) mL/min/1.73 m^2, and median UACR was 32 (IQR, 11-203) mg/g. The participant characteristics across baseline quartiles of plasma ADMA, SDMA, and TMAO concentrations are shown in Tables S2, S3, and S4, respectively. Women, those with less education, and White individuals tended to have higher levels of ADMA (Table S2); male sex and Black race were associated with higher SDMA levels (Table S3); male sex and White race were associated with higher TMAO levels (Table S4).

Biomarker Correlations

Pairwise age- and sex-adjusted Spearman correlations between plasma biomarker concentrations, ratio of urine to plasma biomarker concentrations, eGFR, and UACR are shown in Table 1. For all 3 uremic solutes, plasma levels inversely and moderately correlated with eGFR, with SDMA having the strongest correlation. The plasma concentrations were also directly correlated with UACR, but these correlations were weaker than with eGFR. For the ratio of urine to plasma levels of the uremic solutes, very strong pairwise correlations were observed with eGFR and urine creatinine, and moderately strong correlations were observed with UACR (Table 1).

Association of Uremic Solutes With Cardiovascular Mortality

During a mean follow-up period of 6.2 ± 3.5 years, there were 120 cardiovascular deaths (mean rate of 3.31% per year). Composite event numbers are shown in Table S6. The cardiovascular mortality rate was progressively greater with greater uremic solute plasma concentration quartile (Table 2). HRs were attenuated after adjustment or demographic and traditional cardiovascular risk factors including eGFR and UACR. The results did not differ when HRs were adjusted using a race-independent GFR estimating equation (Table S7).[39] A statistically significant association was observed for higher plasma ADMA with cardiovascular mortality in the multivariable-adjusted model, wherein each 1 SD higher ADMA concentration was associated with a 20% higher risk of cardiovascular

Table 1. Spearman Correlations

	Plasma			Urine			
	ADMA	SDMA	TMAO	Albumin	Creatinine	ACR	eGFR
Plasma							
ADMA	1.000	0.351[a]	0.221[a]	0.117[a]	−0.098	0.135[a]	−0.204
SDMA		1.000	0.410[a]	0.290[a]	−0.194	0.334[a]	−0.603
TMAO			1.000	0.135[a]	−0.308	0.213[a]	−0.396
	Urine-plasma ratio			**Urine**			
	ADMA	SDMA	TMAO	Albumin	Creatinine	ACR	eGFR
Urine-plasma ratio							
ADMA	1.000	0.929[a]	0.875[a]	−0.074	0.816[a]	−0.294[a]	0.512[a]
SDMA		1.000	0.893[a]	−0.075	0.810[a]	−0.296[a]	0.519[a]
TMAO			1.000	−0.060	0.789[a]	−0.277[a]	0.473[a]

Abbreviations: ACR, albumin-creatinine ratio; ADMA, asymmetric dimethylarginine; eGFR, estimated glomerular filtration rate; SDMA, symmetric dimethylarginine; TMAO, trimethylamine-*N*-oxide.
[a]Correlation is significant at the $P < 0.01$ level (2-tailed).

