Title

Plasma Trimethylamine-N -Oxide and Incident Ischemic Stroke: The Cardiovascular Health Study and the Multi-Ethnic Study of Atherosclerosis

Journal

Journal of the American Heart Association, 12(16)

ISSN

2047-9980

Authors

Lemaitre, Rozenn N
Jensen, Paul N
Wang, Zeneng
et al.

Publication Date

2023-08-15

DOI

10.1161/jaha.122.029230

Peer reviewed

Journal of the American Heart Association

ORIGINAL RESEARCH

Plasma Trimethylamine-*N*-Oxide and Incident Ischemic Stroke: The Cardiovascular Health Study and the Multi-Ethnic Study of Atherosclerosis

Rozenn N. Lemaitre, PhD; Paul N. Jensen, PhD; Zeneng Wang, PhD; Amanda M. Fretts, PhD; Colleen M. Sitlani, PhD; Ina Nemet, PhD; Nona Sotoodehnia, MD; Marcia C. de Oliveira Otto, PhD; Weifei Zhu, PhD; Matt Budoff, MD; W. T. Longstreth, MD; Bruce M. Psaty, MD, PhD; David S. Siscovick, MD; Stanley L. Hazen, MD, PhD; Dariush Mozaffarian, MD, DrPH

BACKGROUND: The association of circulating trimethylamine-*N*-oxide (TMAO) with stroke has received limited attention. To address this gap, we examined the associations of serial measures of plasma TMAO with incident ischemic stroke.

METHODS AND RESULTS: We used a prospective cohort design with data pooled from 2 cohorts. The settings were the CHS (Cardiovascular Health Study), a cohort of older adults, and the MESA (Multi-Ethnic Study of Atherosclerosis), both in the United States. We measured plasma concentrations of TMAO at baseline and again during the follow-up using high-performance liquid chromatography and mass spectrometry. We assessed the association of plasma TMAO with incident ischemic stroke using proportional hazards regression adjusted for risk factors.

The combined cohorts included 11 785 participants without a history of stroke, on average 73 (CHS) and 62 (MESA) years old at baseline, including 60% (CHS) and 53% (MESA) women. We identified 1031 total incident ischemic strokes during a median 15-year follow-up in the combined cohorts. In multivariable analyses, TMAO was significantly associated with incident ischemic stroke risk (hazard ratios comparing a doubling of TMAO: 1.11 [1.03–1.18], *P*=0.004). The association was linear over the range of TMAO concentrations and appeared restricted to those without diagnosed coronary heart disease. An association with hemorrhagic stroke was not found.

CONCLUSIONS: Plasma TMAO levels are associated with incident ischemic stroke in a diverse population.

REGISTRATION: URL: https://www.clinicaltrials.gov. Unique identifier: NCT00005133.

Key Words: epidemiology ■ risk factors ■ stroke ■ TMAO

Despite a falling mortality rate in recent years, stroke remains a heavy burden, especially in the elderly.[1] Identification of novel modifiable biomarkers associated with stroke risk is of considerable public health importance.

Plasma trimethylamine-*N*-oxide (TMAO) is a biomarker of diet and metabolism, originating from the dietary precursors carnitine, choline, and betaine,[2] or directly from fish and shellfish.[3] Carnitine, choline, and betaine are converted to trimethylamine by the gut

Correspondence to: Rozenn N. Lemaitre, PhD, University of Washington, Box 359458, 4333 Brooklyn Ave NE, Seattle, WA 98195. Email: rozenl@uw.edu

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.122.029230

This manuscript was sent to Jose R. Romero, MD, Associate Editor, for review by expert referees, editorial decision, and final disposition.

For Sources of Funding and Disclosures, see page 8.

JAHA is available at: www.ahajournals.org/journal/jaha

CLINICAL PERSPECTIVE

What Is New?

- We report that higher levels of plasma trimethylamine-*N*-oxide are associated with a greater risk of incident ischemic stroke in a diverse, population-based study consisting of 2 large prospective cohorts.

What Are the Clinical Implications?

- In addition to its previously reported association with stroke severity, trimethylamine-*N*-oxide may play a role in the development of ischemic stroke.
- While future studies need to determine whether interventions aimed at lowering circulating trimethylamine-*N*-oxide reduce the risk of stroke, it would be prudent to limit known trimethylamine-*N*-oxide sources such as red meat consumption.

Nonstandard Abbreviations and Acronyms

CHS	Cardiovascular Health Study
MESA	Multi-Ethnic Study of Atherosclerosis
TMAO	trimethylamine-*N*-oxide

microbiota, and trimethylamine is converted to TMAO postabsorption by the liver flavin monooxygenases.[4,5] Foods rich in TMAO precursors, such as red meat (carnitine, choline) and eggs (choline), as well as seafood increase TMAO levels in dietary interventions.[2,6,7]

In experimental studies, TMAO promotes atherosclerosis and related pathways.[8–11] In particular, dietary choline or TMAO in mice result in increased plasma TMAO and increased atherosclerotic plaques, and in subjects undergoing coronary angiography, TMAO levels correlate with the extent of vessel disease.[8,12] TMAO also increases foam cell formation in macrophages and impairs reverse cholesterol transport in mice.[13] Therefore we hypothesize that TMAO will be associated with risk of incident ischemic stroke.

Plasma levels of TMAO are associated with incident atherosclerotic cardiovascular disease[14] and all-cause mortality[15] in the CHS (Cardiovascular Health Study), a population-based cohort study. Similarly, in patients with prevalent risk factors, TMAO is associated with risk of cardiovascular disease and death.[16–19] However, the association of TMAO with incident stroke in population studies has received limited attention. To address this gap, we obtained serial measurements of TMAO in 2 cohort studies, CHS and MESA (Multi-Ethnic Study of Atherosclerosis), and investigated the association of TMAO with the risk of incident ischemic stroke in the combined sample.

METHODS

Data Availability

The Data and Material Distribution Agreements we signed with CHS and MESA do not allow us to transfer data to outside investigators. Further information on the CHS study can be found at https://chs-nhlbi.org/, and on the MESA study at https://www.mesa-nhlbi.org. Source code for the analysis is in Data S1.

Study Design

We used a prospective cohort design with data pooled from 2 cohorts.

Study Populations

The study population consists of participants in 2 prospective cohorts, CHS and MESA. CHS participants were recruited from 4 US communities as previously described.[20] The cohort consists of 5201 community-dwelling men and women, aged ≥65 years at enrollment, recruited in 1989 to 1990, plus an additional 687 predominantly Black participants recruited in 1992 to 1993. All CHS activities are currently approved and participants appropriately consented under the University of Washington Institutional Review Board approval number STUDY00000109. Plasma levels of TMAO were measured in CHS samples collected from 5418 participants at baseline (1989–1990/1992–1993) and again in 1996 to 1997 for participants with a clinic visit at that time. Participants with a history of stroke (n=273) or antibiotic use (n=72) at the time of their first TMAO measurement were excluded from the study, leaving 5073 CHS participants in the analysis. Of those, 2670 participants had 2 TMAO measurements.

