Trimethylamine *N*-Oxide and Cardiovascular Events in Hemodialysis Patients

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ABSTRACT

Cardiovascular disease causes over 50% of the deaths in dialysis patients, and the risk of death is higher in white than in black patients. The underlying mechanisms for these findings are unknown. We determined the association of the proatherogenic metabolite trimethylamine *N*-oxide (TMAO) with cardiovascular outcomes in hemodialysis patients and assessed whether this association differs by race. We measured TMAO in stored serum samples obtained 3–6 months after randomization from a total of 1232 white and black patients of the Hemodialysis Study, and analyzed the association of TMAO with cardiovascular outcomes using Cox models adjusted for potential confounders (demographics, clinical characteristics, comorbidities, albumin, and residual kidney function). Mean age of the patients was 58 years; 35% of patients were white. TMAO concentration did not differ between whites and blacks. In whites, 2-fold higher TMAO associated with higher risk (hazard ratio [95% confidence interval]) of cardiac death (1.45 [1.24 to 1.69]), sudden cardiac death [1.70 (1.34 to 2.15)], first cardiovascular event (1.15 [1.01 to 1.32]), and any-cause death (1.22 [1.09 to 1.36]). In blacks, the association was nonlinear and significant only for cardiac death among patients with TMAO concentrations below the median (1.58 [1.03 to 2.44]). Compared with blacks in the same quintile, whites in the highest quintile for TMAO ($\geq$135 μM) had a 4-fold higher risk of cardiac or sudden cardiac death and a 2-fold higher risk of any-cause death. We conclude that TMAO concentration associates with cardiovascular events in hemodialysis patients but the effects differ by race.

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The burden of ESRD in the United States remains high.[1] Over 100,000 patients start dialysis every year and over a million patients will be treated with dialysis in the next decade.[1] Survival on dialysis remains poor; median survival is only 3 years and 5-year survival is <40%.[1] Cardiovascular disease is the leading cause of mortality in dialysis patients, but the factors leading to accelerated cardiovascular disease in dialysis patients are not well defined.[2,3] Traditional cardiovascular disease risk factors do not explain the high risk of accelerated cardiovascular disease in dialysis patients.[4] Survival on dialysis also differs by race, and despite seemingly similar dialysis adequacy, whites have a higher risk of death than blacks treated with dialysis in the United States.[1,5] The reasons for this survival paradox are not fully understood,[6] and very few studies have explored biochemical differences between white and black dialysis patients that may

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contribute to these observed associations.[7–9] Understanding these difference is crucial to improving dialysis care and personalizing uremia management.

Uremic toxins, substances that are normally cleared by the native kidney but are retained in patients with kidney failure, can contribute to cardiovascular disease in dialysis patients.[10] Recent pioneering work has shown that the metabolite trimethylamine *N*-oxide (TMAO) is proatherogenic.[11] In large epidemiologic studies in nondialysis patients, higher blood concentrations of TMAO are associated with 2.5-fold higher risk of cardiovascular events.[12] TMAO accumulates in kidney failure and in dialysis patients TMAO concentrations are >20-fold higher compared with nondialysis patients.[13] However, the association of TMAO with cardiovascular outcomes in dialysis patients has not been well defined.[14–16]

We measured TMAO in specimens of the Hemodialysis (HEMO) Study, a United States multicenter trial of dialysis dose and flux. The goal of our study was to analyze the longitudinal association between TMAO and cardiovascular morbidity and mortality in dialysis patients. The large sample size of the HEMO Study and inclusion of only prevalent dialysis patients with minimal to no residual kidney function afforded a unique opportunity to examine if the association differed by race without potential confounding from residual kidney function.

RESULTS

Baseline Characteristics

Our study sample included 1232 (white, 431 [35%] and black, 801 [65%]) HEMO Study patients that had available stored sera collected between 3–6 months postrandomization, a time-point allowing adequate separation between the trial intervention arms. Table 1 presents the baseline characteristics of the 1232 participants overall and by race. White participants were more likely to be male, have higher comorbidity score, history of gastrointestinal disease, have residual kidney function and had higher predialysis serum urea nitrogen and lower β2-microglobulin concentrations. Mean TMAO concentrations were similar between whites (98±57 μM) and blacks (104±67 μM; P=0.15; Figure 1A). The patients included in this study differed only slightly from all participants of the HEMO Study (Supplemental Table 1).

Table 1. Baseline characteristics of 1232 hemodialysis patients, overall and by race