Table 2. Association of Baseline Plasma ADMA, SDMA, and TMAO With CV Mortality

	Continuous (per 1 SD[a])	Quartile 1	2	3	4
Plasma ADMA					
ADMA range, μM		<0.601	0.601-0.670	0.671-0.760	>0.760
N (no. of events)		151 (21)	136 (30)	132 (32)	136 (37)
Mortality rate, %/y		1.91	3.18	3.87	4.88
HR (95% CI)					
Unadjusted	1.35 (1.17-1.57)	1.00 (reference)	1.68 (0.96-2.94)	2.08 (1.20-3.61)	2.73 (1.59-4.66)
Model 1	1.33 (1.13-1.56)	1.00 (reference)	1.57 (1.15-3.52)	2.01 (1.15-3.52)	2.63 (1.51-4.56)
Model 2	1.20 (1.01-1.43)	1.00 (reference)	1.40 (0.79-2.49)	1.72 (0.97-3.03)	1.93 (1.08-3.45)
Plasma SDMA					
SDMA range, μM		<0.854	0.854-1.045	1.046-1.330	>1.330
N (no. of events)		140 (26)	136 (25)	145 (32)	134 (37)
Mortality rate, %/y		2.56	2.80	3.44	4.71
HR (95% CI)					
Unadjusted	1.33 (1.14-1.55)	1.00 (reference)	1.12 (0.65-1.93)	1.39 (0.83-2.33)	1.94 (1.17-3.21)
Model 1	1.33 (1.15-1.54)	1.00 (reference)	1.07 (0.61-1.87)	1.21 (0.72-2.05)	1.97 (1.17-3.34)
Model 2	1.28 (1.02-1.60)	1.00 (reference)	1.05 (0.60-1.85)	1.03 (0.59-1.81)	1.51 (0.79-2.87)
Plasma TMAO					
TMAO range, μM		<5.900	5.901-9.155	9.156-15.300	>15.300
N (no. of events)		142 (22)	136 (29)	142 (34)	135 (35)
Mortality rate, %/y		2.25	3.37	3.61	4.15
HR (95% CI)					
Unadjusted	1.06 (0.91-1.24)	1.00 (reference)	1.54 (0.88-2.67)	1.64 (0.96-2.80)	1.93 (1.13-3.30)
Model 1	1.07 (0.91-1.27)	1.00 (reference)	1.53 (0.88-2.67)	1.56 (0.91-2.70)	1.96 (1.14-3.39)
Model 2	0.97 (0.78-1.19)	1.00 (reference)	1.41 (0.80-2.50)	1.39 (0.77-2.50)	1.34 (0.73-2.48)

Model 1: age, sex, race, education; Model 2: model 1 + body mass index, systolic blood pressure, hypertension medications, smoking, coronary heart disease, stroke, low-density lipoprotein, high-density lipoprotein, lipid-lowering medications, urinary albumin-creatinine ratio, and estimated glomerular filtration rate. Abbreviations: ADMA, asymmetric dimethylarginine; CV, cardiovascular; HR, hazard ratio; SDMA, symmetric dimethylarginine; TMAO, trimethylamine-*N*-oxide.
[a]SDs for ADMA, SDMA, and TMAO are 0.15 μM, 0.50 μM, and 14.06 μM, respectively.

mortality. The association also became progressively stronger across quartiles of plasma ADMA concentration (Table 2).

Table 3 demonstrates the associations of the ratio of urine to plasma concentrations of the uremic solutes with cardiovascular death. In this analysis, lower ratios of all 3 solutes were independently associated with cardiovascular mortality. The strengths of association ranged from 38% higher risk per 2-fold lower urine to plasma ratio of TMAO to 69% higher risk for the corresponding SDMA association. Neither the urinary concentrations nor fractional excretion of ADMA, SDMA, and TMAO were significantly associated with cardiovascular death.

Association of Uremic Solutes With All-Cause Mortality

A total of 285 all-cause deaths occurred over the course of the study (7.67% per year). Higher plasma concentrations of all 3 solutes were significantly associated with all-cause mortality (Table 4). Lower ratios of urine to plasma concentrations were also consistently associated with all-cause mortality, and again these relationships appeared monotonic across respective quartiles (Table 5).

Association of Uremic Solutes With Incident KFRT

There were 89 participants who developed KFRT. Ascending quartiles of plasma SDMA concentration were strongly and significantly associated with incident KFRT risk in the multivariable-adjusted models; however, the association did not reach significance in the continuous model (Table 6). Higher plasma ADMA was also associated with incident KFRT in the multivariable-adjusted model by inter-quartile comparisons only. There were no statistically significant associations of KFRT with plasma TMAO concentration (Table 6), or with any of the urine to plasma solute concentration ratios (Table S8).

Discussion

We examined the associations between plasma and urine concentrations of ADMA, SDMA, and TMAO—collectively termed uremic solutes—and cardiovascular death in community-living individuals with both diabetes and eGFR <60 mL/min/1.73 m^2. We found that higher plasma ADMA concentration and lower ratios of urine to plasma concentrations for all 3 uremic solutes were significantly associated with cardiovascular death, even after adjusting for demographic and traditional cardiovascular risk factors, eGFR, and UACR. Plasma concentrations and ratios of urine

Table 3. Association of Baseline Ratio of Urine to Plasma Concentrations of ADMA, SDMA, and TMAO With CV Mortality