MESA participants were recruited from 6 US communities as previously described.[21] The cohort consists of 6814 men and women, aged 45 to 84 years at enrollment and free of clinical cardiovascular disease, who were recruited in 2000 to 2002. The MESA cohort includes 38% White, 28% Black, 22% Hispanic, and 12% Chinese American participants. All MESA activities are currently approved and participants appropriately consented under the University of Washington Institutional Review Board approval numbers STUDY00009029 and STUDY00014523. Plasma levels of TMAO were measured in MESA plasma samples collected at baseline (2000–2002) and again in samples collected during a follow-up visit (2005–2007). Participants with a history of stroke (n=1), antibiotic use (n=71) at the time of their first TMAO measurement, and those with no follow-up past their TMAO (n=30) were excluded from the study, leaving 6712 MESA participants in the analysis. Of those, 5265 participants had 2 TMAO measurements.

Data Collection

CHS

Information on lifestyle and clinical risk factors was collected at baseline and annual study clinic visits.[20] Medication use was assessed by a validated medication inventory.[22] Plasma glucose, insulin, lipids, and inflammatory biomarkers were assessed using enzymatic methods.[20] Body mass index (BMI) was calculated from body weight (kg) divided by height squared (m^2). Estimated glomerular filtration rate (eGFR) was calculated with an equation from the Chronic Kidney Disease Epidemiology Collaboration using serum creatinine and cystatin C.[23] Physical activity (kcal/wk) was assessed using a modified Minnesota Leisure-Time Activities questionnaire.[24] Diabetes was defined by fasting (≥8 h) plasma glucose ≥126 mg/dL or random (<8 h fasting) glucose ≥200 mg/dL or new use of insulin or oral hypoglycemic medication (assessed annually). Diabetes was also identified using the Centers for Medicare & Medicaid Services records. Diabetes from the Centers for Medicare & Medicaid Services records was defined by at least 2 inpatient (hospital, nursing home, or home health services) or 3 outpatient (outpatient or carrier health services) or ≥1 inpatient and ≥1 outpatient *International Classification of Diseases, Ninth Revision* (*ICD-9*) claim codes for diabetes diagnosis (with the prefix 250.xx) over a 2-year period.[25]

MESA

The design of MESA has been previously described.[21] Demographics and lifestyle factors were assessed by interview and questionnaires. Physical activity, metabolic equivalent of task-minutes/wk, was converted to kcal/wk to harmonize with the CHS physical activity assessment. Blood pressure was the average of the last 2 of 3 measurements using a Dinamap automated blood pressure device. Total cholesterol, high-density lipoprotein cholesterol, and triglyceride were measured at a central core laboratory. Low-density lipoprotein cholesterol was calculated using the Friedewald equation. Diabetes was defined as a fasting glucose ≥126 mg/dL, use of diabetes medication or insulin, or self-report. Medication use, BMI, and eGFR were assessed by the same methods as in CHS.

Measurement of TMAO

TMAO was measured in EDTA-plasma samples that were stored at −80 °C. TMAO is stable during long-term storage at −80 °C and several freeze–thaw cycles.[26] TMAO was quantified by stable isotope dilution high-performance liquid chromatography with online tandem mass spectrometry as previously reported.[2] The coefficients of variation of the laboratory assay across the study period varied over batches to a maximum of 5.8%. The same method and laboratory were used to measure TMAO in the 2 cohorts' samples.

Ascertainment of Stroke

CHS

Medical records, information from interviews, physician questionnaires, death certificates, medical examiner forms, Health Care Financing Administration hospitalizations, and available computed tomographic or magnetic resonance imaging scans were reviewed by centralized cardiac and stroke adjudication committees to classify events.[27,28] Stroke was defined as a neurological deficit of rapid onset lasting longer than 24 hours unless death supervened or as a subarachnoid hemorrhage. Strokes were classified as ischemic if focal brain deficit without evidence of primary hemorrhage was evident; as hemorrhagic if there was bloody spinal fluid on lumbar puncture or evidence of blood in the subarachnoid space, ventricles, or parenchyma on brain imaging or at surgery or autopsy that did not appear consistent with hemorrhage into an infarction; or as unknown type if information was insufficient for classification. The committee subdivided ischemic stroke into 4 subtypes: small vessel, large vessel, cardioembolic, and other that included mostly uncertain subtypes. Details of the adjudication protocol can be found in Longstreth et al.[28]

MESA

Stroke events in MESA were ascertained from the MESA baseline to December 2018. Every 9 to 12 months, interim hospitalization was inquired from study participants or their next of kin. Medical records and death certifications were reviewed, and stroke events were adjudicated by a MESA cerebrovascular adjudication committee. The ischemic stroke subtypes were classified as large vessel, extracranial; large vessel, intracranial; cardioembolic; small vessel; other specific mechanisms; multiple mechanisms; or undetermined. A detailed description of the stroke subtype classification and adjudication protocols has been previously reported.[29]

Statistical Analysis

In primary analyses, we combined the CHS and MESA data sets with TMAO measures and harmonized covariate adjustments. Cox proportional-hazards regression was used with the option strata (cohort) to estimate the hazard ratios (HR) and 95% CI for incident ischemic stroke with log2[TMAO concentration]. The time variable in these analyses was time since the first TMAO measurement. Subjects remained at risk until first incident stroke, or in absence of stroke, the earliest of death, loss to follow-up, or

latest date of event ascertainment (June 2015 for CHS and December 2018 for MESA). For participants with 2 TMAO measurements, the value of the first measurement was used as the exposure up until the time of the second measurement, after which time the cumulative average of the 2 measurements was used as the exposure. Adjustment covariates were updated at the time of each TMAO measurement. We fit 3 prespecified models of adjustment. Model 1 (minimally adjusted) included adjustment for age, sex, race or ethnicity, and enrollment site. Model 2 (primary model) additionally adjusted for BMI, waist circumference, smoking status, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein cholesterol, prevalent coronary heart disease (CHD), and prevalent atrial fibrillation. Model 3 included further adjustment for eGFR. The proportional hazards assumption was not violated as assessed with Schoenfeld residuals.[30] To test the association of TMAO with ischemic stroke for departure from linearity, we used the Wald test and cubic splines.

We explored potential effect modification by age (years), sex (binary), evidence of kidney disease based on eGFR <60 mL/min (yes/no), CHD (binary), and BMI (kg/m^2) in models that included a multiplicative interaction term between TMAO (linear variable) and the effect modifier Model 2 (primary model). Secondary analyses were conducted separately in the 2 cohorts, stratified on race and ethnicity and stratified on the presence or absence of diagnosed CHD.

Most variables had <5% missing values. Missing covariate data were imputed using demographic and risk variables using single multivariable imputation, which has been shown to produce very similar results as multiple imputation in prior analyses in MESA and CHS.[31]

The significance level of the primary test was set as 2-tailed-alpha = 0.05; and the significance level of interaction tests was set at 0.05/5 (5 effect modifiers) = 0.01. Analyses were performed using Stata version 16.0 (StataCorp, College Station, TX).

