

ORIGINAL RESEARCH

Longitudinal Plasma Measures of Trimethylamine N-Oxide and Risk of Atherosclerotic Cardiovascular Disease Events in Community-Based Older Adults

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BACKGROUND: Trimethylamine N-oxide (TMAO) is a gut microbiota-dependent metabolite of dietary choline, L-carnitine, and phosphatidylcholine-rich foods. On the basis of experimental studies and patients with prevalent disease, elevated plasma TMAO may increase risk of atherosclerotic cardiovascular disease (ASCVD). TMAO is also renally cleared and may interact with and causally contribute to renal dysfunction. Yet, how serial TMAO levels relate to incident and recurrent ASCVD in community-based populations and the potential mediating or modifying role of renal function are not established.

METHODS AND RESULTS: We investigated associations of serial measures of plasma TMAO, assessed at baseline and 7 years, with incident and recurrent ASCVD in a community-based cohort of 4131 (incident) and 1449 (recurrent) older US adults. TMAO was measured using stable isotope dilution liquid chromatography–tandem mass spectrometry (laboratory coefficient of variation, <6%). Incident ASCVD (myocardial infarction, fatal coronary heart disease, stroke, sudden cardiac death, or other atherosclerotic death) was centrally adjudicated using medical records. Risk was assessed by multivariable Cox proportional hazards regression, including time-varying demographics, lifestyle factors, medical history, laboratory measures, and dietary habits. Potential mediating effects and interaction by estimated glomerular filtration rate (eGFR) were assessed. During prospective follow-up, 1766 incident and 897 recurrent ASCVD events occurred. After multivariable adjustment, higher levels of TMAO were associated with a higher risk of incident ASCVD, with extreme quintile hazard ratio (HR) compared with the lowest quintile=1.21 (95% CI, 1.02–1.42; *P*-trend=0.029). This relationship appeared mediated or confounded by eGFR (eGFR-adjusted HR, 1.07; 95% CI, 0.90–1.27), as well as modified by eGFR (*P*-interaction <0.001). High levels of TMAO were associated with higher incidence of ASCVD in the presence of impaired renal function (eGFR <60 mL/min per 1.73 m²: HR, 1.56 [95% CI, 1.13–2.14]; *P*-trend=0.007), but not normal or mildly reduced renal function (eGFR ≥60 mL/min per 1.73 m²: HR, 1.03 [95% CI, 0.85–1.25]; *P*-trend=0.668). Among individuals with prior ASCVD, TMAO associated with higher risk of recurrent ASCVD (HR, 1.25 [95% CI, 1.01–1.56]; *P*-trend=0.009), without significant modification by eGFR.

CONCLUSIONS: In this large community-based cohort of older US adults, serial measures of TMAO were associated with higher risk of incident ASCVD, with apparent modification by presence of impaired renal function and with higher risk of recurrent ASCVD.

Key Words: animal food ■ atherosclerotic cardiovascular disease ■ renal function ■ trimethylamine N-oxide

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Supplementary Material for this article is available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.120.020646>

For Sources of Funding and Disclosures, see page 10.

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CLINICAL PERSPECTIVE

What Is New?

- Long-term elevated levels of trimethylamine N-oxide were positively associated with increased incident atherosclerotic cardiovascular disease in older US adults with impaired renal function or with documented atherosclerotic cardiovascular disease.

What Are the Clinical Implications?

- Serial measurements of trimethylamine N-oxide can be used as a marker of (recurrent) cardiovascular disease risk in those with impaired renal function or with prior atherosclerotic cardiovascular disease.
- If these associations prove to be causal, trimethylamine N-oxide could be targeted to reduce risk of atherosclerotic cardiovascular disease.

Nonstandard Abbreviations and Acronyms

CHS	Cardiovascular Health Study
TMAO	trimethylamine N-oxide

Trimethylamine N-oxide (TMAO) is a gut microbiota-dependent metabolite of dietary choline, L-carnitine, and phosphatidylcholine-rich animal foods (eg, red meat, eggs, fish, and poultry) and endogenously produced γ -butyrobetaine and crotonobetaine.^{1–3} TMAO, a small (75.1-Dalton) amine compound, is cleared by the kidneys (>95% clearance in healthy individuals)⁴ and, independently, causes renal damage and elevated cystatin C in animal models.⁵ Experimental studies support a causal role of TMAO in development and progression of atherosclerotic cardiovascular disease (ASCVD) via multiple mechanisms, including decreased reverse cholesterol efflux, altered bile acid biosynthesis, increased accumulation of cholesterol in macrophages, foam cell formation in artery walls, stimulation of vascular inflammation, platelet aggregation and thrombosis, and renal tubulointerstitial fibrosis and dysfunction.^{1,5–9} In humans, higher plasma TMAO levels have been associated with risk of ASCVD events^{1,6,10} and chronic kidney disease.^{5,11–13} However, such studies have generally been performed in patients with prevalent disease (coronary artery disease, heart failure, diabetes mellitus, and chronic kidney disease) referred to tertiary medical centers,^{10–12,14–17} where reverse causation and selection bias may be problematic. A relationship between plasma TMAO and incident or recurrent ASCVD in community-based populations

has not been established. In addition, given TMAO's renal clearance and impacts on renal dysfunction, the potential mediating or modifying roles of renal function on these relationships are uncertain. Given the health burdens of ASCVD, accounting for 17.8 million annual deaths worldwide,¹⁸ it is crucial to understand the potential interplay between TMAO, renal function, and incident and recurrent ASCVD, which may be leveraged for novel prevention and treatment efforts.

Plasma TMAO levels can also vary over time caused by shifts in dietary intake, microbiome composition, and/or metabolism. Yet, prior studies of clinical events used a single measurement of TMAO or change in TMAO at baseline, rather than serial measures with time-varying updating, which reduces measurement error and misclassification over time. To address these key gaps in knowledge, we prospectively investigated the associations of serial measures of TMAO with incident and recurrent ASCVD in a community-based population of older US adults, including the potential mediating or modifying role of renal function. We hypothesized that higher long-term levels of TMAO would be associated with increased risk of incident and recurrent ASCVD, and the association would be modified by presence of impaired renal function.

METHODS

Data, analytical methods, and study materials will not be made available to other researchers for the purpose of reproducing the results or replicating the procedure. The authors are not authorized to share CHS (Cardiovascular Health Study) data.

Study Population

The CHS is a prospective multicenter community-based cohort study to identify risk factors and consequences of cardiovascular diseases in older adults. A total of 5888 participants were randomly selected and enrolled from Medicare eligibility lists in 4 US communities (Sacramento County, California; Washington County, Maryland; Forsyth County, North Carolina; and Allegheny County, Pennsylvania). Details of the study design and recruitment have been reported.^{19,20} In brief, 5201 men and women were recruited in 1989 to 1990, with an additional 687 predominantly Black participants added in 1992 to 1993. Eligibility criteria included being aged ≥ 65 years, noninstitutionalized, expected to remain in their current community for >3 years, and not under active hospice or cancer treatment. Each center's institutional review committee approved the study protocols, and all participants gave written informed consent. Participants were followed up by means of annual clinic visits through 1999 with interim 6-month telephone contacts; and regular

6-month telephone contacts thereafter. At each annual study visit, participants underwent comprehensive clinic examinations, including a standardized interview, physical examination, laboratory assessment, and diagnostic tests. Information on medical history, alcohol consumption, smoking, and other lifestyle risk factors was also regularly collected (see below).¹⁹

Quantification of Plasma TMAO

Plasma TMAO concentrations were serially measured in all CHS participants with available stored blood samples at baseline (1989–1990 for the main cohort [$n=4407$; 84.7% of living participants]; 1992–1993 for the minority cohort [$n=612$; 89.1%]) and in 1996 to 1997 ($n=3393$; 76.9%). At these visits, 12-hour fasting plasma blood samples were collected using EDTA tubes, and immediately processed and stored at -80°C until analysis. Detailed description of quantification of plasma TMAO has been reported.²¹ Briefly, we used stable isotope dilution liquid chromatography with online LC–MS/MS on a triple quadrupole mass spectrometer (LCMS-8050; Shimadzu Corporation, Kyoto, Japan) at the Cleveland Clinic Lerner Research Institute using $d_9(\text{trimethyl})\text{TMAO}$ as internal standards. TMAO and $d_9(\text{trimethyl})\text{TMAO}$ were monitored using electrospray ionization in positive-ion mode with multiple reaction monitoring of parent and characteristic daughter ion transitions: m/z 76→58 and 85→66, respectively. Laboratory coefficients of variation were $<6\%$ for TMAO at baseline and 1996 to 1997, lower than typical coefficients of variation (6.1%–15.6%) for TMAO measurements performed as part of multiplex ‘omics assays.^{15,22–24}