	Continuous (per Halving[a])	Quartile 1	2	3	4
Ratio of Urine to Plasma ADMA Concentrations					
Ratio range		<17.49	17.49-28.78	28.79-46.35	>46.35
N (no. of events)		137 (39)	140 (37)	138 (24)	140 (20)
Mortality rate, %/y		4.90	4.20	2.57	1.97
HR (95% CI)					
Unadjusted	1.45 (1.24-1.71)	2.67 (1.55-4.58)	2.24 (1.30-3.86)	1.36 (0.75-2.46)	1.00 (reference)
Model 1	1.52 (1.29-1.79)	3.16 (1.83-5.45)	2.44 (1.41-4.24)	1.48 (0.81-2.69)	1.00 (reference)
Model 2	1.53 (1.23-1.90)	2.57 (1.33-4.96)	2.37 (1.33-4.24)	1.37 (0.75-2.52)	1.00 (reference)
Ratio of Urine to Plasma SDMA Concentrations					
Ratio range		<17.34	17.34-31.19	31.20-49.65	>49.65
N (no. of events)		137 (42)	139 (36)	140 (25)	139 (17)
Mortality rate, %/y		5.53	4.01	2.63	1.67
HR (95% CI)					
Unadjusted	1.52 (1.29-1.78)	3.56 (2.03-6.27)	2.49 (1.40-4.44)	1.60 (0.87-2.97)	1.00 (reference)
Model 1	1.61 (1.37-1.91)	4.04 (2.28-7.15)	2.42 (1.36-4.32)	1.57 (0.84-2.91)	1.00 (reference)
Model 2	1.69 (1.37-2.09)	3.94 (2.03-7.62)	2.56 (1.40-4.67)	1.41 (0.75-2.64)	1.00 (reference)
Ratio of Urine to Plasma TMAO Concentrations					
Ratio range		<22.62	22.62-42.13	42.14-71.16	>71.16
N (no. of events)		139 (43)	139 (28)	149 (30)	138 (19)
Mortality rate, %/y		5.47	3.19	3.08	1.92
HR (95% CI)					
Unadjusted	1.34 (1.20-1.49)	3.09 (1.80-5.31)	1.74 (0.97-3.12)	1.64 (0.92-2.92)	1.00 (reference)
Model 1	1.36 (1.22-1.52)	3.26 (1.89-5.63)	1.71 (0.95-3.06)	1.65 (0.93-2.93)	1.00 (reference)
Model 2	1.38 (1.21-1.57)	3.31 (1.76-6.23)	1.86 (1.02-3.42)	1.77 (0.98-3.18)	1.00 (reference)

Model 1: age, sex, race, education, urine creatinine; Model 2: model 1 + body mass index, systolic blood pressure, hypertension medications, smoking, coronary heart disease, stroke, low-density lipoprotein, high-density lipoprotein, lipid-lowering medications, urine albumin, and estimated glomerular filtration rate. Abbreviations: ADMA, asymmetric dimethylarginine; CV, cardiovascular; HR, hazard ratio; SDMA, symmetric dimethylarginine; TMAO, trimethylamine-*N*-oxide.
[a]That is, analysis after $\log_2$ transformation.

to plasma concentrations for all 3 solutes were also significantly associated with the secondary outcome all-cause mortality. Plasma concentrations of ADMA and SDMA were associated with KFRT by inter-quartile comparisons only, and in no case was the ratio of urine to plasma concentrations associated with KFRT. Our observations that urine to plasma ratios of all solutes have stronger associations with cardiovascular mortality than do plasma levels of these solutes suggest that renal handling and clearance of uremic solutes may influence CVD pathogenesis.

The associations between concentrations of plasma uremic solutes and cardiovascular death in this REGARDS DKD population confirmed prior published findings. TMAO and to a lesser extent ADMA and SDMA have been linked to CVD in the general population,[16-18,24-26] and plasma concentrations of all 3 solutes are increased in the context of CKD.[13-15,27,29] We observed associations of increased plasma ADMA concentration with cardiovascular death, and all 3 plasma solutes with all-cause mortality. However, significant associations were more consistently observed for low urine to plasma ratios of the uremic solutes.