RESULTS

The study included 5073 CHS participants and 6712 MESA participants. At baseline, participant mean (SD) age was 73 (6) years in CHS and 62 (10) years in MESA. Women comprised 60% of CHS and 53% of MESA cohorts. In CHS, 15% of participants were Black, while in MESA 28% were Black, 12% were Chinese American, and 22% were Hispanic. Characteristics of the 11 785 combined participants in quartiles of plasma TMAO are shown in Figure 1. Higher TMAO concentrations were associated with older age, male sex, lower eGFR, and generally unfavorable health profile at baseline. The associations of TMAO with participant characteristics were similar in the different race and ethnic groups (Figure S1).

Mean and median plasma TMAO concentrations were higher in CHS than MESA, likely due to older age and lower eGFR in CHS (Table 1). Among the race and ethnic groups, mean TMAO concentrations were highest in White (combined CHS and MESA) and Chinese American participants in MESA. However, adjustment for age and sex reduced the mean concentration in Chinese American participants, while the concentration in White participants remained elevated. Spearman correlations of TMAO at baseline with TMAO concentration in plasma collected 5 to 6 years later were 0.27 in CHS and 0.32 in MESA.

We identified 1031 cases of incident ischemic stroke during up to 26 years of follow-up (median 15.3 years), 741 in CHS and 290 in MESA. Plasma concentrations of TMAO were associated with incident ischemic stroke in the primary analysis (Table 2; Model 2). After multivariable adjustments, the HR of ischemic stroke associated with a 2-fold increase in TMAO concentrations was 1.11 (95% CI, 1.03–1.18; P=0.004). Further adjustment for eGFR decreased the association (Table 2; Model 3). Further adjustment for plasma C-reactive protein, aspirin, statin, and anticoagulant use did not change the study results (Table S1). Point estimates were similar for the associations of TMAO with cardioembolic (HR, 1.13 [95% CI, 1.00–1.28], P=0.06) and noncardioembolic ischemic stroke (HR, 1.10 [95% CI, 1.01–1.19], P=0.03). We did not observe departure from linearity for the TMAO-stroke association (Figure S2). The overall association was not significantly different by age, sex, BMI, or eGFR (not shown). However, we observed an interaction with baseline CHD (P value for interaction: 0.009), such that the association of TMAO with incident ischemic stroke was only observed in participants without baseline CHD (Table 3).

We conducted prespecified analyses stratified on the 4 different race or ethnicities to explore whether the TMAO-stroke association was similar across the race and ethnic groups (Figure 2 and Table S2). The association of TMAO with ischemic stroke in Hispanic participants appeared to be null; however, the P value for interaction of TMAO with race and ethnicity based on 3 degrees of freedom was 0.44.

During follow-up, 167 hemorrhagic strokes occurred, 110 in CHS and 57 in MESA. In secondary analyses, we observed no association of plasma TMAO with incident hemorrhagic stroke (Table S3). For example, the HR of hemorrhagic stroke associated with a 2-fold increase in TMAO concentrations was

	CHS	MESA	TMAO quartiles	
	Mean or %	Mean or %	Trend	Q1, Q4
Age	73 (6)	62 (10)		63, 70
Male	40%	47%		40, 47
Race or Ethnicity				
White	84%	39%		46, 67
Black	15%	28%		26, 19
Chinese American	0%	12%		10, 5
Hispanic	0%	22%		18, 9
Other	1%	0%		0, 0
BMI	27 (5)	28 (5)		27, 28
Waist circumference	95 (13)	98 (14)		95, 97
HDL	55 (16)	51 (15)		53, 52
LDL	130 (35)	117 (32)		122, 123
Alcohol drinks/week	3 (7)	3 (5)		2, 3
Smoking history				
Never	47%	50%		52, 45
Past	42%	37%		34, 42
Current	12%	13%		14, 12
Physical activity (kcal/week)	1149 (1553)	2142 (3363)		1837, 1533
Prevalent diabetes	15%	13%		10, 18
eGFR (creatinine/cystatin C formula)	69 (17)	90 (18)		91, 71
Glucose (mg/dL)	110 (36)	97 (30)		98, 108
Systolic blood pressure	136 (22)	127 (21)		128, 133
Treated hypertension	48%	37%		31, 51
Prevalent myocardial infarction	10%	0%		2, 7
Prevalent CHD	20%	0%		4, 13
Prevalent CHF	5%	0%		1, 4
Fruit (servings/day)	2 (1)	2 (2)		2, 2
Vegetables (servings/day)	3 (1)	2 (1)		2, 3
Milk (servings/day)	0.8 (0.5)	0.9 (1.3)		0.9, 0.8
Cheese (servings/day)	0.4 (0.3)	0.5 (0.7)		0.5, 0.5
Fish (servings/day)	0.3 (0.2)	0.3 (0.3)		0.3, 0.3
Egg (servings/day)	0.2 (0.2)	0.2 (0.3)		0.2, 0.2
Fiber intake (g/day)	27 (10)	6 (7)		12, 18
Non-processed meat (servings/day)	0.5 (0.3)	0.8 (0.7)		0.7, 0.6
Processed meat (servings/day)	0.4 (0.4)	0.2 (0.3)		0.2, 0.3
Energy intake (kcal/day)	1868 (573)	1625 (876)		1696, 1753

Figure 1. Baseline characteristics of 5073 Cardiovascular Health Study and 6712 Multi-Ethnic Study of Atherosclerosis participants in each cohort and across quartiles of baseline plasma trimethylamine-*N*-oxide levels in the combined 11 785 participants*.

*Mean values or % in each cohort, and in the pooled cohorts, trend values across quartiles of trimethylamine-*N*-oxide (TMAO) concentration and mean values or % in the first (Q1) and fourth (Q4) quartiles. Positive trends across TMAO quartiles are shown with blue lines, decreasing trends with red lines, and no trend with gray lines, based on univariate tests and threshold *P* value of 0.0015. BMI indicates body mass index; CHD, coronary heart disease; CHF, chronic heart failure; CHS, Cardiovascular Health Study; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; MESA, Multi-Ethnic Study of Atherosclerosis; and TMAO, trimethylamine-*N*-oxide.

Table 1. Concentration of Plasma Trimethylamine-*N*-oxide (μmol/L) in the Cardiovascular Health Study, the Multi-Ethnic Study of Atherosclerosis, the Pooled Cohorts, and by Race or Ethnicity

	N	Mean (SD)	Median	Min-Max	
Cohort					
CHS	5073	7.59 (11.15)	4.84	0.01–254.99	
MESA	6712	5.28 (7.17)	3.57	0.31–129.16	
Pooled cohorts	11 785	6.27 (9.17)	4.07	0.01–254.99	
Race or ethnicity					Age- and sex-adjusted means (95% CI)
White*	6846	6.88 (9.91)	4.50	0.01–235.63	6.90 (6.67–7.12)
Black*	2634	5.68 (8.46)	3.75	0.56–254.99	5.65 (5.30–6.01)
Hispanic†	6613	5.94 (7.96)	3.91	0.01–160.09	5.07 (4.60–5.54)
Chinese American†	5172	6.70 (10.51)	4.28	0.17–254.99	5.03 (4.39–5.67)

CHS indicates Cardiovascular Health Study; and MESA, Multi-Ethnic Study of Atherosclerosis.
*Cardiovascular Health Study and Multi-Ethnic Study of Atherosclerosis.
†Multi-Ethnic Study of Atherosclerosis only.