Ascertainment of ASCVD

Consistent with American College of Cardiology/American Heart Association guidelines,²⁵ ASCVD was defined as myocardial infarction (MI) (fatal and nonfatal), fatal coronary heart disease, stroke (fatal and nonfatal), sudden cardiac death, and other atherosclerotic death. The standardized CHS definitions of incident MI, stroke, and sudden cardiac death have been previously described in detail.^{26–28} Incident ASCVD events were identified from annual clinic visits, interim 6-month telephone contacts, hospital records, and Centers for Medicare & Medicaid Services and National Death Index data; with centralized adjudication by committees of physicians using standardized criteria based on data from participants, proxy interviews, medical records, physician questionnaires, death certificates, medical examiner forms, Centers for Medicare & Medicaid Services hospitalizations, the National Death Index, and available brain imaging.²⁹

We followed up 4131 participants for incident ASCVD, after excluding 470 participants without TMAO

measurements and 1287 participants with prevalent cardiovascular disease (prevalent angina, coronary revascularization, nonfatal MI, or nonfatal stroke) at the time of their first TMAO measurement (Figure S1). We followed up 1449 participants for recurrent ASCVD, including 522 with prevalent angina or coronary revascularization at baseline (participants with nonfatal MI or nonfatal stroke at baseline were not followed up for recurrent nonfatal MI or stroke in CHS) and 927 who had experienced a new onset of angina, coronary revascularization, nonfatal MI, or nonfatal stroke by 1996 to 1997 and who entered the analysis at that time point, and excluding 125 others without TMAO measures.

Covariates

At each annual visit, medical history was obtained, including smoking status (current, former, or never; lifetime pack-years; and years since quitting in former smokers) and usual frequency and types of alcohol intake. Physical activity was assessed at baseline (1989–1990 or 1992–1993) and in 1996 to 1997 using a modified Minnesota Leisure-Time Activities questionnaire.³⁰ Usual dietary habits were assessed in 1989 to 1990³¹ using a 99-item validated food frequency questionnaire (National Cancer Institute) and in 1996 to 1997³² using a validated 131-item food frequency questionnaire (Willett et al).³³ Fasting blood high-density lipoprotein cholesterol and triglyceride levels were measured using standardized methods,^{34,35} with low-density lipoprotein cholesterol calculated by Friedewald equation, excluding individuals with hypertriglyceridemia. CRP (C-reactive protein) was measured by using a high-sensitivity ELISA.³⁶ Serum creatinine was measured using a colorimetric method (Ektachem 700; Eastman Kodak, Rochester, NY) and calibrated to isotope dilution mass spectrometry.³⁷ Cystatin C was measured from frozen samples using a BNII nephelometer (Siemens, Deerfield, IL).³⁸ Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration creatinine–cystatin C equation that has greater precision and accuracy (calculated as the percentage of estimates that differed from measured eGFR) than equations based on creatinine or cystatin C alone.³⁹

Statistical Analysis

Cox proportional hazards models were used to estimate hazard ratios (HRs) of time to first (first recurrent) ASCVD event associated with TMAO. Time to censoring was derived as the earliest of death, lost to follow-up, and June 30, 2015. The Cox proportional hazards assumption was evaluated using a test based on Schoenfeld residuals.⁴⁰ We found no evidence of violation of the proportional hazard assumption. To leverage

serial TMAO measures and evaluate habitual exposure, we used time-varying cumulative averaging: TMAO measures at baseline were related to risk of ASCVD until 1996 to 1997; and the average of levels at baseline and 1996 to 1997 to risk after 1996 to 1997. For participants with missing TMAO levels at the second time point (19.7%), baseline measurements were carried forward. For participants entering the analysis at the second time point for analyses of recurrent ASCVD, only the second TMAO measure was used, as the first TMAO measure occurred before their initial event. TMAO levels were evaluated categorically in quintiles as indicator variables, with quintile cut points based on cohort baseline measures. To assess linear trends, quintiles were assessed as linear variables after assigning participants the median value in each quintile.

To minimize potential confounding, we considered prespecified covariates based on biologic interest, risk factors for ASCVD in older adults, or associations with TMAO or ASCVD. Covariates included sex, race, enrollment site, education, income, and time-varying risk factors, including age, self-reported health status, smoking status, physical activity, body mass index (BMI), waist circumference, prevalent diabetes mellitus, alcohol intake, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, CRP, systolic and diastolic blood pressure, use of lipid-lowering medication, use of antihypertensive medication, use of antibiotics in the past 2 weeks of TMAO measurement, and habitual diet, including intakes of animal source foods (the sum of unprocessed red meat, processed meat, eggs, chicken, and fish), fruits, vegetables, and dietary fiber. We further adjusted for eGFR as a potential mediator or confounder. Missing covariate data (0.1%–19.5% at baseline; 1.7%–20.2% in 1996–1997) were imputed using multiple demographic and risk variables using single multivariable imputation, which prior analyses in CHS have shown produces similar results as multiple imputation.⁴¹

Potential effect modification of the relationship between TMAO and ASCVD was explored for eGFR (≥ 60 or < 60 mL/min per 1.73 m²) as well as age (≤ 71 or > 71 years), sex (men/women), and BMI (kg/m²). Statistical significance of each multiplicative interaction term was assessed using the Wald test, with *P* values for these exploratory analyses. Bonferroni adjusted for multiple comparisons ($\alpha = 0.05/4 = 0.0125$). Potential nonlinear associations of log-transformed TMAO with ASCVD were explored using restricted cubic splines. Sensitivity analyses were conducted: (1) excluding participants who had taken antibiotics in the 2 weeks before TMAO measurement; (2) excluding early events (within the first 2 years) to minimize any effect of preexisting subclinical disease leading to changes in TMAO levels; (3) excluding those who self-reported their general health as poor at study entry; and (4) assessing

the relationship between TMAO and ASCVD by simple updating (plasma TMAO levels at baseline were related to risk of ASCVD until 1996–1997, and plasma TMAO levels in 1996–1997 were related to risk of ASCVD after 1996–1997). Analyses used a 2-tailed α of 0.05 and were performed using Stata 14.2 (Stata Corp, College Station, TX).

RESULTS

Baseline Characteristics

At baseline, mean (SD) age was 72.2 (5.3) years, 64% of participants were women, and 16% were black people race (Table 1). Most (80%) reported good, very good, or excellent general health, 40% were former smokers, and 12% were current smokers. Mean BMI (SD) was 26.7 (4.8) kg/m², and 11% had prevalent diabetes mellitus. Only 3% had taken antibiotics within the prior 2 weeks. Among those with prevalent cardiovascular disease, most (73%) reported fair or poor general health and about two thirds (60%) were taking hypertension medication.

The median (interquartile range) concentration of TMAO was 4.7 (3.2–7.7) $\mu\text{mol/L}$ at baseline. In crude (unadjusted) analyses, participants with higher TMAO levels were more likely to be White race, report poor self-reported health, have lower high-density lipoprotein cholesterol and higher triglyceride levels, and be taking hypertension medication (Table S1). In addition, eGFR was lower, whereas dietary intake of total animal products was higher, in those with higher levels of TMAO. TMAO levels were not significantly associated with BMI, physical activity, alcohol consumption, blood pressure, or CRP. The Spearman correlation for repeated within-individual measures of TMAO over 7 years (4 years for the minority cohort) was 0.254 (Table S2).

Plasma TMAO and Risk of Incident ASCVD

During 54 447 person-years of follow-up (median, 15 years; maximum, 26 years), 1766 ASCVD events occurred. After multivariable adjustment for ASCVD, demographics, and lifestyle risk factors, higher TMAO levels were positively associated with risk of incident ASCVD (extreme-quintile HR [95% CI]: 1.21 [1.02–1.42]; *P*-trend=0.029; Table 2). Further adjustment for dietary factors had little effect (HR [95% CI]: 1.21 [1.02–1.43]; *P*-trend=0.028). In contrast, after additional adjustment for eGFR, which could be either a mediator or confounder of TMAO's effects, the HR was attenuated, with an extreme quintile HR of 1.07 (95% CI, 0.90–1.27; *P*-trend=0.516).