Urine to plasma ratios of urea and creatinine have typically been employed as measures of urine concentrating capacity and as an index of tubular integrity. Applications to renal pathophysiology have included distinguishing between reduced kidney perfusion and tubular injury as the etiology of acute kidney injury,[40] as well as predicting polycystic kidney disease progression.[41] All 3 solutes are small, uncharged, and not protein-bound, implying that they should undergo glomerular filtration. But because urine to plasma ratios of all 3 solutes remained significantly associated with cardiovascular mortality after adjusting for eGFR, we propose that additional mechanisms of impaired renal clearance are plausible.

TMAO may undergo proximal tubule secretion via organic anion transporters[42,43] though reports are contradictory regarding whether TMAO achieves net secretion (clearance exceeds GFR).[27,28,42,44] Furthermore, evidence for impaired secretion contributing to elevated plasma levels of TMAO in CKD is lacking.[45] The renal tubular contributions to ADMA and SDMA excretions are even less clear because fractional excretion of both molecules is less than 100% in rats[13] and ADMA undergoes significant extrarenal clearance.[15] An alternative explanation for the significant association of urine to plasma ratios with cardiovascular outcomes after eGFR adjustment is that the these ratios could represent markers of filtration that are not fully captured by the creatinine-based eGFR equation. Establishing renal handling with more precision and

Table 4. Association of Baseline Plasma ADMA, SDMA, and TMAO With All-Cause Mortality

	Continuous (per 1 SD[a])	Quartile 1	2	3	4
Plasma ADMA					
ADMA range, μM		<0.601	0.601-0.670	0.671-0.760	>0.760
N (no. of events)		151 (57)	136 (64)	132 (74)	136 (90)
Mortality rate, %/y		5.05	6.63	8.70	11.63
HR (95% CI)					
Unadjusted	1.37 (1.24-1.50)	1.00 (reference)	1.34 (0.94-1.91)	1.78 (1.26-2.52)	2.50 (1.79-3.49)
Model 1	1.38 (1.24-1.53)	1.00 (reference)	1.31 (0.91-1.87)	1.77 (1.25-2.52)	2.52 (1.25-2.52)
Model 2	1.31 (1.17-1.47)	1.00 (reference)	1.17 (0.81-1.69)	1.60 (1.12-2.29)	2.00 (1.39-2.86)
Plasma SDMA					
SDMA range, μM		<0.854	0.854-1.045	1.046-1.330	>1.330
N (no. of events)		140 (47)	136 (65)	145 (83)	134 (90)
Mortality rate, %/y		4.48	7.00	8.77	11.33
HR (95% CI)					
Unadjusted	1.44 (1.33-1.56)	1.00 (reference)	1.61 (1.11-2.35)	2.02 (1.41-2.89)	2.68 (1.88-3.81)
Model 1	1.45 (1.33-1.57)	1.00 (reference)	1.31 (0.91-1.87)	1.77 (1.25-2.52)	2.68 (1.88-3.81)
Model 2	1.42 (1.26-1.60)	1.00 (reference)	1.17 (0.81-1.69)	1.60 (1.12-2.29)	2.05 (1.31-2.20)
Plasma TMAO					
TMAO range, μM		<5.900	5.901-9.155	9.156-15.300	>15.300
N (no. of events)		142 (51)	136 (65)	142 (83)	135 (86)
Mortality rate, %/y		4.99	7.46	8.60	10.00
HR (95% CI)					
Unadjusted	1.15 (1.07-1.24)	1.00 (reference)	1.55 (1.07-2.23)	1.76 (1.24-2.50)	2.10 (1.48-2.96)
Model 1	1.18 (1.08-1.29)	1.00 (reference)	1.59 (1.10-2.30)	1.76 (1.23-2.51)	2.13 (1.50-3.04)
Model 2	1.13 (1.02-1.24)	1.00 (reference)	1.46 (1.00-2.12)	1.47 (1.01-2.14)	1.62 (1.09-2.40)

Model 1: age, sex, race, education; Model 2: Model 1 + body mass index, systolic blood pressure, hypertension medications, smoking, coronary heart disease, stroke, low-density lipoprotein, high-density lipoprotein, lipid-lowering medications, urinary albumin-creatinine ratio, and estimated glomerular filtration rate. Abbreviations: ADMA, asymmetric dimethylarginine; HR, hazard ratio; SDMA, symmetric dimethylarginine; TMAO, trimethylamine-*N*-oxide.
[a]SDs for ADMA, SDMA, and TMAO are 0.15 μM, 0.50 μM, and 14.06 μM, respectively.

determining whether ratios of urine to plasma concentrations of uremic solutes represent mechanistic biomarkers will require further investigation.