0.97 (95% CI, 0.81–1.15; *P*=0.71) with Model 2 multivariate adjustment.

DISCUSSION

In this large, prospective study combining 2 community-based cohorts with diverse populations, we observed a higher risk of incident ischemic stroke associated with elevated plasma TMAO concentrations. The association appeared to be restricted to those without prevalent CHD at the time of TMAO measurement, and in this subgroup, the association was not attenuated by adjustment for eGFR. Evidence of association of TMAO with incident hemorrhagic stroke was not found in the study.

Prior evidence for an association of TMAO with stroke in population studies is limited. Patients with prevalent stroke reportedly have higher plasma TMAO than healthy controls, and among those with stroke, TMAO is associated with the stroke severity.[32–34] However, the association of TMAO with incident ischemic stroke has received limited attention. In a prospective, case–control study nested in a 5-year trial, the China Stroke Primary Prevention Trial, increased baseline TMAO levels were associated with higher risks of incident total and ischemic stroke.[35] In their multivariable analysis, an increase of 1 log unit of TMAO was associated with an odds ratio of ischemic stroke of 1.22 (1.02–1.46). Median serum TMAO levels were noticeably lower than in our study (2.4 versus 4.1 μmol/L in the pooled CHS and MESA cohorts), suggesting the TMAO-stroke association might extend to populations with different ranges of TMAO levels. The elevated point estimate (albeit not significant) for the TMAO-ischemic stroke association in Chinese Americans in our study together with the report from the China Stroke Primary Prevention Trial suggest that further studies on TMAO in Asian populations are warranted.

A possible mechanism underlying the association of TMAO with ischemic stroke might be an effect of TMAO on atherosclerosis. In mice, dietary supplementation with TMAO has been shown to reduce reverse cholesterol transport, promote foam cell formation, and enhance atherosclerotic lesions.[8,13] Levels of TMAO also correlate with atherosclerotic plaque burden in subjects undergoing coronary angiography[8] and with the number of infarcted coronary arteries in patients undergoing surgery,[12] although the temporality of these associations

Table 2. Association of Plasma Trimethylamine-*N*-Oxide With Incident Ischemic Stroke in the Cardiovascular Health Study, the Multi-Ethnic Study of Atherosclerosis, and the Pooled Cohorts

Total N/events		Model 1	Model 2	Model 3
		Hazard ratio for a doubling of TMAO (95% CI), *P* value		
Pooled	11 785/1031	1.15 (1.07–1.23), 5.0×10^{-05}	1.11 (1.03–1.18), 0.004	1.07 (0.99–1.15), 0.07
CHS	5073/741	1.13 (1.05–1.22), 0.002	1.09 (1.01–1.18), 0.03	1.05 (0.97–1.14), 0.23
MESA	6712/290	1.21 (1.05–1.38), 0.007	1.15 (1.00–1.33), 0.05	1.11 (0.96–1.29), 0.17

Model 1 adjustments: age, sex, race or ethnicity, site. Model 2: Model 1 adjustments + body mass index, waist circumference, smoking, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein, prevalent coronary heart disease, and a history of atrial fibrillation. Model 3: Model 2 adjustments + estimated glomerular filtration rate. CHS indicates Cardiovascular Health Study; MESA, Multi-Ethnic Study of Atherosclerosis; and TMAO, trimethylamine-*N*-oxide.

Table 3. Association of Plasma Trimethylamine-*N*-Oxide With Incident Ischemic Stroke in Those With and Without Baseline Coronary Heart Disease

Total N/events		Model 2	Model 3
		Hazard ratio for doubling of TMAO (95% CI), *P* value	
No CHD*	10843/867	1.16 (1.07–1.25), 0.0001	1.13 (1.04–1.22), 0.003
With CHD†	942/164	0.91 (0.77–1.09), 0.32	0.85 (0.71–1.03), 0.10
P for interaction		0.009	0.008

Model 1 adjustments: age, sex, race or ethnicity, site. Model 2: Model 1 adjustments + body mass index, waist circumference, smoking, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein, prevalent coronary heart disease, and a history of atrial fibrillation. Model 3: Model 2 adjustments + estimated glomerular filtration rate. CHD indicates coronary heart disease; and TMAO, trimethylamine-*N*-oxide.

*Cardiovascular Health Study and Multi-Ethnic Study of Atherosclerosis.

†Cardiovascular Health Study only.

cannot be determined. A role of TMAO in atherosclerosis might also underlie reported associations with other incident cardiovascular outcomes in healthy populations. In particular, we previously reported an association of plasma TMAO with the risk of incident atherosclerotic cardiovascular disease, a composite outcome of myocardial infarction, fatal coronary heart disease, stroke, sudden cardiac death, and other atherosclerotic death in CHS.[14]

The nonsignificant association of TMAO with lower risk of ischemic stroke observed in CHS participants with CHD may be due to chance. Another possibility could be that participants with diagnosed CHD modified their diet (eg, reducing their meat intake), resulting in lower TMAO and biasing the TMAO association with stroke.

The overall association of TMAO with incident ischemic stroke was diminished by adjustment for eGFR. As also seen in other studies,[36] we observed that higher circulating levels of TMAO were correlated with lower eGFR (Spearman $\rho = -0.37$), raising the possibility that kidney function may confound the overall association of TMAO with stroke. However, elevated TMAO itself leads to reduced renal filtration and renal dysfunction in mice, and conversely, reduction in TMAO attenuates renal decline in animal models of chronic kidney disease.[36,37] Therefore, eGFR could also be a mediator of the TMAO-stroke association. Of note, in the large subgroup of participants without baseline CHD, the association of TMAO with ischemic stroke showed little attenuation with eGFR adjustment; therefore, eGFR is not likely to confound the TMAO-stroke association in this group.

Strengths of our study include the prospective design that minimizes reverse causation and selection bias; the repeated measurements strengthen the precision of our exposure measurement and validity of findings; we carefully adjusted for many covariates reducing the impact of residual confounding. CHS and MESA are general, community-based, prospective cohorts and the study included participants of several races and ethnicities, enhancing the generalizability of the findings. This study also has limitations. As this is an observational study, we cannot conclude on causality. Analyses stratified on race and ethnicity had limited power to detect difference, especially among Chinese American and Hispanic participants who had a relatively small number of incident ischemic strokes. Due to the long follow-up periods, measurement error may have resulted in an underestimate of the TMAO associations.

In conclusion, levels of plasma TMAO were associated with incident ischemic stroke in 2 large population-based prospective cohorts, suggesting that TMAO may play a role in the development of stroke in addition to the previously reported association with stroke severity.

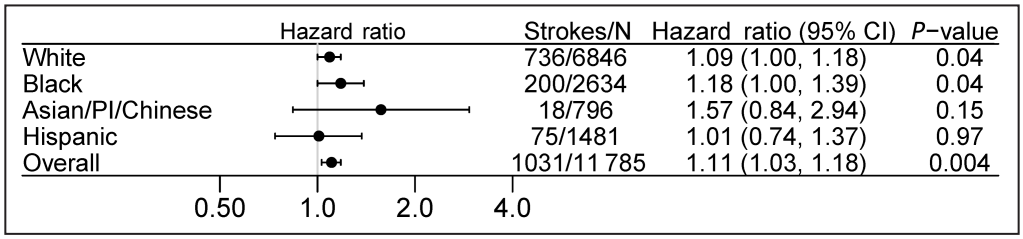


Figure 2. Association of plasma trimethylamine-*N*-oxide with incident ischemic stroke among participants of different race or ethnicity*.