When stratified by baseline eGFR (≥ 60 or < 60 mL/min per 1.73 m²), higher plasma TMAO was associated

Table 1. Baseline Characteristics of Older US Men and Women With Plasma TMAO Measurements in the CHS

Characteristics	Participants free of prevalent cardiovascular disease (n=4131)	Participants with prevalent cardiovascular disease (n=1287)	P value*
TMAO, median (IQR), $\mu\text{mol/L}$	4.72 (3.19–7.69)	5.43 (3.57–8.74)	<0.001
Demographics			
Age, mean (SD), y	72.2 (5.3)	73.6 (5.8)	<0.001
Female sex, n (%)	2623 (64)	603 (47)	<0.001
Race, n (%)			
White	3470 (84)	1055 (82)	0.087
Education, n (%)			
<High school	1121 (27)	427 (33)	0.001
High school	1189 (29)	338 (26)	
Some college	959 (23)	277 (22)	
College graduate	862 (21)	245 (19)	
Income group, n (%)			
<\$11 999	996 (24)	374 (29)	<0.001
\$12 000–\$24 999	1486 (36)	483 (38)	
\$25 000–\$49 999	1106 (27)	302 (23)	
>\$50 000	543 (13)	128 (10)	
Lifestyle and risk factors			
Self-reported health status, n (%)			
Excellent/very good	1751 (42)	281 (22)	<0.001
Good	1562 (38)	475 (37)	
Fair/poor	818 (20)	531 (41)	
Smoking, n (%)			
Never smoked	1979 (48)	559 (43)	<0.001
Former smoker	1639 (40)	595 (46)	
Current smoker	513 (12)	133 (10)	
Physical activity, mean (SD), kcal	1150 (1533)	1143 (1603)	0.405
Alcohol, mean (SD), drinks/wk	1.1 (1.6)	1.0 (1.5)	0.003
BMI, mean (SD), kg/m^2	26.7 (4.8)	26.8 (4.7)	0.481
Waist circumference, mean (SD), cm	94.0 (13.5)	96.1 (12.8)	<0.001
Systolic blood pressure, mean (SD), mm Hg	136 (21)	137 (23)	0.124
Diastolic blood pressure, mean (SD), mm Hg	71 (11)	70 (12)	<0.001
Biochemical variables			
HDL cholesterol, mean (SD), mg/dL	56 (16)	50 (15)	<0.001
LDL cholesterol, mean (SD), mg/dL	130 (35)	131 (36)	0.163
Triglycerides, mean (SD), mg/dL	136 (72)	148 (79)	<0.001
CRP, median (IQR), mg/L	2.4 (1.2–4.4)	3.0 (1.5–5.6)	<0.001
Estimated glomerular filtration rate, mean (SD), mL/min per 1.73 m^2 [†]	70.1 (16.2)	63.8 (17.9)	<0.001
Medical history, n (%)			
Prevalent diabetes mellitus	439 (11)	167 (13)	0.02
Lipid-lowering medication	196 (5)	115 (9)	<0.001
Hypertension medication	1649 (40)	902 (70)	<0.001
Antibiotics in past 2 wk	116 (3)	46 (4)	0.12
Dietary habits, mean (SD)			
Fruits, servings/d	2.1 (1.1)	2.2 (1.0)	0.216
Vegetables, servings/d	2.6 (1.4)	2.4 (1.3)	0.018
Fiber, g/d	29.3 (11.8)	29.8 (12.4)	0.683
Total animal product, servings/wk [‡]	1.7 (1.0)	1.7 (1.0)	0.503

Values represent mean (SD) for continuous variables and frequency (percentage) for categorical variables, unless otherwise stated. For analyses of incident cardiovascular disease, 3877 participants entered the study at baseline (1989–1990 for main cohort; 1992–1993 for minority cohort) and 254 participants entered in 1996 to 1997, the time of their first TMAO measure. BMI indicates body mass index; CHS, Cardiovascular Health Study; CRP, C-reactive protein; HDL, high-density lipoprotein; IQR, interquartile range (25th–75th percentile); LDL, low-density lipoprotein; and TMAO, trimethylamine N-oxide.

*For continuous variables, independent *t*-test was performed; and for TMAO and CRP, Mann–Whitney *U* test was performed because of the distribution of data. For indicator variables, the χ^2 test was performed.

[†]Estimated glomerular filtration rate (mL/min per 1.73 m^2) is calculated on the basis of the Chronic Kidney Disease Epidemiology Collaboration creatinine–cystatin C equation.³⁹

[‡]Total animal source food is the sum of intakes of unprocessed red meat, processed meat, fish, chicken, and eggs.

with higher incidence of ASCVD among individuals with impaired renal function (eGFR <60 mL/min per 1.73 m²) (extreme-quintile HR [95% CI]: 1.56 [1.13–2.14]; *P*-trend=0.007; Table 2), but not with normal or mildly reduced renal function (eGFR ≥60 mL/min per 1.73 m²) (HR [95% CI], 1.03 [0.85–1.25]; *P*-trend=0.668). This interaction was statistically significant (*P*-interaction <0.001). Further adjustment for eGFR attenuated the association in those with impaired renal function (HR [95% CI], 1.37 [0.98–1.90]; *P*-trend=0.084). There was little

evidence for differential associations between TMAO and incident ASCVD by age, sex, or BMI (Table S3). There was also little evidence for nonlinear associations between log-transformed TMAO and ASCVD (Figure).

Plasma TMAO and Risk of Recurrent ASCVD

Among 1449 individuals with prevalent cardiovascular disease, a total of 897 recurrent ASCVD events

Table 2. Risk of Incident ASCVD Associated With Long-Term Levels of Plasma TMAO Among 4131 Older Adults in the CHS, Stratified by Renal Function

Variable	Quintiles of plasma TMAO					<i>P</i> -trend
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	
Overall individuals (n=4131)						
Median TMAO, μmol/L	2.3	3.5	4.7	6.8	13.2	
Cases/total	251/826	324/827	408/825	426/827	357/826	
Person-years	9587	11 217	11 589	12 092	9963	
Age, sex adjusted	1.00 (Reference)	1.03 (0.87–1.21)	1.20 (1.03–1.41)	1.18 (1.01–1.38)	1.22 (1.04–1.44)	0.015
Multivariable*	1.00 (Reference)	1.01 (0.86–1.19)	1.21 (1.03–1.42)	1.14 (0.97–1.33)	1.21 (1.02–1.42)	0.029
Multivariable and diet [†]	1.00 (Reference)	1.01 (0.86–1.19)	1.20 (1.03–1.41)	1.13 (0.96–1.32)	1.21 (1.02–1.43)	0.028
Multivariable, diet, and continuous eGFR (confounder or mediator) [‡]	1.00 (Reference)	0.97 (0.82–1.15)	1.13 (0.96–1.33)	1.03 (0.88–1.22)	1.07 (0.90–1.27)	0.516
Individuals with eGFR <60 mL/min per 1.73 m ² (n=1104)						
Median TMAO, μmol/L	3.1	4.7	6.4	9.2	17.7	
Cases/total	67/220	99/221	109/221	103/222	115/220	
Person-years	2124	2485	2336	2308	2135	
Age, sex adjusted	1.00 (Reference)	1.12 (0.82–1.53)	1.34 (0.99–1.82)	1.27 (0.93–1.72)	1.57 (1.16–2.13)	0.003
Multivariable*	1.00 (Reference)	1.16 (0.84–1.59)	1.30 (0.95–1.77)	1.25 (0.91–1.71)	1.56 (1.13–2.14)	0.007
Multivariable and diet [†]	1.00 (Reference)	1.15 (0.84–1.58)	1.27 (0.93–1.75)	1.24 (0.90–1.70)	1.53 (1.11–2.10)	0.011
Multivariable, diet, and continuous eGFR (confounder or mediator) [‡]	1.00 (Reference)	1.12 (0.82–1.55)	1.22 (0.89–1.68)	1.14 (0.83–1.58)	1.37 (0.98–1.90)	0.084
Individuals with eGFR ≥60 mL/min per 1.73 m ² (n=3027)						
Median TMAO, μmol/L	2.1	3.2	4.2	5.9	11.5	
Cases/total	181/604	233/607	296/606	307/605	256/605	
Person-years	6804	8586	9326	9659	8684	
Age, sex adjusted	1.00 (Reference)	0.92 (0.76–1.12)	1.02 (0.85–1.23)	1.02 (0.85–1.22)	0.96 (0.79–1.16)	0.836
Multivariable*	1.00 (Reference)	0.95 (0.78–1.15)	1.08 (0.89–1.30)	1.05 (0.87–1.27)	1.03 (0.85–1.25)	0.668
Multivariable and diet [†]	1.00 (Reference)	0.95 (0.78–1.16)	1.08 (0.90–1.30)	1.05 (0.87–1.26)	1.03 (0.84–1.25)	0.733
Multivariable, diet, and continuous eGFR (confounder or mediator) [‡]	1.00 (Reference)	0.94 (0.77–1.14)	1.05 (0.87–1.27)	1.01 (0.84–1.23)	0.98 (0.80–1.20)	0.903

ASCVD indicates atherosclerotic cardiovascular disease; CHS, Cardiovascular Health Study; eGFR, estimated glomerular filtration rate; and TMAO, trimethylamine N-oxide.