We found a greater plasma ADMA concentration to be significantly associated with both cardiovascular and all-cause mortality. These data are consistent with most prior studies evaluating these markers in the general, non-KFRT CKD, and KFRT populations,[16,19,20,22] although few studies have been conducted in high-risk diabetic CKD populations.[21,23] The potential pathophysiologic mechanisms of ADMA-induced CVD include inhibition of nitric oxide synthase, thereby contributing to vasoconstriction, hypertension, and ischemia, as well as inflammation, oxidative stress, and altered macrophage lipid metabolism.[12,46]

Of the uremic solutes we examined, plasma levels of SDMA were associated with incident KFRT. Two small studies support a relationship between elevated plasma SDMA in CKD[47] and progression to KFRT.[48] Compared to ADMA, SDMA is a relatively weak inhibitor of nitric oxide synthase,[49] but it may exert other noxious effects on the vasculature, including activation of inflammatory and pro-oxidant pathways.[12,50] Additional studies will be required to both corroborate the association of SDMA with KFRT, and to determine the pathobiological mechanisms of SDMA-mediated DKD progression.

The strengths of this study include the large, well-curated REGARDS diabetic CKD subcohort, with robust representation by Black persons, and assays of 3 specific uremic solutes with high-quality control standards. The study also has important limitations. First, despite adjusting for multiple covariates, residual confounding by GFR remains a possibility because the correlations of uremic solutes with eGFR were strong and eGFR has known imprecision relative to measured GFR and can vary over time. Second, participants with relatively advanced CKD were evaluated, which may predispose the results to index event bias that may not be fully addressed with adjustment for the baseline eGFR. Third, only samples from a REGARDS diabetic subcohort were analyzed; confirmation in other cohorts is therefore warranted. Fourth, our findings could be impacted by the stability of the 3 solutes, which may not be reflected by the 1-week freeze-thaw experiments (Table S2). However, measurement bias generally influences findings toward the null hypothesis, so our observed findings should be robust. Fifth, dietary intake data, specifically for L-carnitine and choline precursors to TMAO, were not obtained in REGARDS. Finally, uremic solutes were measured at 1 point in time. Future studies evaluating trajectories of changes of

Table 5. Association of Baseline Ratio of Urine to Plasma Concentrations of ADMA, SDMA, and TMAO with All-Cause Mortality

	Continuous (per Halving[a])	Quartile 1	2	3	4
Ratio of Urine to Plasma ADMA Concentrations					
Ratio range		<17.49	17.49-28.78	28.79-46.35	>46.35
N (no. of events)		137 (90)	140 (75)	138 (65)	140 (55)
Mortality rate, %/y		11.08	8.32	6.78	5.27
HR (95% CI)					
Unadjusted	1.37 (1.23-1.52)	2.23 (1.59-3.12)	1.65 (1.16-2.33)	1.32 (0.92-1.89)	1.00 (reference)
Model 1	1.43 (1.29-1.59)	2.67 (1.90-3.75)	1.87 (1.32-2.67)	1.44 (1.01-2.07)	1.00 (reference)
Model 2	1.34 (1.17-1.55)	1.96 (1.29-2.97)	1.63 (1.12-2.36)	1.31 (0.90-1.89)	1.00 (reference)
Ratio of Urine to Plasma SDMA Concentrations					
Ratio range		<17.34	17.34-31.19	31.20-49.65	>49.65
N (no. of events)		137 (95)	139 (73)	140 (65)	139 (52)
Mortality rate, %/y		12.27	7.94	6.70	4.93
HR (95% CI)					
Unadjusted	1.39 (1.26-1.55)	2.66 (1.90-3.73)	1.66 (1.17-2.37)	1.37 (0.95-1.98)	1.00 (reference)
Model 1	1.46 (1.31-1.62)	2.88 (2.04-4.06)	1.64 (1.15-2.35)	1.31 (0.91-1.90)	1.00 (reference)
Model 2	1.37 (1.19-1.58)	2.25 (1.50-3.39)	1.53 (1.05-2.24)	1.19 (0.82-1.72)	1.00 (reference)
Ratio of Urine to Plasma TMAO Concentrations					
Ratio range		<22.62	22.62-42.13	42.14-71.16	>71.16
N (no. of events)		139 (93)	139 (74)	149 (63)	138 (55)
Mortality rate, %/y		11.62	8.11	6.39	5.40
HR (95% CI)					
Unadjusted	1.27 (1.18-1.37)	2.30 (164-3.21)	1.54 (1.09-2.19)	1.20 (0.84-1.72)	1.00 (reference)
Model 1	1.31 (1.21-1.42)	2.41 (1.72-3.37)	1.52 (1.07-2.16)	1.18 (0.82-1.69)	1.00 (reference)
Model 2	1.26 (1.15-1.39)	2.01 (1.35-3.00)	1.47 (1.02-2.13)	1.23 (0.85-1.78)	1.00 (reference)