Characteristic	Overall	Race		*P* Value
		Whites	Blacks	
N	1232	431	801	
Trimethylamine oxide (μM)				
Mean±SD.	101.9±63.9	98.4±57.0	103.8±67.3	0.15
Median [25th to 75th percentiles]	88 [62–124]	87 [63–120]	88 [62–125]	
Demographics				
Age, yr	57.7±13.8	58.5±14.9	57.3±13.2	0.12
Female sex	699 (56.7)	205 (47.6)	494 (61.7)	<0.001
Clinical characteristics				
ICED score	2.0±0.8	2.1±0.9	1.9±0.8	<0.001
Diabetes	555 (45.0)	181 (42.0)	374 (46.7)	0.12
Cardiac disease	976 (79.2)	330 (76.6)	646 (80.6)	0.11
Gastrointestinal disease	466 (37.8)	183 (42.5)	283 (35.3)	0.02
Attributed cause of ESRD				<0.001
Diabetes	463 (37.96)	159 (36.9)	304 (38.0)	
Hypertension	397 (32.2)	74 (17.2)	323 (40.3)	
Polycystic kidney disease	32 (2.6)	18 (4.2)	14 (1.7)	
Other	316 (25.6)	169 (39.2)	147 (18.4)	
Residual kidney urea clearance >0	413 (33.5)	166 (38.5)	247 (30.8)	0.01
Dialysis characteristics[a]				
Years of prior dialysis	3.5±4.2	3.5±4.5	3.5±4.0	0.74
Predialysis systolic BP, mmHg[a]	152.2±25.7	151.1±25.8	152.8±25.7	0.27
Postdialysis weight, Kg	70.3±15.2	69.9±15.9	70.5±14.8	0.53
Body mass index, Kg/m^2	25.8±5.4	25.7±5.7	25.8±5.3	0.73
Relative volume removed, %	4.1±1.7	4.1±1.7	4.1±1.7	0.95
High-dose group	613 (49.8)	223 (51.7)	390 (48.7)	0.32
High-flux group	620 (50.3)	222 (51.5)	398 (49.7)	0.55
Predialysis laboratory tests[a]				
BUN, mg/dl	59.4±18.7	61.5±17.8	58.3±19.0	0.004
Single-pool Kt/V	1.5±0.3	1.5±0.3	1.5±0.3	0.15
Serum albumin, g/dl	3.6±0.4	3.6±0.4	3.6±0.4	0.78
Serum β2-microglobulin, mg/L	36.7±14.3	34.8±12.4	37.7±15.2	<0.001
enPCR, g/kg/day	1.0±0.3	1.1±0.2	1.0±0.3	<0.001
Dietary recall at baseline				
Total protein, g/kg-ABW/day	0.9±0.3	0.9±0.3	0.9±0.4	0.62
Fat%	35.6±7.5	34.8±7.7	36.1±7.3	0.003
Carbohydrates%	48.3±9.2	49.4±9.3	47.7±9.1	0.002
Dietary recall at yr 1				
Total protein, g/kg-ABW/day	0.9±0.4	0.9±0.4	0.9±0.4	0.32
Fat, %	36.5±7.8	35.7±7.9	36.9±7.7	0.02
Carbohydrates, %	47.2±9.3	48.5±9.3	46.5±9.2	0.001

Values for categorical variables are given as number (percentage); values for continuous variables are given as mean±SD.
enPCR, equilibrated normalized protein catabolic rate. ABW, adjusted body weight.
[a]Data from the same date as the TMAO sample.

Association between TMAO and Outcomes

The primary outcomes for our analyses were cardiac death, sudden cardiac death, and first cardiovascular event (composite of first cardiovascular hospitalization or death from any cause). Secondary outcome was all-cause mortality. In Poisson regression models adjusted for age and sex, we noticed a

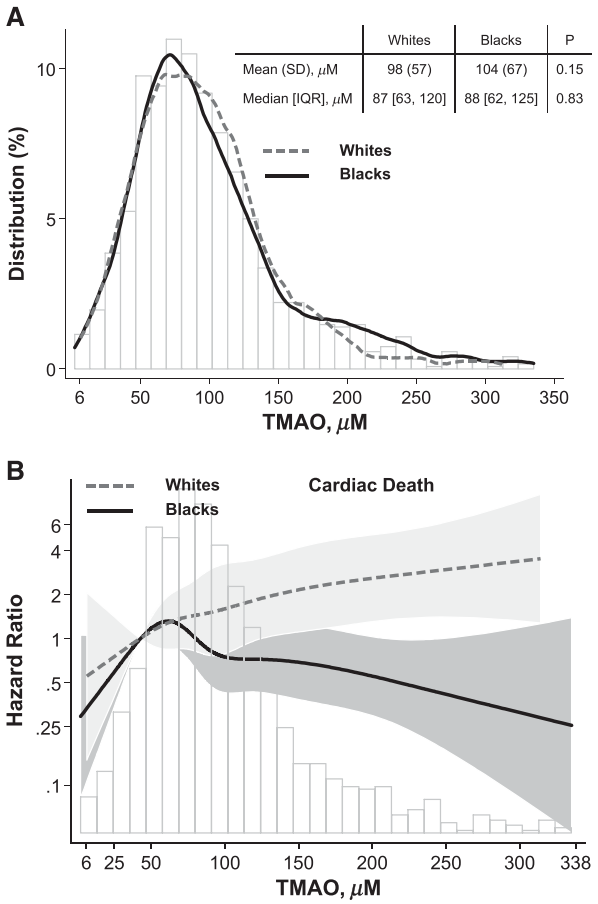

Figure 1. Distribution of TMAO concentrations are similar in white and black patients of the HEMO Study but the risk of cardiac death is linear in whites and non-linear in blacks. (A) Distribution of TMAO in the HEMO Study. Histogram depicts the overall distribution (vertical gray bars). Lines depict the distribution in whites (broken line) and blacks (solid line). Extreme observations, defined as TMAO >99th percentile (335 μM), are excluded (n=12). (B) Adjusted hazard of cardiac death. Relative hazard predicted using Cox proportional hazards regression adjusted for age, sex, ICED severity score, cause of ESRD, body mass index (categorized as <18, 18–25 and >25 kg/m^2), systolic BP (categorized as <130, 130–160 and >160 mmHg), albumin, relative volume removed on dialysis, and residual kidney function (urinary stdKt/V_{UREA} calculated from urinary urea clearance). TMAO is modeled as a restricted cubic spline with 5 knots (5th, 27.5th, 50th, 72.5th, and 95th percentiles); 10th percentile is used as the reference (HR=1). The lines represent the adjusted HR in whites (broken line) and blacks (solid line). The shaded area is the 95% CI of the HR (whites, light gray; blacks, dark gray). Vertical bars are the frequency histogram, showing the distribution of TMAO. Extreme observations, defined as TMAO >99th percentile (335 μM), are excluded (n=12).

significant interaction between race and TMAO and outcomes; p-interactions were 0.001 for cardiac death and sudden cardiac death, 0.05 for cardiovascular event or death and 0.06 for any-cause death (Supplemental Figure 1). Based on these data, we conducted further analyses stratified by race.