*Multivariable adjustments include sex (men/women), race (White/black people), study site (Bowman Gray, Davis, Hopkins, or Pittsburgh), education (<high school, high school, some college, or college graduate), income (<\$11 999, \$12 000–\$24 999, \$25 000–\$49 999, or >\$50 000/y), and time-varying risk factors, including age (65–74, 75–84, or ≥85 years), self-reported health status (excellent, very good, or good/fair/poor), smoking status (never, former, or current), alcohol intake (0, <1, 1–2.49, 2.5–7.49, 7.5–14.49, or >14.5 drinks/wk), physical activity (<500, 500–1000, 1000–1500, or >1500 kcal/wk), body mass index (<18.5, 18.5–25, 25.1–30, or >30 kg/m²), waist circumference (cm), lipid-lowering medication (yes/no), antihypertensive medication (yes/no), antibiotics (yes/no), prevalent diabetes mellitus (yes/no), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), CRP (C-reactive protein) (mg/L), systolic blood pressure (mm Hg), and diastolic blood pressure (mm Hg).

[†]Diet adjustments include fruits (servings/d), vegetables (servings/d), fiber (g/d), and total animal source food (servings/wk).

[‡]Further adjusted for eGFR (mL/min/1.73 m²), given experimental evidence that TMAO causes renal dysfunction and reduced clearance. eGFR could represent a confounder or mediator (ie, in the causal pathway of effect) for the association between TMAO and ASCVD.

occurred during 14 118 person-years of follow-up. After multivariable adjustment, plasma TMAO levels were associated with higher recurrence of ASCVD, with an extreme-quintile HR of 1.25 (95% CI, 1.01–1.56; *P*-trend=0.009; Table 3). Further adjustment for dietary factors did not appreciably alter these results. As seen for incident ASCVD, further adjustment for eGFR as a mediator or confounder attenuated the association (HR [95% CI], 1.10 [0.87–1.39]; *P*-trend=0.179). No statistically significant effect modification was identified for the relationship between plasma TMAO and recurrent ASCVD by differences in eGFR (*P*-interaction=0.021).

Sensitivity Analyses

Findings were similar excluding participants who had taken antibiotics in the past 2 weeks before the study entry, excluding early events in the first 2 years of follow-up, excluding individuals with poor self-reported general health, and using simple updated (recently measured) rather than cumulative updated TMAO levels (Tables S6 and S7).

DISCUSSION

In this large, community-based prospective cohort of older US adults, habitual levels of plasma TMAO were

positively associated with incident ASCVD. This relationship was modified by renal function, with significantly higher risk only among individuals with impaired renal function (eGFR <60 mL/min per 1.73 m²), but not among those with normal or mildly reduced renal function (eGFR ≥60 mL/min per 1.73 m²). Plasma TMAO was also associated with higher risk of recurrent ASCVD. Renal function also appeared to be an important mediator or confounder of these relationships. To our knowledge, this is the first community-based study to prospectively assess the associations of habitual levels of TMAO, a gut microbiota metabolite of certain animal source foods, with onset of ASCVD; and the first study to focus on older adults, the general population at highest risk.

TMAO is an intriguing molecule representing the interplay between diet, the microbiome, and endogenous metabolism. In humans, intestinal microbiota metabolize choline, phosphatidylcholine, and carnitine into trimethylamine, which is further oxidized by the liver to TMAO by hepatic flavin-containing monooxygenase isoform 3.⁴² Endogenously produced γ -butyrobetaine and crotonobetaine, gut microbiota metabolites of L-carnitine, are converted to trimethylamine and further oxidized to TMAO.^{3,43} Mechanistic studies support proatherosclerotic effects of plasma TMAO, including experimental accumulation of cholesterol in macrophages and foam cell formation in artery walls by

Table 3. Risk of Recurrent ASCVD Associated With Long-Term Levels of Plasma TMAO in the CHS Among 1449 Older Adults With Prevalent CVD at Baseline

Variable	Quintiles of plasma TMAO					<i>P</i> -trend
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	
Median TMAO, μ mol/L	2.4	3.7	5.3	7.5	14.5	
Cases/total	156/291	178/288	181/290	191/290	191/290	
Person-years	2767	2999	2998	2810	2544	
Age, sex adjusted	1.00 (Reference)	0.98 (0.79–1.22)	0.99 (0.80–1.23)	1.09 (0.88–1.34)	1.23 (1.00–1.53)	0.012
Multivariable*	1.00 (Reference)	0.98 (0.79–1.22)	0.98 (0.79–1.22)	1.07 (0.86–1.33)	1.25 (1.01–1.56)	0.009
Multivariable and diet [†]	1.00 (Reference)	0.99 (0.80–1.23)	0.99 (0.79–1.23)	1.09 (0.88–1.36)	1.26 (1.01–1.57)	0.008
Multivariable, diet, and continuous eGFR (confounder or mediator) [‡]	1.00 (Reference)	0.95 (0.76–1.18)	0.93 (0.74–1.16)	0.98 (0.78–1.23)	1.10 (0.87–1.39)	0.179

Included individuals with prevalent CVD, including angina or coronary revascularization, at baseline (n=522) and those who had a new onset of CVD (new angina, coronary revascularization, nonfatal myocardial infarction, or nonfatal stroke) between baseline and 1996 to 1997 and who entered the analysis in 1996 to 1997 (n=927). Individuals were followed up for recurrent ASCVD (myocardial infarction, fatal coronary heart disease, stroke, sudden cardiac death, or other atherosclerotic death), whichever occurred first. ASCVD indicates atherosclerotic CVD; CHS, Cardiovascular Health Study; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; and TMAO, trimethylamine N-oxide.

*Multivariable adjustments include sex (male/female), race (White/black people), study site (Bowman Gray, Davis, Hopkins, or Pittsburgh), education (<high school, high school, some college, or college graduate), annual income (<\$11 999, \$12 000–\$24 999, \$25 000–\$49 999, or >\$50 000), and time-varying risk factors, including age (65–74, 75–84, or ≥85 years), self-reported health status (excellent, very good, or good/fair/poor), smoking status (never, former, or current), alcohol intake (0, <1, 1–2.49, 2.5–7.49, 7.5–14.49, or >14.5 drinks/wk), physical activity (<500, 500–1000, 1000–1500, or >1500 kcal/wk), body mass index (<18.5, 18.5–25, 25.1–30, or >30 kg/m²), waist circumference (cm), lipid-lowering medication (yes/no), antihypertensive medication (yes/no), antibiotics (yes/no), prevalent diabetes mellitus (yes/no), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), CRP (C-reactive protein) (mg/L), systolic blood pressure (mm Hg), and diastolic blood pressure (mm Hg).

[†]Diet adjustments include fruits (servings/d), vegetables (servings/d), fiber (g/d), and total animal source food (servings/wk).

[‡]Further adjusted for eGFR (mL/min per 1.73 m²), given experimental evidence that TMAO causes renal dysfunction and reduced clearance. eGFR could represent a confounder or mediator (ie, in the causal pathway of effect) for the association between TMAO and ASCVD.

increasing cell surface expression of proatherogenic scavenger receptors (cluster of differentiation 36 and scavenger receptor A)^{1,44,45}; reduced reverse cholesterol efflux and bile acid biosynthesis⁶; increased vascular inflammation via signaling of mitogen-activated protein kinase and nuclear factor- κ B^{8,46}; and enhanced platelet reactivity and thrombosis via increased Ca^{2+} release from intracellular stores.⁷ Together, these processes could collectively influence the development, incidence, and recurrence of clinical ASCVD.