Model 1: age, sex, race, education, urine creatinine; Model 2: model 1 + body mass index, systolic blood pressure, hypertension medications, smoking, coronary heart disease, stroke, low-density lipoprotein, high-density lipoprotein, lipid-lowering medications, urine albumin, and estimated glomerular filtration rate. Abbreviations: ADMA, asymmetric dimethylarginine; HR, hazard ratio; SDMA, symmetric dimethylarginine; TMAO, trimethylamine-*N*-oxide.
[a]That is, analysis after $\log_2$ transformation.

uremic solutes with cardiovascular outcomes are required.

In conclusion, greater plasma ADMA concentration and lower ratios of urine to plasma concentrations of ADMA, SDMA, and TMAO are independently associated with the primary outcome of cardiovascular death among community-living persons with both diabetes and CKD. Because the strongest associations were observed for lower urine to protein ratios of the uremic solutes, which may reflect diminished GFR as well as tubular dysfunction, we suggest that the ratio of urine to plasma concentrations of ADMA, SDMA, and TMAO represents unique links between DKD and cardiovascular death.

Each uremic solute has been implicated in CVD pathophysiology, implying that in instances where plasma levels were elevated, the biomarkers might be mechanistic and contribute to the risk of DKD for CVD by enhancing atherogenesis and thrombosis, although this requires future study. Nevertheless, these 3 biomarkers have not yet achieved clinical utility. If validated in other cohorts, lower ratios of urine to plasma concentrations of ADMA, SDMA, and TMAO could be used to identify the subset of patients with diabetes and CKD who are at particularly high risk for cardiovascular and all-cause mortality.

Supplementary Material

Supplementary File (PDF)

Figure S1: Spline graphs to demonstrate linearity between plasma biomarkers and cardiovascular mortality.

Table S1: Baseline characteristics of participants who were included and excluded from analyses.

Table S2: Freeze-thaw data for samples from 4 different patients with varying degrees of CKD.

Table S3: Baseline characteristics of study participants in the random subcohort by quartile of plasma ADMA.

Table S4: Baseline characteristics of study participants in the random subcohort by quartile of plasma SDMA.

Table S5: Baseline characteristics of study participants in the random subcohort by quartile of plasma TMAO.

Table S6: Event numbers for CV death.

Table S7: Association of baseline plasma ADMA, SDMA, and TMAO with CV mortality.

Table S8: Association of baseline urine to plasma ratio of ADMA, SDMA, and TMAO with KFRT.

Article Information

CKD Biomarkers Consortium: The collaborators in phase II of the CKD Biomarkers Consortium are (asterisk indicates principal investigator): Joseph Massaro, PhD (Boston University School of