*Hazard ratios of ischemic stroke, 95% CIs, and *P* values for a doubling of trimethylamine-*N*-oxide (TMAO) concentrations in models stratified by race and ethnicity and overall. The models were adjusted for age, sex, race, or ethnicity, site, body mass index, waist circumference, smoking, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein cholesterol, prevalent coronary heart disease, and a history of atrial fibrillation. Strokes/N are the number of incident strokes and the total number of participants in each stratum and overall. The *P* value for interaction of TMAO with race and ethnicity based on 3 degrees of freedom was 0.44.

ARTICLE INFORMATION

Received January 25, 2023; accepted July 17, 2023.

Affiliations

Cardiovascular Health Research Unit, Department of Medicine, University of Washington, Seattle, WA (R.N.L., P.N.J., C.M.S., N.S., B.M.P.); Department of Cardiovascular & Metabolic Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH (Z.W., I.N., W.Z., S.L.H.); Department of Epidemiology (A.M.F., B.M.P.) and Division of Cardiology (N.S.), University of Washington, Seattle, WA Division of Epidemiology, Human Genetics and Environmental Science, The University of Texas Health Science Center at Houston School of Public Health, Houston, TX (M.C.d.O.O.); Department of Medicine, University of California at Los Angeles, Los Angeles, CA (M.B.); Department of Neurology, University of Washington, Seattle, WA (W.T.L.); Kaiser Permanente Washington Health Research Institute, Seattle, WA (B.M.P.); New York Academy of Medicine, New York, NY (D.S.S.); Department of Cardiovascular Medicine, Heart, Vascular and Thoracic Institute, Cleveland Clinic, Cleveland, OH (S.L.H.); and Friedman School of Nutrition Science and Policy, Tufts University, Boston, MA (D.M.).

Acknowledgments

The authors thank the other investigators, the staff, and the participants in the CHS and MESA studies for their valuable contributions. A full list of principal CHS investigators and institutions can be found at CHS-NHLBI.org. A full list of participating MESA investigators and institutions can be found at http://www.mesa-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.

Sources of Funding

CHS: this research was supported by R01-HL135920 from the National Heart, Lung, and Blood Institute (NHLBI) and R01 HL103866 from the NIH Office of Dietary Supplements. These sources funded but did not influence the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication. The CHS cohort was supported by contracts HHSN268201200036C, HHSN268200800007C, HHSN268201800001C, N01HC55222, N01HC85079, N01HC85080, N01HC85081, N01HC85082, N01HC85083, N01HC85086, 75N92021D00006, and grants U01HL080295 and U01HL130114 from the NHLBI, with additional contribution from the National Institute of Neurological Disorders and Stroke (NINDS). Additional support was provided by R01AG023629 from the National Institute on Aging (NIA). MESA: the MESA study was supported by contracts 75N92020D00001, HHSN268201500003I, N01-HC-95159, 75N92020D00005, N01-HC-95160, 75N92020D00002, N01-HC-95161, 75N92020D00003, N01-HC-95162, 75N92020D00006, N01-HC-95163, 75N92020D00004, N01-HC-95164, R01 HL127659, 75N92020D00007, N01-HC-95165, N01-HC-95166, N01-HC-95167, N01-HC-95168, and N01-HC-95169 from the National Heart, Lung, and Blood Institute, and by grants UL1-TR-000040, UL1-TR-001079, and UL1-TR-001420 from the National Center for Advancing Translational Sciences (NCATS).

Disclosures

Dr Psaty serves on the Steering Committee of the Yale Open Data Access project funded by Johnson & Johnson. Dr Mozaffarian reports research funding from the National Institutes of Health, the Gates Foundation, The Rockefeller Foundation, Vail Innovative Global Research, and the Kaiser Permanente Fund at East Bay Community Foundation; personal fees from Acasti Pharma and Barilla; scientific advisory board, Beren Therapeutics, Brightseed, Calibrate, Elysium Health, Filtricine, HumanCo, Instacart, January Inc., Perfect Day, Tiny Organics, and (ended) Day Two, Discern Dx, and Season Health; stock ownership in Calibrate and HumanCo; and chapter royalties from UpToDate. Dr Hazen and Dr Wang report being named as co-inventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics, and being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Procter & Gamble, Zehna Therapeutics, and Cleveland HeartLab, a wholly owned subsidiary of Quest Diagnostics. Dr Hazen also reports being a paid consultant formerly for Procter & Gamble and currently with Zehna Therapeutics. He also reports having received research funds from Procter & Gamble, Zehna Therapeutics, and Roche Diagnostics. Dr Lemaitre reports research funding from the National Institutes of Health. The remaining authors have no disclosures to report.

Supplemental Material

Data S1
Tables S1–S3
Figures S1–S2

REFERENCES

1. Tsao CW, Aday AW, Almarzooq ZI, Alonso A, Beaton AZ, Bittencourt MS, Boehme AK, Buxton AE, Carson AP, Commodore-Mensah Y, et al. Heart disease and stroke statistics-2022 update: a report from the American Heart Association. *Circulation*. 2022;145:e153–e639. doi: 10.1161/CIR.0000000000001052
2. Wang Z, Bergeron N, Levison BS, Li XS, Chiu S, Jia X, Koeth RA, Li L, Wu Y, Tang WHW, et al. Impact of chronic dietary red meat, white meat, or non-meat protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women. *Eur Heart J*. 2019;40:583–594. doi: 10.1093/eurheartj/ehy799
3. Wang Z, Tang WHW, O'Connell T, Garcia E, Jeyarajah EJ, Li XS, Jia X, Weeks TL, Hazen SL. Circulating trimethylamine N-oxide levels following fish or seafood consumption. *Eur J Nutr*. 2022;61:2357–2364. doi: 10.1007/s00394-022-02803-4
4. Tang WH, Hazen SL. The contributory role of gut microbiota in cardiovascular disease. *J Clin Invest*. 2014;124:4204–4211. doi: 10.1172/JCI72331
5. Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, Allayee H, Lee R, Graham M, Crooke R, et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab*. 2013;17:49–60. doi: 10.1016/j.cmet.2012.12.011
6. Wang M, Wang Z, Lee Y, Lai HTM, de Oliveira Otto MC, Lemaitre RN, Fretts A, Sotoodehnia N, Budoff M, DiDonato JA, et al. Dietary meat, trimethylamine N-oxide-related metabolites, and incident cardiovascular disease among older adults: the cardiovascular health study. *Arterioscler Thromb Vasc Biol*. 2022;42:e273–e288. doi: 10.1161/ATVBAHA.121.316533
7. Miller CA, Corbin KD, da Costa KA, Zhang S, Zhao X, Galanko JA, Blevins T, Bennett BJ, O'Connor A, Zeisel SH. Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose-response study. *Am J Clin Nutr*. 2014;100:778–786. doi: 10.3945/ajcn.114.087692
8. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472:57–63. doi: 10.1038/nature09922
9. Wang Z, Roberts AB, Buffa JA, Levison BS, Zhu W, Org E, Gu X, Huang Y, Zamanian-Daryoush M, Culley MK, et al. Non-lethal inhibition of gut microbial trimethylamine production for the treatment of atherosclerosis. *Cell*. 2015;163:1585–1595. doi: 10.1016/j.cell.2015.11.055
10. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, Li L, Fu X, Wu Y, Mehrabian M, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. *Cell*. 2016;165:111–124. doi: 10.1016/j.cell.2016.02.011
11. Schugar RC, Shih DM, Warrier M, Helsley RN, Burrows A, Ferguson D, Brown AL, Gromovsky AD, Heine M, Chatterjee A, et al. The TMAO-producing enzyme flavin-containing monooxygenase 3 regulates obesity and the beiging of white adipose tissue. *Cell Rep*. 2017;20:279. doi: 10.1016/j.celrep.2017.06.053
12. Mafune A, Iwamoto T, Tsutsumi Y, Nakashima A, Yamamoto I, Yokoyama K, Yokoo T, Urashima M. Associations among serum trimethylamine-N-oxide (TMAO) levels, kidney function and infarcted coronary artery number in patients undergoing cardiovascular surgery: a cross-sectional study. *Clin Exp Nephrol*. 2016;20:731–739. doi: 10.1007/s10157-015-1207-y
13. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med*. 2013;19:576–585. doi: 10.1038/nm.3145
14. Lee Y, Nemet I, Wang Z, Lai HTM, de Oliveira Otto MC, Lemaitre RN, Fretts AM, Sotoodehnia N, Budoff M, DiDonato JA, et al. Longitudinal plasma measures of trimethylamine N-oxide and risk of atherosclerotic cardiovascular disease events in community-based older adults. *J Am Heart Assoc*. 2021;10:e020646. doi: 10.1161/JAHA.120.020646