In addition, plasma TMAO appears influenced by and to directly affect renal function. Experimentally, elevated plasma TMAO induces renal fibrosis, reduces renal filtration, and increases plasma cystatin C levels.⁵ In a circular manner, impaired kidney function leads to higher levels of plasma TMAO and potentially greater atherosclerotic effects,^{5,47} supported by findings from our present and prior observational studies^{22,48} that plasma TMAO inversely associates with glomerular filtration rate ($r=-0.62$ to -0.31). The design of our investigation cannot disentangle the extent to which eGFR is a mediator or confounder of the association between plasma TMAO levels and incident or recurrent ASCVD. For example, it is possible that normal renal filtration of TMAO helps protect against adverse effects of its dietary-microbiome production, clearing this molecule from the plasma before it can cause clinically relevant harm. Conversely, when renal function is reduced, higher plasma TMAO is not rapidly cleared, leading to increasing levels that both damage the cardiovascular system and reduce kidney function over time. Our

novel results support the need for further investigation of the potential interplay between diet, the microbiome, renal function, and ASCVD in experimental and interventional models.

Our finding of a positive association between TMAO and recurrent ASCVD in this community-based study is consistent with prior observational studies enrolling patients with preexisting cardiovascular disease seen at tertiary medical centers.^{49–51} Mechanistic studies suggest that several pathways of risk for TMAO may be especially problematic among individuals with preexisting vascular disease. This includes, for example, induction by TMAO of platelet hyperresponsiveness and propensity for thrombosis *in vivo*^{7,52,53} and promotion of vascular inflammation and upregulation of adhesion molecules in the artery wall.^{8,54} Such effects would be most problematic in the presence of preexisting plaque and vascular disease (and distinct from other pathways that might promote early onset of atherosclerosis). In our investigation, risk was most evident among older adults with modestly reduced eGFR, and among those with preexisting ASCVD (independent of eGFR). These groups represent individuals with clinically evident vascular disease. Our results highlight the need for further experimental assessment of pathways of risk for TMAO among individuals with and without vascular disease.

Prior observational studies of TMAO and ASCVD events generally assessed referred patients with significant underlying health conditions (eg, coronary artery disease, heart failure, diabetes mellitus, and stroke) seen at tertiary medical centers.^{17,49–51} Such patients would

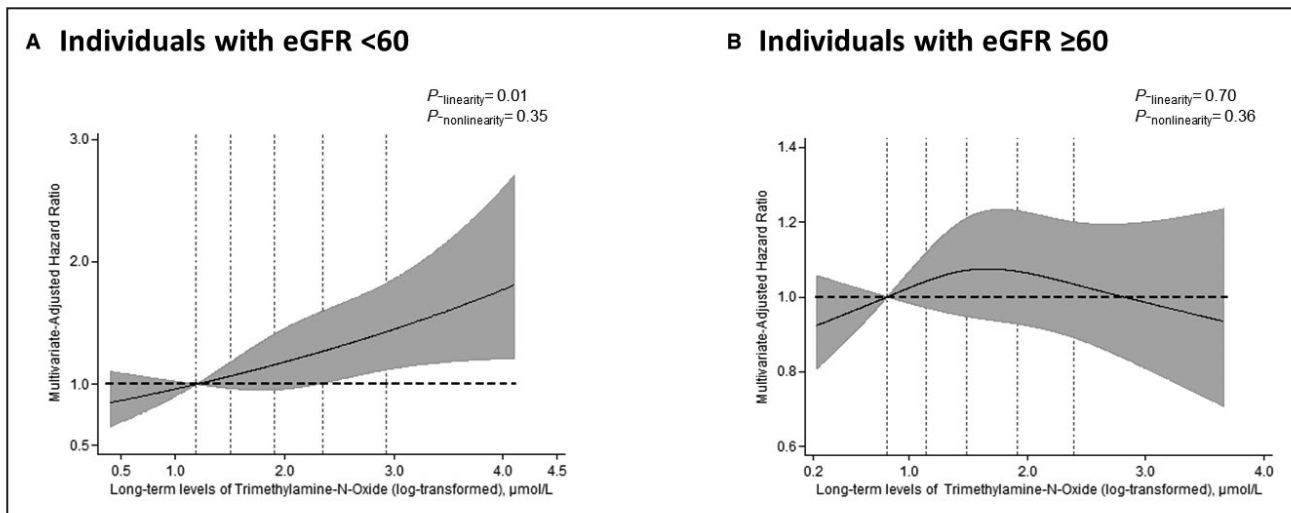


Figure. Multivariable-adjusted relationship of long-term levels of log-transformed trimethylamine N-oxide with risk of atherosclerotic cardiovascular disease by renal function, with estimated glomerular filtration rate (eGFR) <60 mL/min per 1.73 m^2 (A) or eGFR ≥ 60 mL/min per 1.73 m^2 (B).

The solid lines and shaded area represent the central risk estimate and 95% CI, respectively. The dotted vertical lines correspond to the 10th, 25th, 50th, 75th, and 90th percentiles. The top and bottom 1% of participants were omitted as outliers to provide better visualization. *P* values for linear associations are presented. Evidence for nonlinearity was calculated by performing a likelihood ratio test between a multivariable model with all spline terms vs a multivariable model with only the linear term. Significant linearity was found in individuals with eGFR <60 mL/min per 1.73 m^2 (P -linearity=0.01). Estimates were adjusted for potential confounders (see the footnote in Table 2).

generally have lower eGFR and higher prevalent burdens of atherosclerosis, more similar to the participants in our study followed up for recurrent ASCVD. In 2 prior studies focused on patients with significantly impaired renal function (eg, stage 3b and 4 chronic kidney disease),^{16,55} positive associations between a single baseline measure of TMAO and risk of all-cause mortality or ischemic events were attenuated and no longer statistically significant after adjustment for eGFR. Our findings build on and extend these prior results by documenting a significant association between plasma TMAO and recurrent ASCVD, and among those with lower eGFR and incident ASCVD in a community-based cohort. Our findings also suggest that healthier older adults with lower underlying burdens of atherosclerosis and renal dysfunction may not be similarly affected by plasma TMAO. A recent study among middle-aged female US nurses found that changes (increases) in TMAO levels over time were associated with a higher risk of subsequent coronary heart disease.¹⁴ This study did not report the association of baseline or habitual TMAO levels with coronary heart disease, nor present data on renal function. It seems possible that increasing TMAO levels in this study of nurses could represent, at least in part, worsening eGFR over time, given the interlinkages between plasma TMAO, renal function, and ASCVD seen in our investigation.

We documented a high within-individual variability in plasma TMAO levels over 7 years ($\rho=0.254$), consistent with a prior small study among 100 German subjects over 1 year.⁵⁶ This raises the importance of using serial TMAO measures over time to facilitate the investigation of habitual or usual TMAO levels and health outcomes. TMAO levels are influenced by several factors, including consumption of animal products, abundance of trimethylamine-producing bacteria in the gut, endogenous production of γ -butyrobetaine and crotonobetaine, enhanced hepatic flavin-containing monooxygenase isoform 3 enzyme activity, and impaired renal function.⁵⁷ Each of these factors also represents potential lifestyle and therapeutic targets to be evaluated in interventions to reduce the TMAO-associated risk of ASCVD.

Our investigation has several strengths. We used repeated measures of plasma TMAO, reducing misclassification attributable to changes over time. The community-based recruitment increases generalizability, whereas low loss to follow-up reduces potential for selection bias. The large sample size and extended follow-up provided a large number of events and statistical power to detect relevant associations. Information on a wide array of ASCVD risk factors, laboratory measures, demographic characteristics, and lifestyle habits was prospectively measured using standardized methods,^{19,20,37} facilitating multivariable adjustment to reduce confounding. Both creatinine and cystatin C

were measured, allowing direct calculation of eGFR. ASCVD outcomes were identified and confirmed by a comprehensive review and centralized adjudication process, reducing the potential for missed or misclassified outcomes.

Potential limitations should be considered. Our study population included older adults, mostly White race; thus, our findings may not be generalizable to younger populations or other races. Because of the observational nature of our study, residual confounding by unknown or unmeasured factors may be present. However, our results were robust to adjustment for multiple risk factors for ASCVD as well as demographics and dietary and other lifestyle habits. Data on factors associated with TMAO production, such as composition of gut microbiota and hepatic flavin-containing monooxygenase isoform 3 activity, were not collected, which should be considered in future studies. Participants with nonfatal MI or nonfatal stroke at baseline were not followed up for recurrent nonfatal MI or stroke in CHS, which may influence the generalizability of the study result. About 20% of participants had missing TMAO levels at the second time point, for whom baseline measurements were carried forward. Although this may cause some misclassification of TMAO levels during follow-up, 80% of participants did have serial measures, and our investigation represents by far the largest prospective study using serial measurements of TMAO over time to assess risk of ASCVD.