Cardiac Death

There were 216 deaths due to cardiac causes during 3,175 person years of follow-up (median, 2.3 years; 25th to 75th percentiles, 1.1–3.9) with a crude mortality rate of 68 per 1000 person years. The adjudicated causes of death were: ischemic heart disease (61.6%), congestive heart failure (11.6%), arrhythmias and other conduction disorders (15.3%), and other heart diseases (11.6%). In fully-adjusted models (Figure 1B, Table 2), each 2-fold higher TMAO was associated with a 45% higher risk of cardiac mortality in whites (hazard ratio [HR] 1.45; 95% confidence interval [95% CI], 1.24 to 1.69; P<0.001) but not in blacks (HR, 0.90; p-interaction <0.001). We further explored the nonlinear association among blacks noted in Figure 1B. With TMAO modeled as quintiles (Supplemental Table 2), with the lowest quintile (<56 μM) as the reference category, there was a graded increase in risk of cardiac death in whites (p-trend <0.001), whereas in blacks there was higher risk only in the second quintile. With TMAO modeled as a linear spline with a knot at the 50th percentile, there was a linear increase in the risk of cardiac mortality in whites (p-change in slope=0.55). In blacks, the association was nonlinear (p-change in slope=0.05); among patients with TMAO concentrations below the median (88 μM), each 2-fold higher TMAO was associated with 58% higher risk of cardiac death (HR, 1.58; 95% CI, 1.03 to 2.44; P=0.04). Above the median, there was no association with cardiac death.

Sudden Cardiac Death, First Cardiovascular Event and Any-Cause Death

Higher TMAO concentrations were associated with a higher risk of sudden cardiac death, first cardiovascular event, and any-cause death, particularly in whites (Table 2). The effect modification with race was notable particularly for sudden cardiac death (P <0.001) and any-cause death (P=0.02). The nonlinear association between TMAO and outcomes in blacks was notable for sudden cardiac death although it did not reach statistical significance (Supplemental Figure 2A, Supplemental Table 2).

Outcomes among Whites and Blacks

To further explore the racial differences, we compared the hazard for outcomes in whites compared with blacks, stratified by quintiles of TMAO (Table 3). The higher risk of death in whites was particularly evident at higher concentrations of TMAO. Among patients in the highest quintile for TMAO (≥135 μM) whites had an almost 4-fold higher risk of cardiac death (HR, 3.89; 95% CI, 2.47 to 6.13; P<0.001), sudden cardiac death (HR, 3.95; 95% CI, 2.15 to 7.25; P<0.001) and almost 2-fold higher risk of any-cause death (HR, 1.92; 95% CI, 1.28 to 2.86; P=0.001).

Subgroup Analyses

Supplemental Table 3 presents the results of subgroup analyses. The results should be interpreted with caution due to

Table 2. Association of TMAO with outcomes in 1232 patients of the hemodialysis study

Outcomes	*N*	Events	IR Per 1000 PY	Model 1 Unadjusted		Model 2 Adjusted: Age, Sex		Model 3 Adjusted: Model 2 + Comorbidity + Clinical + Labs		Model 4 Model 3 + Residual Kidney Function	
				HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Cardiac death											
All patients	1232	216	68.0	1.08 (0.95 to 1.23)	0.24	1.04 (1.03 to 1.04)	<0.001	1.10 (0.96 to 1.25)	0.17	1.09 (0.96 to 1.24)	0.19
Whites	431	96	101.3	1.46 (1.28 to 1.66)	<0.001	1.46 (1.28 to 1.66)	<0.001	1.46 (1.25 to 1.71)	<0.001	1.45 (1.24 to 1.69)	<0.001
Blacks	801	120	53.9	0.92 (0.79 to 1.06)	0.25	0.92 (0.78 to 1.08)	0.31	0.90 (0.77 to 1.06)	0.21	0.90 (0.77 to 1.06)	0.20
P value interaction					<0.001		<0.001		<0.001		<0.001
Sudden cardiac death											
All patients	1232	124	39.1	1.16 (1.01 to 1.33)	0.03	0.99 (0.82 to 1.20)	0.91	1.17 (1.01 to 1.36)	0.04	1.16 (1.01 to 1.35)	0.04
Whites	431	54	57.0	1.76 (1.36 to 2.28)	<0.001	1.75 (1.39 to 2.20)	<0.001	1.71 (1.34 to 2.18)	<0.001	1.70 (1.34 to 2.15)	<0.001
Blacks	801	70	31.4	0.93 (0.79 to 1.08)	0.33	0.93 (0.79 to 1.09)	0.38	0.92 (0.78 to 1.08)	0.32	0.92 (0.78–1.08)	0.31
P value interaction					<0.001		<0.001		<0.001		<0.001
First cardiovascular event or any cause death											
All patients	1148	626	274.3	1.05 (0.95 to 1.15)	0.35	0.64 (0.51 to 0.79)	<0.001	1.05 (0.97 to 1.14)	0.20	1.05 (0.98 to 1.14)	0.17
Whites	388	220	333.0	1.16 (0.99 to 1.37)	0.07	1.16 (1.00 to 1.34)	0.05	1.15 (1.00 to 1.32)	0.04	1.15 (1.01 to 1.32)	0.04
Blacks	760	406	250.3	1.01 (0.90 to 1.14)	0.87	1.03 (0.92 to 1.15)	0.65	1.01 (0.92 to 1.10)	0.83	1.01 (0.93 to 1.10)	0.80
P value interaction					0.18		0.23		0.09		0.09
Any-cause death											
All Patients	1232	550	173.3	1.02 (0.94 to 1.11)	0.57	1.04 (0.97 to 1.11)	0.32	1.06 (0.98 to 1.14)	0.15	1.06 (0.98 to 1.14)	0.15
Whites	431	217	229.0	1.16 (1.04 to 1.29)	0.01	1.15 (1.05 to 1.27)	0.003	1.22 (1.09 to 1.37)	<0.001	1.22 (1.09 to 1.36)	<0.001
Blacks	801	333	149.5	0.97 (0.86 to 1.10)	0.67	0.98 (0.85 to 1.12)	0.74	0.97 (0.85 to 1.10)	0.65	0.97 (0.85 to 1.10)	0.64
P value interaction					0.07		0.10		0.02		0.02