15. Fretts AM, Hazen SL, Jensen P, Budoff M, Sitlani CM, Wang M, de Oliveira Otto MC, DiDonato JA, Lee Y, Psaty BM, et al. Association of trimethylamine N-oxide and metabolites with mortality in older adults. *JAMA Netw Open*. 2022;5:e2213242. doi: 10.1001/jamanetworkopen.2022.13242
16. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y, Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med*. 2013;368:1575–1584. doi: 10.1056/NEJMoa1109400
17. Schiattarella GG, Sannino A, Toscano E, Giugliano G, Gargiulo G, Franzone A, Trimarco B, Esposito G, Perrino C. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis. *Eur Heart J*. 2017;38:2948–2956. doi: 10.1093/eurheartj/ehx342
18. Qi J, You T, Li J, Pan T, Xiang L, Han Y, Zhu L. Circulating trimethylamine N-oxide and the risk of cardiovascular diseases: a systematic review and meta-analysis of 11 prospective cohort studies. *J Cell Mol Med*. 2018;22:185–194. doi: 10.1111/jcmm.13307
19. Heianza Y, Ma W, Manson JE, Rexrode KM, Qi L. Gut microbiota metabolites and risk of major adverse cardiovascular disease events and death: a systematic review and meta-analysis of prospective studies. *J Am Heart Assoc*. 2017;6:e004947. doi: 10.1161/JAHA.116.004947
20. Fried LP, Borhani NO, Enright P, Furberg CD, Gardin JM, Kronmal RA, Kuller LH, Manolio TA, Mittelmark MB, Newman A, et al. The cardiovascular health study: design and rationale. *Ann Epidemiol*. 1991;1:263–276. doi: 10.1016/1047-2797(91)90005-W
21. Bild DE, Bluemke DA, Burke GL, Detrano R, Diez Roux AV, Folsom AR, Greenland P, Jacob DR Jr, Kronmal R, Liu K, et al. Multi-Ethnic Study of Atherosclerosis: objectives and design. *Am J Epidemiol*. 2002;156:871–881. doi: 10.1093/aje/kwf113
22. Psaty BM, Lee M, Savage PJ, Rutan GH, German PS, Lyles M. Assessing the use of medications in the elderly: methods and initial experience in the Cardiovascular Health Study. The Cardiovascular Health Study Collaborative Research Group. *J Clin Epidemiol*. 1992;45:683–692. doi: 10.1016/0895-4356(92)90143-B
23. Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, Kusek JW, Manzi J, Van Lente F, Zhang YL, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med*. 2012;367:20–29. doi: 10.1056/NEJMoa1114248
24. Taylor HL, Jacobs DR Jr, Schucker B, Knudsen J, Leon AS, Debacker G. A questionnaire for the assessment of leisure time physical activities. *J Chronic Dis*. 1978;31:741–755. doi: 10.1016/0021-9681(78)90058-9
25. Brutsaert EF, Biggs ML, Delaney JA, Djousse L, Gottdiener JS, Ix JH, Kim F, Mukamal KJ, Siscovick DS, Tracy RP, et al. Longitudinal assessment of N-terminal pro-B-type natriuretic peptide and risk of diabetes in older adults: the cardiovascular health study. *Metabolism*. 2016;65:1489–1497. doi: 10.1016/j.metabol.2016.06.002
26. Wang Z, Levison BS, Hazen JE, Donahue L, Li XM, Hazen SL. Measurement of trimethylamine-N-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry. *Anal Biochem*. 2014;455:35–40. doi: 10.1016/j.ab.2014.03.016
27. Ives DG, Fitzpatrick AL, Bild DE, Psaty BM, Kuller LH, Crowley PM, Cruise RG, Theroux S. Surveillance and ascertainment of cardiovascular events. The Cardiovascular Health Study. *Ann Epidemiol*. 1995;5:278–285. doi: 10.1016/1047-2797(94)00093-9
28. Longstreth WT Jr, Bernick C, Fitzpatrick A, Cushman M, Knepper L, Lima J, Furberg CD. Frequency and predictors of stroke death in 5888 participants in the Cardiovascular Health Study. *Neurology*. 2001;56:368–375. doi: 10.1212/WNL.56.3.368
29. Longstreth WT Jr, Gasca NC, Gottesman RF, Pearce JB, Sacco RL. Adjudication of transient ischemic attack and stroke in the Multi-Ethnic Study of Atherosclerosis. *Neuroepidemiology*. 2018;50:23–28. doi: 10.1159/000486174
30. Grambsch P, Therneau T. Proportional hazards tests and diagnostics based on weighted residuals. *Biometrika*. 1994;81:515–526. doi: 10.1093/biomet/81.3.515
31. Arnold AM, Kronmal RA. Multiple imputation of baseline data in the cardiovascular health study. *Am J Epidemiol*. 2003;157:74–84. doi: 10.1093/aje/kwf156
32. Rexidamu M, Li H, Jin H, Huang J. Serum levels of trimethylamine-N-oxide in patients with ischemic stroke. *Biosci Rep*. 2019;39:BSR20190515. doi: 10.1042/BSR20190515
33. Wu C, Xue F, Lian Y, Zhang J, Wu D, Xie N, Chang W, Chen F, Wang L, Wei W, et al. Relationship between elevated plasma trimethylamine N-oxide levels and increased stroke injury. *Neurology*. 2020;94:e667–e677. doi: 10.1212/WNL.0000000000008862
34. Zhu W, Romano KA, Li L, Buffa JA, Sangwan N, Prakash P, Tittle AN, Li XS, Fu X, Androjna C, et al. Gut microbes impact stroke severity via the trimethylamine N-oxide pathway. *Cell Host Microbe*. 2021;29:1199–1208.e5. doi: 10.1016/j.chom.2021.05.002
35. Nie J, Xie L, Zhao BX, Li Y, Qiu B, Zhu F, Li GF, He M, Wang Y, Wang B, et al. Serum trimethylamine N-oxide concentration is positively associated with first stroke in hypertensive patients. *Stroke*. 2018;49:2021–2028. doi: 10.1161/STROKEAHA.118.021997
36. Tang WH, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatisa-Boyle B, Li XS, Levison BS, Hazen SL. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circ Res*. 2015;116:448–455. doi: 10.1161/CIRCRESAHA.116.305360
37. Gupta N, Buffa JA, Roberts AB, Sangwan N, Skye SM, Li L, Ho KJ, Varga J, DiDonato JA, Tang WHW, et al. Targeted inhibition of gut microbial trimethylamine N-oxide production reduces renal tubulointerstitial fibrosis and functional impairment in a murine model of chronic kidney disease. *Arterioscler Thromb Vasc Biol*. 2020;40:1239–1255. doi: 10.1161/ATVBAHA.120.314139

SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Source code for the analysis of main models

```
local xvar tmao
local m1 "age male i.racecat i.site"
local m2 "`m1' i_bmi i_waist i.i_smoke i_kcal_pa p_dm i_glu i_sbp i_htnmed
i_ldl p_chd p_af"
local m3 "`m2' egfr"

local ix "age male i_bmi egfr p_chd"
local nested 3

stset ttocens_yrs, failure(inc_strk) id(idno)
stsplit tsplit if visit2==1, at(0) after(_t=time2_yrs)
drop if _t0==0 & visit1==0
sort idno _t0
bysort idno: g ncnt=_n

foreach x in tmao choline betaine carnitine butyrobetaine crotonobetaine
age i_bmi i_waist i_hdl i_ldl i_alcoh i_smoke i_kcal_pa i_sbp i_htnmed
p_dm p_af p_chd p_chf p_mi p_strk i_antibio i_glu egfr i_crp i_trig asa
warf lipid sttn {
      g `x'=`x'1
      replace `x'=`x'2 if tsplit==0
}

foreach x in `xvar' {
      g l2`x'=log(`x')/log(2)
      replace l2`x'=(l2`x'+(log(`x'1)/log(2)))/2 if tsplit==0 & visit1==1
}

foreach x in `xvar' {
      forvalues i=1/`nested' {
            stcox l2`x' `m`i'', robust strata(cohort)

            if inlist(`i',2,3) {
                  estat phtest, detail
                  matrix c=r(phtest)
                        scalar phstrk`x'`i'=c[1,4]
            }
            if inlist(`i',2,3) {
                  foreach r in `ix' {
                        stcox c.l2`x'##c.`r' `m`i'', robust strata(cohort)
                  }
            }
      }
}
```

Table S1. Association of trimethylamine-N-oxide with incident ischemic stroke with additional adjustments.

	HR	95% CI	p
Main model	1.106	(1.03, 1.18)	0.004
Main + CRP	1.106	(1.03, 1.18)	0.004
Main + aspirin use	1.106	(1.03, 1.18)	0.004
Main + statin use	1.091	(1.01, 1.17)	0.02
Main + anticoagulant use	1.106	(1.03, 1.18)	0.004

Hazard ratios obtained with adjustments for age, sex, race, site, body mass index, waist circumference, smoking, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein, prevalent coronary heart disease, and a history of atrial fibrillation

CRP: C-reactive protein; HR: hazard ratio; CI: confidence interval

Table S2. Association of trimethylamine-N-oxide with incident ischemic stroke stratified on race/ethnicity.

Ethnic group	**Total N/stroke number**	**Model 2** HR (95% CI), p	**Model 3** HR (95% CI), p
Overall	11785/1031	1.11 (1.03, 1.18), 0.004	1.07 (0.99, 1.15), 0.07
White	6846/736	1.09 (1.00, 1.18), 0.04	1.06 (0.97, 1.15), 0.19
Black	2634/200	1.18 (1.00, 1.39), 0.04	1.09 (0.92, 1.30), 0.32
Chinese Am.	796/18	1.57 (0.84, 2.94), 0.15	1.63 (0.85, 3.09), 0.14
Hispanic	1481/75	1.01 (0.74, 1.37), 0.97	0.96 (0.68, 1.34), 0.80

3 degrees of freedom interaction test p=0.44

Model 1 adjustments: age, sex, race or ethnicity, site

Model 2: Model 1 adjustments + body mass index, waist circumference, smoking, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein, prevalent coronary heart disease, and a history of atrial fibrillation

Model 3: Model 2 adjustments + estimated glomerular filtration rate

HR: hazard ratio; CI: confidence interval

Table S3. Association of plasma trimethylamine-N-oxide with incident hemorrhagic stroke.

	HR	95% CI	p
Model 1	0.95	(0.79, 1.13)	0.55
Model 2	0.97	(0.81, 1.15)	0.71
Model 3	1.00	(0.84, 1.20)	0.99

Model 1 adjustments: age, sex, race or ethnicity, site

Model 2: Model 1 adjustments + body mass index, waist circumference, smoking, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein, prevalent coronart heart disease, and a history of atrial fibrillation