In conclusion, in this large, community-based cohort of older US adults, serial measures of TMAO were associated with higher risk of incident ASCVD, with apparent modification by presence of impaired renal function; and with higher risk of recurrent ASCVD. Our findings highlight the need for further experimental and interventional studies to elucidate the interplay between TMAO, renal function, and incident and recurrent ASCVD.

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Received December 21, 2020; accepted June 15, 2021.

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Sources of Funding

The authors received support from the National Institutes of Health grants R01HL135920, P01 HL147823, and R01 HL103866 and a Leducq Foundation Award. CHS was supported by contracts HHSN268201200036C, HHSN268200800007C, HHSN268201800001C, N01HC55222, N01HC85079, N01HC85080, N01HC85081, N01HC85082, N01HC85083, N01HC85086, and grants U01HL080295 and U01HL130114 from the National Heart, Lung, and Blood Institute, with additional contribution from the National Institute of Neurological Disorders and Stroke. Additional support was provided by R01AG023629 from the National Institute on Aging. A full list of principal CHS investigators and institutions can be found at CHS-NHLBI.org. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosures

Dr Mozaffarian reports research funding from the National Institutes of Health, the Gates Foundation, and the Rockefeller Foundation; personal fees from GOED, Bunge, Indigo Agriculture, Motif FoodWorks, Amarin, Acasti Pharma, Cleveland Clinic Foundation, America's Test Kitchen, and Danone; participating on scientific advisory boards of start-up companies focused on innovations for health, including Brightseed, DayTwo, Elysium Health, Filtricine, Foodome, HumanCo, and Tiny Organics; and chapter royalties from UpToDate. Drs Hazen and Wang report being named as coinventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. Dr Hazen also reports being a paid consultant for P&G and having received research funds from P&G and Roche Diagnostics; both Drs Hazen and Wang report being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland HeartLab (a subsidiary of Quest Diagnostics) and P&G. Dr Psaty serves on the Steering Committee of the Yale Open Data Access Project funded by Johnson & Johnson.

Supplementary Material

Tables S1–S7

Figure S1

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SUPPLEMENTAL MATERIAL

Table S1. Baseline characteristics of US men and women in the Cardiovascular Health Study by quintiles of trimethylamine N-oxide*

	Trimethylamine N-oxide					p-value
	Q1 (n=826)	Q2 (n=827)	Q3 (n=825)	Q4 (n=827)	Q5 (n=826)	
Median TMAO (IQR), $\mu\text{mol/L}$	2.29 (1.84, 2.61)	3.49 (3.19, 3.77)	4.72 (4.40, 5.14)	6.77 (6.16, 7.69)	13.2 (10.4, 19.9)	
Demographics						
Age, (SD) years	71.7 (5.0)	71.7 (4.9)	72.3 (5.5)	72.4 (5.3)	72.8 (5.6)	<0.001
Female, n (%)	584 (70.7)	541 (65.4)	511 (61.9)	492 (59.5)	495 (59.9)	<0.001
Race, n (%)						0.001
White	661 (80.0)	686 (83.0)	698 (84.6)	704 (85.1)	721 (87.3)	
Education, n (%)						0.884
<High school	223 (27.0)	227 (27.4)	229 (27.8)	235 (28.4)	207 (25.1)	
High school	245 (29.7)	240 (29.0)	237 (28.7)	233 (28.2)	234 (28.3)	
Some college	188 (22.8)	202 (24.4)	180 (21.8)	186 (22.5)	203 (24.6)	
College graduate	170 (20.6)	158 (19.1)	179 (21.7)	173 (20.9)	182 (22.0)	
Income group, n (%)						0.032
<\$11,999	224 (27.1)	193 (23.3)	190 (23.0)	198 (23.9)	191 (23.1)	
\$12,000-\$24,999	275 (33.3)	315 (38.1)	295 (35.8)	307 (37.1)	294 (35.6)	
\$25,000-\$49,999	226 (27.4)	221 (26.7)	244 (29.6)	212 (25.6)	203 (24.6)	
>\$50,000	101 (12.2)	98 (11.9)	96 (11.6)	110 (13.3)	138 (16.7)	
Lifestyle and risk factors						
Self-reported health status, n (%)						0.121
Excellent/ Very good	356 (43.1)	355 (42.9)	361 (43.8)	345 (41.7)	334 (40.4)	
Good	303 (36.7)	317 (38.3)	326 (39.5)	317 (38.3)	299 (36.2)	
Fair/poor	167 (20.2)	155 (18.7)	138 (16.7)	165 (20.0)	193 (23.4)	
Smoking, n (%)						0.002
Never smoked	447 (54.1)	387 (46.8)	396 (48.0)	362 (43.7)	377 (45.6)	
Former smoker	282 (34.1)	326 (39.4)	336 (40.7)	356 (43.0)	349 (42.3)	
Current smoker	97 (11.7)	114 (13.8)	93 (11.3)	109 (13.2)	100 (12.1)	
Physical activity, (SD) kcal	1,181 (1,599)	1,136 (1,480)	1,162 (1,555)	1,122 (1,537)	1,147 (1,493)	0.947
Alcohol, (SD) drinks/wk	2.2 (6.2)	2.8 (7.3)	2.2 (5.6)	2.9 (7.0)	2.9 (9.7)	0.091
BMI, (SD) kg/m^2	26.2 (4.8)	26.7 (4.9)	26.9 (4.6)	27.2 (4.7)	26.6 (5.0)	<0.001

	Trimethylamine N-oxide					
	Q1 (n=826)	Q2 (n=827)	Q3 (n=825)	Q4 (n=827)	Q5 (n=826)	p-value
Waist circumference, (SD) cm	92.4 (13.7)	93.4 (13.3)	94.6 (13.2)	95.7 (13.4)	94.4 (13.8)	<0.001
Systolic blood pressure, (SD) mmHg	137 (21)	136 (22)	135 (21)	137 (22)	135 (21)	0.070
Diastolic blood pressure, (SD) mmHg	72 (11)	72 (11)	71 (11)	72 (11)	70 (11)	0.004
Biochemical, (SD)						
HDL cholesterol, mg/dL	57.8 (15.9)	56.7 (16.0)	55.0 (15.1)	54.5 (15.7)	55.0 (15.8)	<0.001
LDL cholesterol, mg/dL	131 (36)	130 (34)	130 (36)	128 (33)	128 (36)	0.333
Triglycerides, mg/dL	130 (63)	134 (66)	136 (70)	145 (85)	139 (75)	<0.001
C-reactive protein, mg/L	4.3 (7.2)	4.6 (8.4)	4.5 (6.7)	4.8 (8.7)	4.6 (7.2)	0.690
Estimated glomerular filtration rate, mL/min/1.73m ²	77.3 (14.0)	73.5 (14.1)	70.0 (15.1)	65.6 (16.1)	62.4 (18.4)	<0.001
Medical history, n (%)						
Prevalent diabetes	84 (10.2)	85 (10.3)	101 (12.2)	95 (11.5)	85 (10.3)	0.570
Lipid lowering medication	35 (4.2)	36 (4.4)	39 (4.7)	52 (6.3)	43 (5.2)	0.303
Hypertension medication	282 (34.1)	307 (37.1)	331 (40.1)	376 (45.5)	386 (46.7)	<0.001
Antibiotics in past 2 weeks	22 (2.7)	26 (3.1)	21 (2.5)	22 (2.7)	25 (3.0)	0.935
Dietary habits, (SD)						
Fruits, servings/d	2.2 (1.0)	2.1 (1.0)	2.2 (1.0)	2.1 (1.1)	2.2 (1.0)	0.623
Vegetables, servings/d	2.6 (1.3)	2.5 (1.3)	2.6 (1.4)	2.5 (1.4)	2.6 (1.4)	0.475
Fiber, g/d	29.6 (11.8)	30.0 (11.8)	29.4 (11.8)	28.6 (11.6)	28.7 (11.9)	0.074
Total animal product, servings/d	1.65 (0.92)	1.67 (0.96)	1.71 (1.02)	1.79 (0.96)	1.77 (1.00)	0.012

* Values reported as mean (SD) for continuous variables, and frequency, percentage (%) for categorical variables, unless otherwise stated. 3,877 participants entered the study at baseline (1989-90 or 1992-93) and 254 participants at 1996-97. IQR= interquartile range (25th, 75th)

Table S2. Spearman correlation coefficient for repeated within-individual measures of trimethylamine-N-oxide over two time points*

	TMAO measured at baseline (1989-90 or 1992-93)
TMAO measured in 1996-97	0.254

*2,470 participants who had two TMAO measures at baseline (1989-90 or 1992-93) and 1996-97.