HR represents increase in risk per 2-fold increase in TMAO concentrations. Modeled as natural log transformed TMAO/natural log of 2. Model 1 was unadjusted. Model 2 adjusted for age and sex. Model 3 adjusted for variables in Model 2 + ICED severity score, cause of ESRD, body mass index (categorized as <18, 18–25 and >25 kg/m2), systolic BP (categorized as <130, 130–160 and >160 mmHg), albumin, and relative volume removed on dialysis. Model 4 adjusted for variables in Model 3 + residual kidney function (urinary stdKt/V_{UREA} calculated from urinary urea clearance). IR, incidence rate; PY, person years.

Table 3. Risk of outcomes in white compared with black patients of the HEMO study stratified by quintiles of TMAO

Outcomes	Range, μM	*N*	Events	IR Per 1000 PY	Model 1		Model 2		Model 3		Model 4	
					HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Cardiac death												
Quintile 1	2.25–56.2	246	37	58.9	1.49 (0.93 to 2.40)	0.10	1.50 (0.91 to 2.47)	0.11	1.71 (1.00 to 2.92)	0.05	1.70 (1.00 to 2.89)	0.05
Quintile 2	56.3–76.7	248	46	72.9	0.92 (0.50 to 1.68)	0.79	0.87 (0.46 to 1.65)	0.66	0.96 (0.51 to 1.79)	0.89	0.98 (0.52 to 1.86)	0.95
Quintile 3	76.8–100	243	40	63.1	1.89 (1.03 to 3.46)	0.04	1.83 (1.01 to 3.34)	0.05	2.08 (1.17 to 3.69)	0.01	2.12 (1.21 to 3.69)	0.01
Quintile 4	101–134	251	53	81.4	2.80 (1.70 to 4.62)	<0.001	2.62 (1.56 to 4.41)	<0.001	3.27 (2.08 to 5.13)	<0.001	3.29 (2.10 to 5.17)	<0.001
Quintile 5	135–682	244	40	63.4	3.74 (2.54 to 5.50)	<0.001	3.65 (2.42 to 5.52)	<0.001	3.94 (2.48 to 6.27)	<0.001	3.89 (2.47 to 6.13)	<0.001
P trend						<0.001		<0.001		<0.001		<0.001
Sudden cardiac death												
Quintile 1	2.25–56.2	246	19	30.2	1.02 (0.38 to 2.75)	0.97	1.06 (0.40 to 2.75)	0.91	1.15 (0.45 to 2.95)	0.78	1.14 (0.45 to 2.93)	0.78
Quintile 2	56.3–76.7	248	20	31.7	0.86 (0.36 to 2.04)	0.72	0.81 (0.32 to 2.07)	0.67	0.85 (0.33 to 2.21)	0.75	0.87 (0.32 to 2.35)	0.78
Quintile 3	76.8–100	243	25	39.4	1.43 (0.74 to 2.76)	0.29	1.38 (0.70 to 2.75)	0.36	1.47 (0.73 to 2.96)	0.281	1.49 (0.74 to 3.01)	0.27
Quintile 4	101–134	251	35	53.8	2.66 (1.60 to 4.41)	<0.001	2.55 (1.48 to 4.41)	<0.001	2.84 (1.78 to 4.52)	<0.001	2.86 (1.80 to 4.55)	<0.001
Quintile 5	135–682	244	25	39.6	4.15 (2.14 to 8.04)	<0.001	4.08 (2.14 to 7.79)	<0.001	3.98 (2.12 to 7.50)	<0.001	3.95 (2.15 to 7.25)	<0.001
P trend						<0.001		<0.001		<0.001		<0.001
First cardiovascular event or any-cause death												
Quintile 1	2.25–56.2	225	121	276.9	0.92 (0.61 to 1.39)	0.70	0.97 (0.61 to 1.53)	0.89	0.91 (0.61 to 1.35)	0.65	0.91 (0.61 to 1.36)	0.66
Quintile 2	56.3–76.7	232	120	262.1	1.50 (1.02 to 2.21)	0.04	1.49 (1.08 to 2.06)	0.02	1.56 (1.19 to 2.04)	0.001	1.55 (1.19 to 2.02)	0.001
Quintile 3	76.8–100	229	115	240.3	1.85 (1.27 to 2.69)	0.001	1.85 (1.26 to 2.71)	0.002	1.77 (1.23 to 2.53)	0.002	1.75 (1.22 to 2.51)	0.002
Quintile 4	101–134	235	134	280.8	1.34 (1.05 to 1.73)	0.02	1.28 (1.00 to 1.64)	0.05	1.37 (1.12 to 1.67)	0.002	1.36 (1.12 to 1.66)	0.002
Quintile 5	135–682	227	136	314.8	1.43 (1.09 to 1.87)	0.01	1.45 (1.09 to 1.94)	0.01	1.29 (0.93 to 1.79)	0.12	1.30 (0.94 to 1.80)	0.12
P trend						0.33		0.30		0.47		0.46
Any-cause death												
Quintile 1	2.25–56.2	246	111	176.6	1.20 (0.85 to 1.69)	0.30	1.20 (0.85 to 1.70)	0.30	1.14 (0.87 to 1.50)	0.35	1.14 (0.87 to 1.50)	0.35
Quintile 2	56.3–76.7	248	106	168.1	1.22 (0.89 to 1.67)	0.22	1.16 (0.83 to 1.62)	0.37	1.26 (0.89 to 1.78)	0.19	1.27 (0.90 to 1.78)	0.18
Quintile 3	76.8–100	243	100	157.7	2.35 (1.37 to 4.06)	0.002	2.30 (1.33 to 3.96)	0.003	2.39 (1.40 to 4.07)	0.001	2.40 (1.42 to 4.07)	0.001
Quintile 4	101–134	251	121	185.9	1.72 (1.24 to 2.37)	0.001	1.58 (1.17 to 2.15)	0.003	1.85 (1.41 to 2.44)	<0.001	1.86 (1.41 to 2.45)	<0.001
Quintile 5	135–682	244	112	177.6	1.97 (1.39 to 2.80)	<0.001	1.92 (1.31 to 2.80)	<0.001	1.92 (1.29 to 2.87)	0.001	1.92 (1.28 to 2.86)	0.001
P trend						0.02		0.03		0.04		0.04