Model 3: Model 2 adjustments + estimated glomerular filtration rate

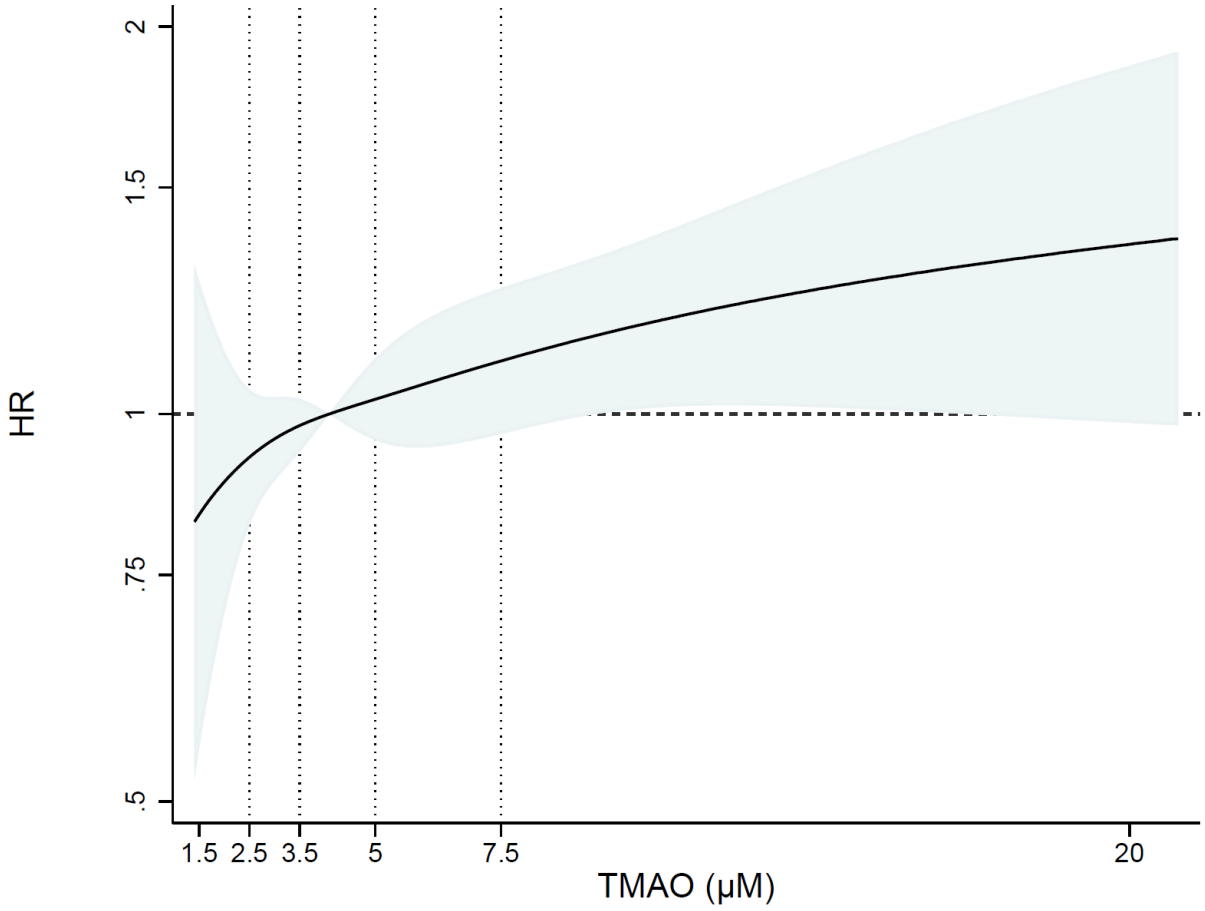
HR: hazard ratio, CI: confidence interval

Figure S1. Participant characteristics in quartiles of trimethylamine-N-oxide, stratified on race and ethnic group.

	White			Black			Chinese American			Hispanic		
	Mean or %	Trend	Q1, Q4	Mean or %	Trend	Q1, Q4	Mean or %	Trend	Q1, Q4	Mean or %	Trend	Q1, Q4
Age (years)	69 (9)		66, 72	65 (10)		62, 68	61 (10)		62, 62	61 (10)		59, 64
Male	43%		38, 46	42%		38, 44	48%		48, 50	48%		44, 56
BMI (kg/m2)	27 (5)		26, 27	30 (6)		30, 30	29 (5)		24, 24	29 (5)		29, 30
Waist circumference (cm)	95 (14)		94, 96	101 (15)		99, 102	101 (13)		86, 88	101 (13)		98, 103
HDL (mg/dL)	53 (16)		55, 52	54 (15)		54, 54	48 (13)		50, 49	48 (13)		49, 47
LDL (mg/dL)	125 (34)		126, 125	120 (34)		119, 119	119 (34)		112, 113	119 (34)		121, 118
Alcohol (drinks/week)	3 (6)		3, 3	2 (6)		2, 2	2 (5)		2, 1	2 (5)		2, 2
Smoking history												
Never	45%		49, 43	46%		46, 46	54%		77, 71	54%		57, 49
Past	43%		38, 46	36%		34, 36	33%		19, 22	33%		31, 38
Current	11%		13, 11	17%		20, 17	14%		4, 7	14%		12, 13
Physical activity (kcal/week)	1641 (2412)		1792, 1526	1970 (3592)		2257, 1545	1841 (3098)		1335, 1164	1841 (3098)		1622, 1810
Prevalent diabetes	11%		7, 15	20%		13, 26	17%		8, 19	17%		12, 25
eGFR (mL/min)	75 (19)		85, 66	87 (21)		97, 76	91 (18)		96, 90	91 (18)		97, 82
Glucose (mg/dL)	102 (29)		97, 107	105 (40)		99, 110	103 (39)		95, 106	103 (39)		99, 107
Systolic blood pressure (mmHg)	131 (22)		128, 133	135 (22)		133, 137	127 (22)		123, 125	127 (22)		124, 130
Treated hypertension	41%		30, 50	53%		42, 63	33%		23, 36	33%		24, 39
Prevalent myocardial infarction	7%		4, 9	3%		1, 5	0%			0%		
Prevalent coronary heart disease	12%		8, 17	6%		3, 9	0%			0%		
Prevalent heart failure	3%		1, 5	2%		1, 3	0%			0%		
Fruit (servings/day)	2 (1)		2, 2	2 (2)		2, 2	2 (2)		1, 1	2 (2)		2, 2
Vegetables (servings/day)	3 (1)		2, 3	2 (2)		2, 2	2 (1)		3, 3	2 (1)		2, 1
Milk (servings/day)	0.9 (0.9)		0.9, 0.8	0.7 (0.9)		0.6, 0.7	1.1 (1.4)		0.7, 0.6	1.1 (1.4)		1.2, 1.1
Cheese (servings/day)	0.5 (0.5)		0.6, 0.5	0.4 (0.5)		0.5, 0.4	0.7 (0.7)		0.1, 0.1	0.7 (0.7)		0.7, 0.7
Fish (servings/day)	0.3 (0.2)		0.2, 0.3	0.4 (0.4)		0.4, 0.3	0.2 (0.3)		0.4, 0.5	0.2 (0.3)		0.2, 0.2
Egg (servings/day)	0.2 (0.2)		0.2, 0.2	0.3 (0.3)		0.2, 0.3	0.2 (0.3)		0.2, 0.2	0.2 (0.3)		0.2, 0.3
Non-processed meat (servings/day)	0.6 (0.4)		0.6, 0.6	0.7 (0.7)		0.8, 0.7	0.9 (0.8)		0.7, 0.8	0.9 (0.8)		0.9, 0.8
Processed meat (servings/day)	0.3 (0.3)		0.3, 0.3	0.4 (0.4)		0.4, 0.4	0.2 (0.3)		0.1, 0.1	0.2 (0.3)		0.1, 0.2
Fiber intake (g/day)	20 (13)		17, 22	13 (12)		11, 15	5 (6)		3, 2	5 (6)		5, 6
Energy intake (kcal/day)	1790 (653)		1768, 1810	1767 (911)		1779, 1734	1692 (932)		1151, 1148	1692 (932)		1705, 1685

BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; eGFR: estimated glomerular filtration rate

Figure S2. Hazard ratio of ischemic stroke associated with plasma trimethylamine-N-oxide – spline analysis.



The reference point in the graph is the median of plasma trimethylamine-N-oxide concentration.

Hazard ratios adjusted for age, sex, race or ethnicity, site, body mass index, waist circumference, smoking, physical activity, prevalent diabetes, fasting glucose, systolic blood pressure, treated hypertension, low-density lipoprotein, prevalent coronary heart disease, and a history of atrial fibrillation

HR: hazard ratio; TMAO: trimethylamine-N-oxide