Table S3. Trimethylamine N-oxide and the risk of atherosclerotic cardiovascular disease in the Cardiovascular Health Study: analysis of potential interaction by age, sex, body mass index, and estimated glomerular filtration rate with respective stratified analyses with Bonferroni correction (significance<0.01)*

(Cases/Total population)	TMAO	
	HR (95% CI)	P-value
Age		
≤71 years (912/2,237)	1.08 (0.98, 1.18)	0.03
>71 years (854/1,894)	1.04 (0.97, 1.12)	
Sex		
Female (1,108/2,623)	1.06 (0.99, 1.14)	0.74
Male (658/1,508)	1.04 (0.95, 1.14)	
BMI		
<25kg/m ² (645/1,597)	1.06 (0.98, 1.15)	0.50
25-29.9 kg/m ² (754/1,708)	1.06 (0.97, 1.16)	
≥30 kg/m ² (367/826)	1.04 (0.88, 1.22)	
eGFR		
≤60 mL/min/1.73m ² (471/1,058)	1.09 (1.02, 1.17)	<0.001
>60 mL/min/1.73m ² (1,295/3,073)	0.97 (0.88, 1.06)	

Multivariable adjustments include age (65-74y, 75-84y, 85y+), sex (male, female), race (white, non-white), study site (Bowman Gray, Davis, Hopkins, Pittsburgh), education (<high school, high school, some college, college graduate), income (<\$11,999, \$12,000-\$24,999, \$25,000-\$49,999, >\$50,000/y), self-reported health status (excellent, very good, good/fair/poor), smoking status (never, former, current), alcohol intake (0, <1, 1-2.49, 2.5-7.49, 7.5-14.49, >14.5 drinks/wk), physical activity (<500, 500-1000, 1000-1500, >1500 kcal/wk), BMI (<18.5, 18.5-25, 25.1-30, >30 kg/m²), waist circumference (cm), lipid lowering medication (yes/no), anti-hypertensive medication (yes/no), antibiotics (yes/no), prevalent diabetes (yes/no), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), C-reactive protein (mg/L), systolic blood pressure (mmHg), diastolic blood pressure (mmHg)

*P-value obtained from continuous interaction term for age, BMI, and eGFR, and categorical interaction term for sex (females as reference).

Table S4. Relative risk of atherosclerotic cardiovascular disease (n=1,739) associated with long-term levels of plasma trimethylamine among 4,066 older men and women in the Cardiovascular Health Study, excluding participants who had taken antibiotics in the 2 weeks prior to study entry (n=65).

	Trimethylamine N-oxide quintiles					p-trend
	Q1	Q2	Q3	Q4	Q5	
Median TMAO, $\mu\text{mol/L}$	2.3	3.5	4.7	6.8	13.1	
Cases/Total	246/814	323/812	397/814	423/812	350/814	
Person-years	9,560	11,123	11,249	11,846	9,846	
Age, sex adjusted	1.00 (Reference)	1.05 (0.89-1.24)	1.24 (1.05-1.45)	1.21 (1.04-1.42)	1.24 (1.06-1.47)	0.014
Multivariable [†]	1.00 (Reference)	1.04 (0.88-1.23)	1.24 (1.06-1.46)	1.17 (1.00-1.38)	1.23 (1.04-1.45)	0.028
Multivariable and diet [‡]	1.00 (Reference)	1.05 (0.89-1.24)	1.24 (1.05-1.45)	1.17 (0.99-1.37)	1.22 (1.04-1.45)	0.035
Multivariable, diet, and renal function [§]	1.00 (Reference)	1.01 (0.86-1.20)	1.16 (0.99-1.37)	1.07 (0.91-1.26)	1.08 (0.91-1.29)	0.579

[†]Multivariable adjustments include age (65-74y, 75-84y, 85y+), sex (male, female), race (white, non-white), study site (Bowman Gray, Davis, Hopkins, Pittsburgh), education (<high school, high school, some college, college graduate), income (<\$11,999, \$12,000-\$24,999, \$25,000-\$49,999, >\$50,000/y), self-reported health status (excellent, very good, good/fair/poor), smoking status (never, former, current), alcohol intake (0, <1, 1-2.49, 2.5-7.49, 7.5-14.49, >14.5 drinks/wk), physical activity (<500, 500-1000, 1000-1500, >1500 kcal/wk), BMI (<18.5, 18.5-25, 25.1-30, >30 kg/m²), waist circumference (cm), lipid lowering medication (yes/no), anti-hypertensive medication (yes/no), antibiotics (yes/no), prevalent diabetes (yes/no), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), C-reactive protein (mg/L), systolic blood pressure (mmHg), diastolic blood pressure (mmHg)

[‡]Diet adjustments include fruits (servings/d), vegetables (servings/d), fiber (g/d), and total animal source food (servings/week).

[§]Estimated glomerular filtration rate (mL/min/1.73m²) is calculated based on the CKD-EPI creatinine-cystatin C equation.³⁹

Table S5. Sensitivity analyses for the associations between long-term levels of plasma trimethylamine among 4,031 older men and women in the Cardiovascular Health Study excluding events within the first 2 years of follow-up*

	Trimethylamine N-oxide quintiles					p-trend
	Q1	Q2	Q3	Q4	Q5	
Median TMAO, $\mu\text{mol/L}$	2.3	3.5	4.7	6.7	13.2	
Cases/Total	238/807	307/805	389/806	395/807	337/806	
Person-years	9,443	11,157	11,613	12,192	9,942	
Age, sex adjusted	1.00 (Reference)	1.00 (0.84-1.18)	1.16 (0.99-1.37)	1.10 (0.94-1.29)	1.19 (1.01-1.41)	0.032
Multivariable [†]	1.00 (Reference)	0.98 (0.83-1.16)	1.15 (0.98-1.36)	1.05 (0.89-1.24)	1.17 (0.99-1.39)	0.053
Multivariable and diet [‡]	1.00 (Reference)	0.98 (0.82-1.16)	1.15 (0.98-1.36)	1.04 (0.89-1.23)	1.17 (0.99-1.39)	0.049
Multivariable, diet, and renal function [§]	1.00 (Reference)	0.95 (0.80-1.12)	1.08 (0.92-1.28)	0.96 (0.81-1.13)	1.04 (0.87-1.25)	0.576

*100 ASCVD events occurred within the first 2 years of follow-up to minimize reverse causation (pre-existing subclinical disease leading to changes in levels of TMAO).

[†]Multivariable adjustments include age (65-74y, 75-84y, 85y+), sex (male, female), race (white, non-white), study site (Bowman Gray, Davis, Hopkins, Pittsburgh), education (<high school, high school, some college, college graduate), income (<\$11,999, \$12,000-\$24,999, \$25,000-\$49,999, >\$50,000/y), self-reported health status (excellent, very good, good/fair/poor), smoking status (never, former, current), alcohol intake (0, <1, 1-2.49, 2.5-7.49, 7.5-14.49, >14.5 drinks/wk), physical activity (<500, 500-1000, 1000-1500, >1500 kcal/wk), BMI (<18.5, 18.5-25, 25.1-30, >30 kg/m²), waist circumference (cm), lipid lowering medication (yes/no), anti-hypertensive medication (yes/no), antibiotics (yes/no), prevalent diabetes (yes/no), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), C-reactive protein (mg/L), systolic blood pressure (mmHg), diastolic blood pressure (mmHg)

[‡]Diet adjustments include fruits (servings/d), vegetables (servings/d), fiber (g/d), and total animal source food (servings/week).