HR are from models stratified by quintiles of TMAO. In each quintile, HR represents increase in risk per 2-fold increase in TMAO concentrations. Modeled as natural log transformed TMAO/natural log of 2. P-trend represents *P* value from an interaction between race and quintile indicator variables in a separate model. Model 1 was unadjusted. Model 2 adjusted for age and sex. Model 3 adjusted for variables in Model 2 + ICED severity score, cause of ESRD, body mass index (categorized as <18, 18–25 and >25 kg/m2), systolic BP (categorized as <130, 130–160 and >160 mmHg), albumin, and relative volume removed on dialysis. Model 4 adjusted for variables in Model 3 + residual kidney function (urinary stdKt/V_{UREA} calculated from urinary urea clearance). IR, incidence rate; PY, person years.

multiple comparisons, and a *P* value of 0.05/11=0.004 is suggested as a significant interaction between the groups. There were trends toward an association between TMAO and cardiac death in those with diabetes (p-interaction=0.03) and between TMAO and first cardiovascular event among those assigned to standard dialysis dose (p-interaction=0.03).

Other Analyses: Predictors of Serum TMAO Concentrations

Table 4 presents the results of univariate and multivariate cross-sectional associations of TMAO. In the multivariate model, using forward selection (p entry ≤0.05), diabetes, gastrointestinal disease, and predialysis urea were associated with higher TMAO concentrations whereas residual kidney function was associated with lower TMAO concentrations. Similar results were noted with TMAO modeled as quintiles (Supplemental Table 4). Mean predialysis TMAO concentrations were lower in the high Kt/V_{UREA} group (97±65 μM) compared with the standard Kt/V_{UREA} group (106±62 μM; *P*=0.01). However, there was marked variability between individuals and the correlation between TMAO and Kt/V_{UREA} was poor (r=–0.09; Figure 2A). There was no effect of dialysis membrane (flux) intervention on TMAO. TMAO concentrations were 104±68 μM and 100±59 μM in the high and low flux groups, respectively (*P*=0.28). Higher normalized protein catabolic rate (assessing dietary protein intake), measured at the same timepoint as TMAO, was associated with a higher concentration of TMAO (Figure 2B); however, the correlation was low (r=0.21) and there was marked variability between individuals. There were no associations between TMAO and protein or fat intake assessed from dietary recall at the time of randomization (3–6 months before the TMAO measurement) or at 12-months (6–9 months after TMAO measurement).

DISCUSSION

In this report from a large, national, multicenter study of prevalent hemodialysis patients, we report an association between TMAO and the risk of outcomes. Notably, the concentrations of TMAO in HEMO patients were 10–20-fold higher than reported in the people with normal renal

Table 4. Univariate and multivariate cross-sectional associations of TMAO

Characteristics	Modeling	Univariate Analysis		Multivariate Analysis	
		TMAO (SEM)	*P* Value	TMAO (SEM)	*P* Value
Demographic					
Age, yr	Per 10 years higher	1.3 (1.3)	0.29		
Sex, female %	Female versus male	0.1 (3.6)	0.98		
Race, black %	Black versus non-black	4.4 (3.7)	0.23	8.0 (3.7)	0.03
Clinical Characteristics					
Diabetes, %	Yes versus no	8.1 (3.6)	0.02	7.9 (3.6)	0.03
Cardiac disease, %	Yes versus no	3.6 (4.4)	0.42		
ICED score, %	<3 versus 3	1.1 (3.8)	0.77		
Gastrointestinal disease, %	Yes versus no	8.5 (3.7)	0.02	10.5 (3.8)	0.01
Residual kidney urea clearance, ml/min/35L TBW	Per 0.5 ml/min/35 L TBW higher	−5.4 (1.8)	0.003	−5.0 (1.8)	0.01
Body mass index, Kg/m^2	≥25 Kg/m^2 versus <25 Kg/m^2	4.3 (3.6)	0.23		
Body surface area, m^2	Per 0.5 m^2 higher	6.8 (4.5)	0.13		
Dialysis Characteristics					
Years of prior dialysis	Per 1 yr higher	0.6 (0.4)	0.18		
Predialysis systolic BP, mmHg	Per 10 mmHg higher	−0.2 (0.7)	0.81		
Post Dialysis Weight, Kg	Per 1 kg higher	0.3 (0.1)	0.04		
Relative volume removed, %	Per 1% higher	1.6 (1.1)	0.13		
Dose intervention	High dose versus standard dose	−9.3 (3.6)	0.01		
Flux intervention	High flux versus low flux	3.9 (3.6)	0.27		
Treatment time, min	Per 30 minute higher	−3.0 (1.9)	0.11		
Blood flow rate, ml/min	Per 50 ml/min higher	−1.3 (1.5)	0.37		
Dialysate flow rate, ml/min	Per 100 ml/min higher	−0.1 (1.4)	0.94		
Predialysis laboratory tests					
BUN, mg/dl	Per 10 mg/dl higher	7.8 (0.9)	<0.001	8.4 (1.0)	<0.001
Single-pool Kt/V_{UREA}	Per 0.2 higher	−1.9 (1.3)	0.14		
Serum albumin, g/dl	Per 0.5 g/dl higher	2.9 (2.3)	0.22		
Serum β2-microglobulin, mg/L	Per 10 mg/L higher	2.2 (1.3)	0.09		
Equilibrated nPCR, g/kg/day	Per 0.2 g/kg/day higher	9.7 (1.4)	<0.001		