Table 6. Association of Baseline Plasma ADMA, SDMA, and TMAO With Incident KFRT

	Continuous (per 1 SD[a])	Quartile 1	2	3	4
Plasma ADMA					
ADMA range, μM		<0.601	0.601-0.670	0.671-0.760	>0.760
N (no. of events)		151 (15)	136 (23)	132 (19)	136 (32)
KFRT incidence, %/y		1.40	2.55	2.35	4.60
HR (95% CI)					
Unadjusted	1.29 (1.08-1.55)	1.00 (reference)	1.80 (0.94-3.46)	1.69 (0.86-3.34)	3.29 (1.78-6.08)
Model 1	1.41 (1.16-1.70)	1.00 (reference)	1.70 (0.88-3.28)	1.95 (0.98-3.87)	3.93 (2.10-7.35)
Model 2	1.22 (0.97-1.52)	1.00 (reference)	1.62 (0.80-3.28)	2.03 (0.95-4.34)	2.75 (1.38-5.48)
Plasma SDMA					
SDMA range, μM		<0.854	0.854-1.045	1.046-1.330	>1.330
N (no. of events)		140 (<10)	136 (11)	145 (24)	134 (48)
KFRT incidence, %/y		0.58	1.20	2.73	7.47
HR (95% CI)					
Unadjusted	1.65 (1.50-1.82)	1.00 (reference)	2.10 (0.78-5.68)	4.75 (1.94-11.63)	13.32 (5.69-31.20)
Model 1	1.66 (1.48-1.86)	1.00 (reference)	2.23 (0.82-6.07)	5.06 (2.06-12.45)	10.89 (4.58-25.88)
Model 2	1.20 (0.99-1.46)	1.00 (reference)	1.67 (0.60-4.65)	2.28 (0.89-5.79)	2.96 (1.10-7.97)
Plasma TMAO					
TMAO range, μM		<5.900	5.901-9.155	9.156-15.300	>15.300
N (no. of events)		142 (13)	136 (14)	142 (27)	135 (35)
KFRT incidence, %/y		1.31	1.65	3.02	4.69
HR (95% CI)					
Unadjusted	1.31 (1.15-1.49)	1.00 (reference)	1.26 (0.59-2.69)	2.28 (1.18-4.42)	3.52 (1.86-6.67)
Model 1	1.32 (1.16-1.51)	1.00 (reference)	1.31 (0.62-2.80)	2.73 (1.40-5.32)	3.84 (2.01-7.32)
Model 2	1.06 (0.88-1.28)	1.00 (reference)	1.03 (0.48-2.23)	0.97 (0.47-2.02)	0.98 (0.45-2.16)

Model 1: age, sex, race, education; Model 2: model 1 + body mass index, systolic blood pressure, hypertension medications, smoking, coronary heart disease, stroke, low-density lipoprotein, high-density lipoprotein, lipid-lowering medications, urinary albumin-creatinine ratio, and estimated glomerular filtration rate. Abbreviations: ADMA, asymmetric dimethylarginine; HR, hazard ratio; KFRT, kidney failure with replacement therapy; SDMA, symmetric dimethylarginine; TMAO, trimethylamine-*N*-oxide.
[a]SDs for ADMA, SDMA, and TMAO are 0.15 μM, 0.50 μM, and 14.06 μM, respectively.

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Association of Uremic Solutes With Cardiovascular Death in Diabetic Kidney Disease

Setting & Participants

Observational cohort study

N = 555 REGARDS participants with diabetes and baseline eGFR <60 mL/min/1.73 m^2

Plasma and urine ADMA, SDMA, and TMAO assayed by LC-MS/MS

Analysis

Outcomes

- **Primary:** CV death
- **Secondary:** kidney failure with replacement therapy (KFRT)

Analysis

Multivariable Cox proportional hazards regression analysis

Results

Plasma	CV Death	KFRT
ADMA	1.20 (1.01, 1.43)	1.22 (0.97, 1.52)
SDMA	1.28 (1.02, 1.60)	1.20 (0.99, 1.46)
TMAO	0.97 (0.78, 1.19)	1.06 (0.88, 1.28)

Adjusted HR (95% CI) per 1-SD greater biomarker concentration

Urine:Plasma	CV Death	KFRT
ADMA	1.53 (1.23, 1.90)	1.15 (0.87, 1.53)
SDMA	1.69 (1.37, 2.09)	1.11 (0.83, 1.48)
TMAO	1.38 (1.21, 1.57)	1.18 (0.97, 1.44)

Adjusted HR (95% CI) per halving of biomarker concentration

CONCLUSION: Higher plasma ADMA and SDMA, as well as lower U/P ratios for all 3 solutes, were independently associated with CV death.

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