[§]Estimated glomerular filtration rate (mL/min/1.73m²) is calculated based on the CKD-EPI creatinine-cystatin C equation.³⁹

Table S6. Sensitivity analyses for the associations between long-term levels of plasma trimethylamine among 4,055 older men and women in the Cardiovascular Health Study excluding those who reported poor self-reported health at study entry*

	Trimethylamine N-oxide quintiles					p-trend
	Q1	Q2	Q3	Q4	Q5	
Median TMAO, $\mu\text{mol/L}$	2.3	3.5	4.7	6.8	13.2	
Cases/Total	249/811	309/810	404/811	418/812	351/811	
Person-years	9,483	11,140	11,406	11,903	9,937	
Age, sex adjusted	1.00 (Reference)	0.99 (0.84-1.17)	1.21 (1.03-1.42)	1.17 (1.00-1.37)	1.21 (1.03-1.42)	0.016
Multivariable [†]	1.00 (Reference)	0.97 (0.82-1.15)	1.22 (1.04-1.43)	1.14 (0.97-1.33)	1.19 (1.01-1.41)	0.032
Multivariable and diet [‡]	1.00 (Reference)	0.97 (0.82-1.15)	1.21 (1.04-1.43)	1.13 (0.96-1.33)	1.19 (1.01-1.41)	0.031
Multivariable, diet, and renal function [§]	1.00 (Reference)	0.94 (0.79-1.11)	1.15 (0.98-1.35)	1.04 (0.88-1.22)	1.06 (0.89-1.26)	0.510

* Excluded 61 individuals who self-reported poor health at baseline (1989-90 or 1992-93) and 15 individuals who entered in 1996-97 and who self-reported poor health.

[†] Multivariable adjustments include age (65-74y, 75-84y, 85y+), sex (male, female), race (white, non-white), study site (Bowman Gray, Davis, Hopkins, Pittsburgh), education (<high school, high school, some college, college graduate), income (<\$11,999, \$12,000-\$24,999, \$25,000-\$49,999, >\$50,000/y), self-reported health status (excellent, very good, good/fair/poor), smoking status (never, former, current), alcohol intake (0, <1, 1-2.49, 2.5-7.49, 7.5-14.49, >14.5 drinks/wk), physical activity (<500, 500-1000, 1000-1500, >1500 kcal/wk), BMI (<18.5, 18.5-25, 25.1-30, >30 kg/m²), waist circumference (cm), lipid lowering medication (yes/no), anti-hypertensive medication (yes/no), antibiotics (yes/no), prevalent diabetes (yes/no), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), C-reactive protein (mg/L), systolic blood pressure (mmHg), and diastolic blood pressure (mmHg)

[‡] Diet adjustments include fruits (servings/d), vegetables (servings/d), fiber (g/d), and total animal source food (servings/week).

[§] Estimated glomerular filtration rate (mL/min/1.73m²) is calculated based on the CKD-EPI creatinine-cystatin C equation.³⁹

Table S7. Relative risk of atherosclerotic cardiovascular disease associated with levels of plasma trimethylamine among 4,131 older men and women in the Cardiovascular Health Study (simple updating approach)*

	Trimethylamine N-oxide quintiles					p-trend
	Q1	Q2	Q3	Q4	Q5	
Overall individuals (n=4,131)						
Median TMAO, $\mu\text{mol/L}$	2.3	3.5	4.7	6.8	13.1	
Cases/Total	280/827	280/826	309/826	370/826	527/826	
Person-years	10,306	10,141	10,402	10,412	13,186	
Age, sex adjusted	1.00 (Reference)	1.00 (0.85-1.18)	1.03 (0.87-1.21)	1.22 (1.04-1.42)	1.21 (1.05-1.41)	0.001
Multivariable [†]	1.00 (Reference)	1.01 (0.85-1.19)	1.04 (0.88-1.23)	1.17 (1.00-1.37)	1.17 (1.01-1.36)	0.013
Multivariable and diet [‡]	1.00 (Reference)	1.01 (0.85-1.19)	1.03 (0.88-1.22)	1.16 (0.99-1.36)	1.16 (1.00-1.35)	0.023
Multivariable, diet, and renal function [§]	1.00 (Reference)	0.98 (0.82-1.15)	0.97 (0.82-1.14)	1.05 (0.89-1.24)	1.01 (0.87-1.19)	0.653

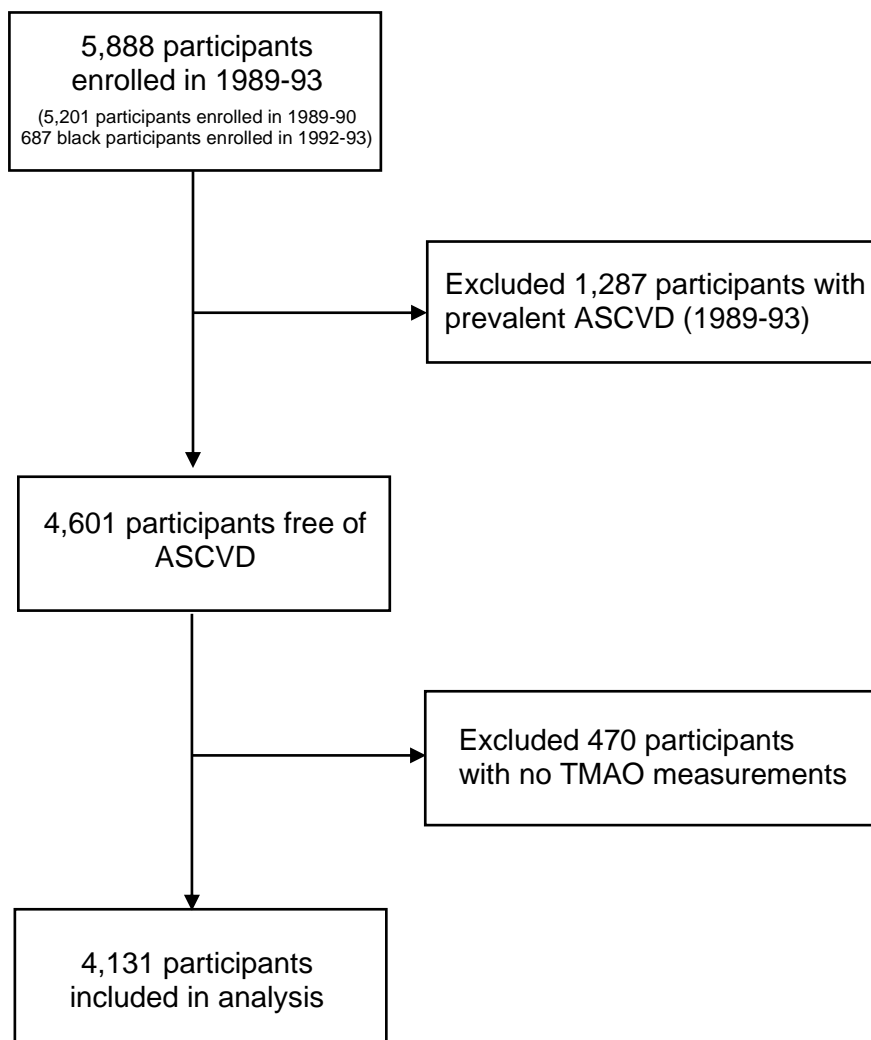
* Plasma TMAO levels at baseline (1989-90 or 1992-93) were related to risk of ASCVD from 1989-90 or 1992-93 to 1996-97, and the plasma TMAO levels in 1996-97 were related to risk of ASCVD from 1996-97 to 2015.

[†]Multivariable adjustments include age (65-74y, 75-84y, 85y+), sex (male, female), race (white, non-white), study site (Bowman Gray, Davis, Hopkins, Pittsburgh), education (<high school, high school, some college, college graduate), income (<\$11,999, \$12,000-\$24,999, \$25,000-\$49,999, >\$50,000/y), self-reported health status (excellent, very good, good/fair/poor), smoking status (never, former, current), alcohol intake (0, <1, 1-2.49, 2.5-7.49, 7.5-14.49, >14.5 drinks/wk), physical activity (<500, 500-1000, 1000-1500, >1500 kcal/wk), BMI (<18.5, 18.5-25, 25.1-30, >30 kg/m²), waist circumference (cm), lipid lowering medication (yes/no), anti-hypertensive medication (yes/no), antibiotics (yes/no), prevalent diabetes (yes/no), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), C-reactive protein (mg/L), systolic blood pressure (mmHg), diastolic blood pressure (mmHg)

[‡]Diet adjustments include fruits (servings/d), vegetables (servings/d), fiber (g/d), and total animal source food (servings/week).

[§] Estimated glomerular filtration rate (mL/min/1.73m²) is calculated based on the CKD-EPI creatinine-cystatin C equation.³⁹

Figure S1. Flow chart of this study.



ASCVD, atherosclerotic cardiovascular disease; TMAO, trimethylamine-N-oxide