Results are from linear regression of TMAO (on natural scale) on predictors in separate models (univariate associations) and in a forward selection model with model entry based on *P* ≤0.05 (multivariate model). For the multivariate model, R^2=7.4% and root mean squared error (RMSE) = 62.1. TBW, total body water; nPCR, normalized protein catabolic rate.

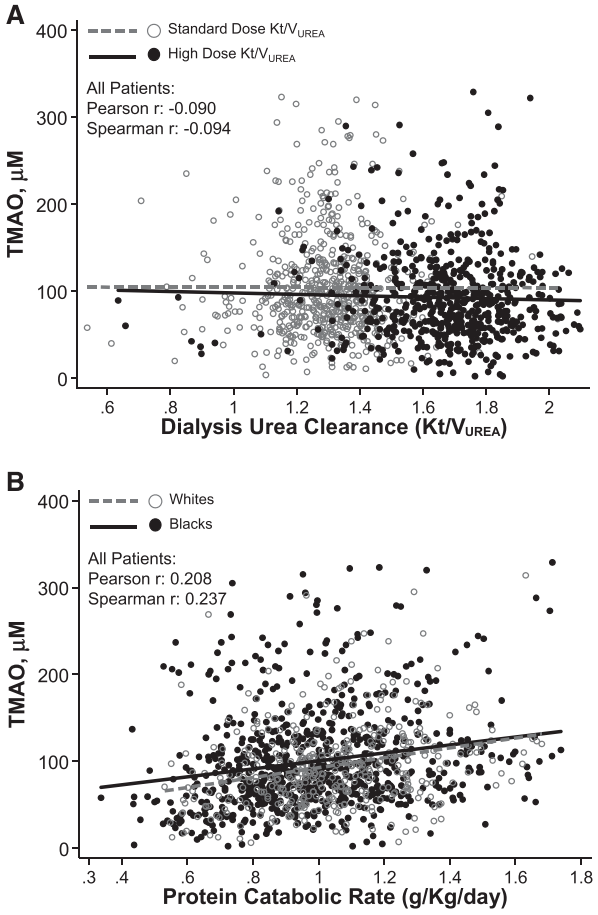

Figure 2. TMAO was poorly correlated with dialysis urea clearance and had a low correlation with nutritional protein intake. (A) Association of TMAO with dialysis urea clearance. Scatterplot of TMAO (*y*-axis) and dialysis urea clearance (Kt/V_{UREA}; *x*-axis). Solid circles represent concentrations in patients randomized to high dialysis dose and open circles represent concentrations in patients randomized to standard dialysis dose. Lines are linear fit from regression of TMAO on Kt/V_{UREA}, separately in the high dose group (solid line) and standard dose group (broken line). Pearson and Spearman correlation coefficients are presented for the overall cohort. Extreme observations (n=43), defined as TMAO or Kt/V_{UREA} >99th percentile are excluded. (B) Association of TMAO with nutritional protein intake. Scatterplot of TMAO (*y*-axis) and equilibrated nitrogen protein catabolic rate (*x*-axis). Open circles represent concentrations in whites and solid circles represent concentration in blacks. Lines are linear fit from regression of TMAO on protein catabolic rate, separately in whites (broken line) and blacks (solid line). Pearson and Spearman correlation coefficients are presented for the overall cohort. Extreme observations (n=44), defined as TMAO or protein catabolic rate >99th percentile are excluded.

function.[13] The association between TMAO and outcomes differed by race. Among white patients, there was a linear increase in risk: a 2-fold increase in TMAO was associated with a 45% higher risk of cardiac death, 70% higher risk of sudden cardiac death, 15% higher risk of first cardiovascular event, and 22% higher risk of all-cause mortality. The association between TMAO concentrations and outcomes was nonlinear in blacks with a 58% higher risk of death per 2-fold increase in TMAO among patients with TMAO concentrations below median. Among patients in the highest quintile of TMAO ($\geq$135 μM), whites had almost 4-fold higher risk of cardiac death and 4-fold higher risk of sudden cardiac death, compared with blacks. These findings suggest that TMAO is associated with vascular toxicity in hemodialysis patients and may contribute to the race-survival paradox described in dialysis patients.

Uremic toxins responsible for morbidity and mortality in dialysis patients remain largely unknown.[17] Consequently, there are no targeted solute lowering therapies for the accelerated cardiovascular disease in dialysis patients.[17] Despite the majority (>95%) of dialysis patients receiving dialysis considered "adequate" by clearance of urea (Kt/V_{UREA}), survival and quality of life remain poor.[18] The HEMO Study hypothesized that increasing dialyzer urea clearance (Kt/V_{UREA}) will also increase the clearance of unknown uremic toxins and reduce the risk of adverse outcomes. The higher Kt/V_{UREA} group in HEMO study received a 30% higher dialyzer urea clearance (Kt/V_{UREA}) compared with the standard dose group. However, the higher dose had no effect on survival.[19] Lack of knowledge about specific uremic toxins has prevented individualization of dialysis prescription.[17] Our study identifies TMAO as a potentially modifiable uremic toxin responsible for cardiovascular toxicity in hemodialysis patients.

Serum TMAO concentrations in dialysis patients reflect the net effect of TMAO production and clearance.[13] TMAO production is a result of the diet-microbiome-host interactions.[11] High-fat foods, such as red meat, liver, and egg yolk contain high concentrations of L-carnitine and phosphatidylcholine (also called lecithin). Choline is released from phosphatidylcholine by the action of phospholipases in the intestine. Choline and L-carnitine are then metabolized to trimethylamine by the gut microbes. Trimethylamine, a gas that smells like decaying fish, is rapidly reabsorbed and is converted to TMAO by hepatic flavin monooxygenase 3 enzymes. TMAO is a 75 Dalton metabolite that is not protein-bound and rapidly cleared by the kidneys, likely by a combination of glomerular filtration and tubular secretion.[13] In animal models, higher TMAO concentrations lead to enhanced macrophage levels of low density lipoprotein scavenger receptors CD36 and SR-A1 and enhanced lipid-laden macrophage (foam cell) development.[11] In nondialysis patients with coronary artery disease, higher TMAO concentrations are associated with greater coronary atherosclerotic burden[11] and a 2–3-fold higher risk of major cardiovascular events.[12] A recent study also noted that higher concentrations of TMAO in patients with chronic kidney disease, including some on dialysis (n=20), correlated with higher coronary atherosclerosis burden.[16] Our results extend these findings to a larger population of dialysis patients. These findings also indicate that even though TMAO levels are greatly influenced by kidney function, TMAO levels

associate with cardiovascular events even in the absence of renal function. Notably, in dialysis patients, predialysis TMAO concentrations (median, 88 μM) were >20-fold higher than noted in the general population studies and higher concentrations were associated with increased risk of cardiac death, sudden cardiac death, first cardiovascular event, and any-cause death.

Our findings of the risk of cardiovascular outcomes with TMAO differ from two prior dialysis studies. Kaysen *et al.*[15] determined the association of serum TMAO in 235 incident hemodialysis patients of the Comprehensive Dialysis Study. TMAO concentrations (median, 43 μM) were 50% lower than our study, although still markedly higher than patients with normal kidney function. There were no associations between TMAO and all-cause mortality or cardiovascular mortality, the latter assessed by claims data. Kalim *et al.*[14] also did not find a significant association between TMAO and cardiovascular mortality, assessed by claims data, using a case-control design in the incident hemodialysis Accelerated Mortality on Renal Replacement cohort. In both these cohorts, race interactions were not reported and, importantly, residual kidney function, a major contributor to TMAO clearance, was not assessed. Our study cohort was significantly different from these studies. The HEMO Study recruited prevalent dialysis patients and excluded patients with significant residual kidney function (urinary urea clearance >1.5 ml/min). Kidney function is a major contributor to TMAO clearance[13] and a significant predictor of mortality in incident dialysis patients.[20] The lack of association noted in the prior studies could be due to uncontrolled confounding by unmeasured residual kidney function. Additionally, in the HEMO Study, cardiovascular outcomes were physician-adjudicated and likely have a greater specificity for cardiovascular events compared with claims-based outcomes.

We noted differences in association between TMAO and outcomes in white versus black dialysis patients (effect modification). In whites, TMAO had a linear increase in risk with cardiovascular mortality but in blacks the association was nonlinear. These findings are intriguing and hypothesis generating. Functional polymorphisms in flavin monooxygenase 3, the enzyme converting trimethylamine to TMAO, are associated with varying enzyme catalytic efficiency and vary between different race and ethnic groups.[21,22] However, the distribution of TMAO concentrations did not differ by race in the HEMO Study. For similar reasons, racial differences in microbiome may not be a contributing factor. The exact mechanisms by which TMAO leads to expression of macrophage LDL influx receptors, such as CD36 and SR-A1, are unknown. In animal models, genetic differences in macrophage cholesterol efflux genes can also contribute to foam-cell accumulation.[23] It is plausible that there are genetic differences that contribute to these differences in whites and blacks as has been noted for plasma lipid composition.[24,25] The nonlinear association noted in blacks is also intriguing and could be from a threshold effect above which higher concentrations do not increase toxicity or a possibility of selection bias with blacks "resistant" to the toxicity of TMAO surviving longer. Our findings suggest that the higher risk of mortality in white versus black dialysis patients, referred to as the race-survival paradox in dialysis, may partly be due to the effects of TMAO.[6]

It is not clear if the racial differences noted in our study are unique to dialysis patients. In patients not on dialysis, higher TMAO concentrations are associated with cardiovascular events,[12,26] coronary atherosclerosis burden[11] and chronic kidney disease progression.[16,27] However, to our knowledge, differences in TMAO concentrations or presence of effect modification by race has not previously been described. Some studies have suggested that the inflammatory and nutritional milieu in dialysis patients has a differentially lethal effect by race, with blacks being more resilient to inflammation.[7,28] Further research is needed to explore mechanisms including whether genetic factors may play a role.[29,30]

There are some limitations to our study. TMAO was measured at only one time point and changes in diet and microbiome over time may change serum TMAO concentrations. HEMO samples have been stored for a long time. However, TMAO is quite robust to multiple freeze-thaw cycles.[31] We did not have data on C-reactive protein and were not able to adjust for inflammation in our models. These limitations are balanced by the major strengths of our study including its large sample size, national prospective study design, exclusion of patients with significant residual kidney function, careful collection of samples, long duration of follow-up, and carefully adjudicated cardiovascular outcomes.

In conclusion, TMAO is associated with cardiovascular morbidity and mortality in HEMO patients although its effects differ among whites and blacks. These racial differences in association between TMAO and outcomes may explain the race-survival paradox in dialysis patients. Our findings call for a carefully designed clinical trial to determine the effect of lowering TMAO concentrations on outcomes in dialysis patients.

CONCISE METHODS

Study Design

The HEMO Study was a clinical trial that randomized 1846 prevalent HEMO patients to standard or high dialyzer urea clearance (assessed by Kt/V_{UREA}, an index of urea clearance by dialysis) and to low- or high-flux dialysis membranes (assessed by β2-microglobulin clearance).[19,32] The patients were enrolled from May 1995 to February 2001 from 15 clinical centers in the United States comprising 72 dialysis units and followed for outcomes until death, kidney transplantation, or end of study in December 2001. Major exclusion criteria included residual urea clearance >1.5 ml/min per 35 L urea volume of distribution, unstable angina, active systemic infection, New York Heart Association class IV congestive heart failure, and severe

hypoalbuminemia (<2.6 g/dl). The participating institutions' review boards reviewed and approved the study.

Data Collection

Laboratory Measurements

We measured TMAO by liquid chromatography/mass spectrometry using TMAO-d9 (Cambridge Isotope Laboratories, Andover, MA) as internal standards. Plasma was deproteinized by mixture with an internal standard solution and methanol (2:1:20 vol:vol:vol). Five μL of each sample supernatant was injected in a Shimadzu Prominence LC-20A system (Kyoto, Japan) and analytes were separated on a silica column (150×2.1 mm, 3 Om Luna silica; Phenomenex, Torrance, CA) at room temperature. The mobile phase was 90% methanol containing 10 mM ammonium formate and 0.2% formic acid (v/v) at a flow rate of 0.2 ml/min. MS was performed on an API 4000 Triple Quadrupole Mass Spectrometer (AB Sciex, Framingham, MA) with electrospray ionization in the positive mode. Ion transitions used for quantitation were m/z 76→58 for TMAO with corresponding transitions for the internal standards. Recoveries averaged 113±6% for TMAO and the coefficient of variation for quality control samples run with each assay was 5%. When measured values fell below 80% of the lowest standard, a value half way between zero and the low end of the standard curve was imputed. For other laboratory tests including urea, albumin, and β2-microglobulin, we used data collected as part of the HEMO Study.

Outcomes

Cardiac death was defined as deaths due to coronary events, arrhythmias, sudden cardiac death, congestive heart failure, or cerebrovascular events. Sudden cardiac death was defined as witnessed death with preceding duration of symptoms less than 24 hours or unwitnessed death, or unwitnessed unexpected death with symptom duration less than the interval since the last dialysis session.[33] Cardiovascular hospitalizations were defined as hospitalizations for ischemic heart disease, heart failure, arrhythmias, other cardiac conditions, hypertension, and peripheral vascular disease. Causes for death and hospitalizations in the HEMO Study were determined locally and then adjudicated by an outcomes committee that was unaware of treatment-group assignments.[34]

Other Covariates

Demographics and clinical information was obtained for all participants at baseline. Comorbidity was assessed using the Index of Coexisting Disease (ICED) at baseline and then annually. The final ICED score ranged from 0–3 with higher numbers indicating greater comorbidity. Detailed dietary information was collected at baseline and then annually using 2-day assisted recall. Residual kidney function was assessed at baseline by a timed urine collection with measurement of urinary urea clearance. Systolic blood pressure, weight, and volume removed on dialysis were collected as per the dialysis unit routine and were recorded on the monthly HEMO kinetic modeling day, the same date as the blood sample collection. Relative volume removed was calculated as predialysis weight minus post dialysis weight divided by predialysis weight. Body mass index was calculated by dividing target weight in kilograms by height in meters squared. Data for Kt/V_{UREA} and normalized protein catabolic rate (an index of protein intake) were obtained from the HEMO database.

Statistical Analyses

We analyzed the baseline characteristics of the patients overall and by race, comparing differences using chi-squared test for categorical variables and linear regression for continuous variables. Covariates with missing values included cause of ESRD (1.9%), systolic BP (0.1%), albumin (0.5%), and residual kidney function (0.1%). To avoid listwise deletion,[35] we imputed missing data with 10 data replicates. For survival analyses, we set the time origin as the date of dialysis initiation with at-risk time starting at the date of sample collection (left censoring: accounts for duration of dialysis prior to enrollment). We censored participants at kidney transplantation or end of the study for mortality analyses and also for transfer to nonparticipating clinical centers for hospitalization analyses, as the hospitalization information was not collected after transfer. To visualize the association between TMAO and outcomes, we displayed age and sex adjusted incidence rates for outcomes using a Poisson regression model with TMAO modeled as restricted cubic spline with 5 knots. We used Cox proportional hazards models to analyze the association between TMAO and outcomes modeling TMAO as a natural log and as quintiles. We tested proportionality assumptions by Schoenfeld residual plots. We adjusted the Cox models for the following prespecified factors: age, sex, race, ICED score, cause of ESRD, body mass index (categorized as <18, 18–25 and >25 kg/m^2), systolic BP categorized as <130, 130–160 and >160 mmHg, relative volume removed, serum albumin and residual kidney function (urinary standard Kt/V_{UREA} calculated from urinary urea clearance). We generated plots for the adjusted hazard of outcomes with TMAO modeled as a restricted cubic spline with 5 knots to allow visual assessment of the association. We prespecified analyses in the following subgroups: age (above or below median), sex, race (whites versus blacks), diabetes, cardiac disease, gastrointestinal disease, body mass index (<18 or 18–25 or >25), albumin (above or below median), residual kidney function (any versus none) and trial interventions. We conducted further stratified analyses in subgroups with significant interactions. In additional analyses, we explored predictors of serum TMAO concentrations using univariate and multivariate linear regression. We considered two sided $P<0.05$ as statistically significant. We conducted all analyses using SAS 9.4 (SAS Institute Inc., Cary, NC) and STATA 13.0.

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DISCLOSURE

None.

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